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Case study for the integration of in vitro data in the developmental neurotoxicity hazard identification and characterisation using deltamethrin as a prototype chemical

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Case study for the integration of in vitro data in the developmental neurotoxicity hazard identification and characterisation using deltamethrin as a prototype chemical

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Paris 2022

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Foreword

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by Iris Mangas[1)], Andrea Terron[1)], Martina Panzarea[1)], Laura Martino[2)], Federica Barrucci[2)], Elisa Aiassa[2)], Irene Munoz Guajardo[2)], Kevin Crofton[3)], Martin Wilks[3)], Susanne Hougaard Bennekou[3)], Martin Paparella[3)], Ioanna Tzoulaki[3)], Antonio Hernandez-Jerez[3)] for illustrating practical use of IATA and submitted to the 2021 review cycle of the IATA Case Studies Project.

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The case study was reviewed by the project team, and endorsed at the 6th meeting of the Working Party on Hazard Assessment in June 2022.

The case study is illustrative examples, and their publication as OECD monographs does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, the cases study should not be interpreted as official regulatory decisions made by the authoring member countries.

This document is published under the responsibility of the Chemicals and Biotechnology Committee of the OECD.

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Abbreviations and acronyms

3-PBA	3-phenoxybenzoic acid
5-HT	5-hydroxytryptamine
ADHD	Attention-deficit/hyperactivity disorder
ADME	Absorption, distribution, metabolism and excretion
ADOS	Autism Diagnostic Observation
AMPARs	alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AO	Adverse Outcome
AOP	Adverse Outcome Pathway
ASD	Autism Spectrum Disorder
BASC	Behavioural Assessment System for Children
BDNF	Brain-Derived Neurotrophic Factor
BMC	Benchmark Concentration
BMR	Benchmark Response
BRIEF	Behaviour Rating Inventory of Executive Function
BSID-II	Bayley Scales of Infant Development, 2nd edition
BSID-III	Bayley Scales of Infant Development, 3rd edition
BSID-IIS	Bayley Scales for Infant Development—Spanish version
BV2	Cells derived from raf/myc-immortalised murine neonatal microglia
Bw	Body weight
Camk1g	Calcium/Calmodulin Dependent Protein Kinase IG
CBCL	Child Behaviour Check List
cis-DBCA	cis-3-(2,2-dibromo-vinyl)-2,2-dimethylcyclopropane carboxylic acid
Cl2CA	cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid
Cmax	Peak concentration
CNN	Convolutional neuronal network
CNS	Central Nervous System
CREB	cAMP response element-binding protein
CWM	Cincinnati water maze
DAP	Dialkylphosphates organophosphorus pesticides metabolity
DAP	dialkylphosphate
DCCA	3-2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichloro-diphenyl-trichloroethane
DEP	Diethylphosphate
DETP	Diethylthiophosphate
DIV	Days In Vitro
DMDP	Dimethyldiphosphate
DMP	Dimethylphosphate
DMTP	Dimethyldithiophosphate
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity

DNT-IVB	DNT in vitro battery
DQ	Development quotient
DST	Development Screen Test
EKE	Expert knowledge elicitation
ESA	Electrical spike activity
GABA	g-Aminobutyric acid
GD	Gestation Day
GLP	Good Laboratory Practice
HCI	High Content imaging
hiPSC	human induced pluripotent stem cells
HNNF	Human neuronal network formation
hNPC	Human neural progenitor cells
HOS	Human observational studies
IATA	Integrated Approaches for Testing and Assessment
IUF	Leibniz Research Institute for Environmental Medicine
IVB	In vitro battery
IVIVE	in vitro to in vivo extrapolation
KE	Key Event
KER	Key Event Relationship
LDH	lactate dehydrogenase
LOAEL	lowest-observed-adverse-effect leve
LUHMES	Lund human mesencephalic cells
MAP2	Microtubule Associated Protein
MBR	Mean burst rate
MDI	Cognitive impairment
MEA	Microelectrode arrays
MFR	Mean Firing Rate
MI	Mental development index
MIE	Molecular Initiating Event
MISIB	Mean Interspike Interval In Burst
MoA	Mode of Action
MRI	Magnetic resonance imaging
MSEL	Mullen Scales of Early Learning
MWM	Morris Water Maze behavioural test
NCC	Neural crest cells
NDD	Neurodevelopmental disorder
NMDA	N-Methyl-d-aspartic acid
NMDAR	N-Methyl-d-aspartic acid receptor
NNF	Neuronal network formation
non-TD	non-typical development
NPC	Neural Progenitor Cell
NPC1 test	Primary hNPC Proliferation Assay
NPC2 test	Primary hNPC Migration Assay
NPC3 test	Primary hNPC Neuronal Differentiation Assay
NPC4 test	Neuronal Morphology (neurite length and area) of young neurons differentiated from hNPC
NPC5 test	Oligodendrocyte differentiation
OHAT/NTP	The Office of Health Assessment and Translation/National Toxicology Program
OL	Oligodendrocyte
OPC	OL progenitor cell
OP	organophosphate pesticide
PBPK	Physiologically-based pharmacokinetic
pCREB	cAMP response element-binding protein phosphorylated
PDI	Impaired psychomotor development

PND	Postnatal day
PoD	Point-of-departure
pre-OLs	Pre-myelinating oligodendrocytes
PTrkB	Tropomyosin receptor kinase B phosphorylated
r/HNNf	Rat/human neuronal network formation
RoB	Risk of bias
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SCOs	Synchronous Calcium oscillations
SDQ	Strengths and Difficulties Questionnaire
SNAP25	Synaptosomal-Associated Protein, 25kDa
TCPy	3,5,6-Trichloro-2-pyridinol
TG426	OECD Test Guideline 426
TK	Toxicokinetics
TNF	Tumour Necrosis Factor alpha
TrkB	Tropomyosin receptor kinase B
TUBB3	Tubulin Beta 3 Class III
UF	Uncertainty Factor
UKN2 test	The cMINC Neural Crest Cell Migration Assay
UKN4 test	The NeuriTox Neurite Outgrowth of CNS Neurons Test (UKN4 Test)
UKN5 test	The PeriTox Neurite Outgrowth of PNS Neurons Test
VGCCs	Voltage-gated Ca2+ channel
VGCC	Voltage-gated Ca2+ channel
WISC-IV	Wechsler Intelligence Scale for Children
WoE	Weight of evidence

Executive Summary

This case study was developed to show the applicability of an *in vitro* battery (IVB) for developmental neurotoxicity (DNT) testing in the context of the European Pesticide Regulation (EU) 283/2013 and 1107/2009. Deltamethrin, a type II pyrethroid insecticide, was chosen as a model chemical to perform the process including the novel IVB. Deltamethrin can cause acute and repeated neurotoxicity effects after oral administration, and a developmental neurotoxicity study was performed by the data owner. Therefore, regulatory *in vivo* guideline DNT study (OECD TG 426) is available as well as *in vivo* experimental studies from literature with overall equivocal results. Moreover, human epidemiological data showed a potential concern for neurodevelopmental disorders for the pyrethroid chemical class. This led to the hypothesis that integrating mechanistic understanding determined by relevant *in vitro* assays could reduce the uncertainty on the DNT hazard identification/characterisation of deltamethrin.

This integrated approaches for testing and assessment (IATA) case study is comprised by several data streams and methodologies. A systematic literature review of *in vivo* and *in vitro* experimental evidence as well as human observational studies (HOS) was performed. The studies retrieved were submitted to a critical appraisal and quantitative analysis of uncertainty by expert knowledge elicitation together with the DNT-IVB that covers basic neurodevelopmental key events, e.g. functional and/or morphological alterations in brain cells and tissue. The adverse outcome pathway (AOP) concept has been applied as a framework to develop the IATA to structure all the evidence gathered and characterise the individual biological and toxicological relevance of the IVB in predicting an adverse effect.

The core element of the case study was an integrated and extensive uncertainty analysis of all the lines of *in vivo* and *in vitro* evidence as well as the HOS used as supporting evidence. Since no AOP was available so far, an AOP network was developed consisting of two molecular initiating events (MIE) leading to altered neurobehavioral function by including data for which it was judged by experts consensus that there was more than a 66% probability of a causal association together with a range/probability distribution expressing the uncertainty on the lowest concentration/dose triggering the causal relationship. The strength of the key event relationship (KER) was quantified using a Bayesian network analysis approach. It was found that the marginal probability of an AOP string in the AOP network was always above 50%, indicating that it is most likely that the particular MIE, key event (KE), adverse outcome (AO) would be triggered by deltamethrin at a minimum concentration/dose. The critical evidence gap, driving most of the uncertainty, was the lack of empirical support providing biological understanding of what is occurring between the KE before the AO and the AO. The dose/concordance of the AOP was underpinned by applying a PBPK model for deltamethrin and performing reverse dosimetry. Furthermore, the equivocal *in vivo* effects data amongst different studies were contextualised by indicating that only direct dosing of pups (as opposed the exposure by the mother) would achieve an exposure level high enough to trigger the AOP network. This approach can support regulatory decision making, since it established the certainty of a dose-range that potentially can cause the AO.

Overall, the case study shows the applicability of the DNT-IVB in a hazard characterisation context. Furthermore, the case study illustrates the usefulness of a postulated AOP network and probabilistic quantification of weight of evidence to improve regulatory decision making. The overall process and the mechanistic understanding also increased the ability of interpreting the HOS by providing a plausible

mechanistic link to a human-relevant AO, therefore supporting the contextualisation of these studies in the future risk assessment process.

1 Introduction

1.1. Background

The consequences of human developmental neurotoxicity (DNT) liabilities have a relevant socio-economic impact and the current increase in prevalence of neurodevelopmental disorders cannot be solely explained by genetic factors. The developing nervous system is especially vulnerable to certain chemicals and exposure may result in altered neural development with consequences that may be different from the one observed with the chemical in an adult nervous system. This is due to a plethora of neurodevelopmental processes that play only marginal roles in the adult brain or distinct roles of e.g. neurotransmitter signalling in the developing compared with the adult nervous system (Nguyen et al., 2001). Moreover, certain developing brain cell types like oligodendrocyte precursor cells are more sensitive to stressors compared with their adult counterparts, rendering white matter development as a susceptible target. Despite the sensitivity of the developing nervous system, DNT hazard identification remains a 'hidden hazard' and the main reasons for this are due to the limited data requirements for DNT across the different jurisdictions and the uncertainties associated with the current *in vivo* DNT models (Paparella et al., 2020).

Annex II of the **Regulation No 1107/2009** on the placing of plant protection products on the market in the European Union, considers DNT as a critical effect of particular significance, and studies may be carried out when there is indication for potential developmental neurotoxicity from others studies, or when the concern arises from the systematic literature review (Regulation 283/2013). In the European Union, recognised testing methods include those listed in the Commission **Communication No 2013/C 95/01** (1998 US EPA DNT Guideline, OECD TG 426 and OECD TG 443, with DNT cohort) (US EPA, 1998; OECD 2007 and 2018b).

In vivo DNT studies are complex in both study design and data interpretation, frequently resulting in diverging conclusions (Makris et al., 2009). A variety of *in vitro* methods (DNT *in vitro* battery, DNT-IVB) are now available, which utilise human-relevant cell systems capable of testing chemicals for the potential to disrupt key neurodevelopmental processes (Frank et al., 2017; Bal-Price et al., 2018; Masjosthusmann et al., 2020). Here the assumption is that dysregulation of a fundamental process in brain development has the potential to lead to an adverse exophenotype (i.e. behaviour or physical appearance; Glahn et al., 2014). Therefore, by means of mechanistic understanding, this integrated approaches to testing and assessment (IATA) case study has been developed to acknowledge the contribution of the DNT-IVB in the DNT hazard characterisation of chemical substances with the intent of satisfying the regulatory need i.e. when a weight of evidence (WoE) analysis in chemical hazard assessments is necessary because available *in vivo* DNT data exist but are inconclusive, or a new concern has been raised from new published data.

1.2. Problem formulation

This case study is using deltamethrin as a model chemical to test the regulatory problem formulation on DNT hazard characterisation (see below). Deltamethrin is an approved pesticide active substance under Reg. 1107/2009. Deltamethrin is a type II pyrethroid showing neurotoxicity effects in acute and long-term toxicity studies and was tested in an OECD 426 DNT study. Scientific peer-reviewed open literature studies were also submitted and appraised in line with Regulation 1107/2009.

Recent scientific literature on *in vivo* and *in vitro* experimental studies and human observational studies (HOS) showed DNT concerns for deltamethrin or its chemical class in general (Dalsager et al., 2019, Pitzer et al., 2019; Zhang et al., 2018). Therefore, deltamethrin represents an excellent case study evaluating to what extent the DNT-IVB informs a WoE approach quantified through an uncertainty analysis and how it can provide mechanistic fit-for-purpose information on the DNT hazard characterisation.

The specific problem formulation is therefore the following: How certain are we that the pesticide active substance deltamethrin is a developmental neurotoxicant in humans based on the data collected, appraised, synthesised and integrated using an operational protocol in line with the IATA and adverse outcome pathway (AOP) framework?

2 Purpose

2.1. Purpose of use

This IATA case study was developed to be included in the OECD Guidance under development by the DNT expert group of the OECD on the use and interpretation of DNT-IVB. Additional case studies will be included in the OECD Guidance; specifically, this case study is intended to provide an example for the use and application of the DNT-IVB for single substance DNT hazard assessment.

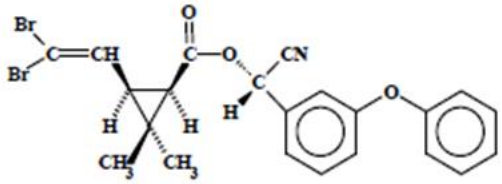
This IATA addresses DNT hazard characterisation of the pesticide active substance deltamethrin in line with the drafted problem formulation: How certain are we that the pesticide active substance deltamethrin is a developmental neurotoxicant in humans based on the data collected, appraised, synthesised and integrated using an operational protocol in line with the IATA and adverse outcome pathway (AOP) framework?

A structured scientific assessment approach using a predefined protocol for a systematic literature review, including screening for relevance, critical appraisal of the evidence and data extraction and analysis, was therefore applied. An uncertainty analysis was performed to support the selection of molecular initiating events (MIEs), key events (KEs) and adverse outcome (AO) and a Bayesian network analysis was used to quantify the uncertainties in the key events relationships (KERs) and for the quantification of the overall WoE. This approach was also used to quantify the impact of the DNT-IVB on the mechanistic understanding to conclude on the IATA case study.

2.2. Target chemical(s)

The target chemical for this case study was the pesticide deltamethrin which is the common name for (S)-alpha-cyano-3-phenoxybenzyl (1R, 3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate and specifically refer to cis-deltamethrin as the active isomer (CAS 52918–63–5; C22H19Br2NO3; molecular weight 505.2 g/mol). Other isomers of deltamethrin are considered to be impurities. Its chemical structure is shown in Figure 1.

Figure 1. Chemical structure of deltamethrin



Deltamethrin was chosen as a prototypical chemical with a well known mechanism of action (binding to the voltage-gated sodium channel), for which existing *in vivo* DNT data was deemed equivocal, and testing with the DNT IVB can be used to inform the Weight of Evidence (WoE)-based assessment for DNT.

Deltamethrin is a type II pyrethroid insecticide. The insecticidal actions of pyrethroids depend on their ability to bind to and disrupt voltage-gated sodium channels (VGSC) of insect nerves. Deltamethrin can cause acute and repeated neurotoxicity effects after oral administration, and a developmental neurotoxicity study was performed by the data owner. Therefore, regulatory *in vivo* guideline DNT study (OECD TG 426) is available as well as *in vivo* experimental studies from literature with overall equivocal results. Moreover, human observational repidemiological data showed a potential concern for neurodevelopmental disorders for the pyrethroid chemical class. This led to the hypothesis that integrating mechanistic understanding determined by relevant *in vitro* assays could reduce the uncertainty on the DNT hazard identification/characterisation of deltamethrin.

2.3. Endpoint(s)

This IATA is performed for the assessment of the endpoint DNT following exposure to deltamethrin. Any adverse effect on the normal development of nervous system structure and/or function, predefined by different specific endpoints measured *in vivo*, *in vitro* and in HOS, are presented in Table 2 (details in Appendix A: Protocol of the IATA).

2.4. Exposure information

This IATA case study concerns hazard characterisation; therefore, exposure considerations are not included. Nevertheless, deltamethrin is a pesticide active substance and information on dietary and non-dietary exposures will be used in the risk assessment process while considering all routes of exposure.

However, the IATA case study is compiled using evidence derived from in vitro, *in vivo* and HOS. All this evidence is structured in a postulated AOP network when the empirical support for the KERs will focus on dose and temporal concordance for each KER and for the overall pathway (see Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)).

Neurotoxicity of deltamethrin is consistent with the pyrethroid mechanism of action observed in acute toxicity studies conducted in young rats before weaning and adult rats. These studies frequently provide the most sensitive endpoints for this class of chemicals. Apart from zeta-cypermethrin, DNT studies (and their endpoints) were not used to identify reference points (RP; also known as point-of-departure, PoD). ADME data on pyrethroids (including deltamethrin) indicates that pyrethroids are rapidly absorbed, distributed and cleared from the body and the acute neurotoxic effect is correlated with peak concentration (Cmax). Therefore, studies conducted by direct dosing through oral gavage in pups would provide the best model for the assessment of the dose and temporal concordance of deltamethrin toxicity. Several studies were conducted with deltamethrin for the development of a physiologically-based pharmacokinetic (PBPK) model to evaluate target tissue dosimetry in rats ranging from postnatal day (PND) 10 to PND 90. These studies were not systematically reviewed and were only used in the context of this IATA to support the dose and temporal concordance in the postulated AOP network and to estimate the correspondence between the nominal concentration used in the *in vitro* studies, the external dose administered in the *in vivo* studies and the expected concentration in the brain (Song et al., 2019; Mortuza et al., 2018; Kim et al., 2010; US EPA, 2017).

The deltamethrin PBPK models for predicting the disposition after oral exposure at any age from birth and adulthood is therefore available but does not include the gestational and lactation periods a (Song et al., 2019). The two life-stage models were developed to investigate and validate '*in vitro* to *in vivo* extrapolation' (IVIVE) approaches based on *in vitro* metabolism data to predict the time course of plasma and brain concentrations of deltamethrin for rats at different ages following single dose exposure. *In vivo* data were produced to validate the IVIVE model and used in this IATA case study to support the concentration/dose concordance in the postulated AOP. *In vivo* data were therefore generated to estimate the deltamethrin level in plasma and brain obtained in a range of doses, volumes of administration and dissolving vehicle relevant for the comparative analysis with the dose administered *in vivo* in the studies used to derive the empirical support for the AOs. Similarity in the administered volume and used vehicle are relevant as it is known that they can significantly affect chemical kinetic in general and of deltamethrin in the specific (Kim et al., 2008; Mortuza et al., 2018).

Deltamethrin kinetic data were produced and reported (Mortuza et al., 2018). Because of the correlation between neurotoxicity and Cmax, the Cmax in plasma and brain was used as a measure of internal and target organ exposure, respectively. Single oral doses of 0.1, 0.25 and 0.5 mg/kg deltamethrin dissolved in 5 ml/kg corn oil were administered by gavage to rats on PND 15 and 90. Plasma and brain were collected up to 48 h following single dose administration and were analysed upon serial sacrifice for total (free and bound) deltamethrin by gas chromatography-negative chemical ionisation-mass spectrometry. Plasma unbound fraction was evaluated using C14-labelled deltamethrin (Mortuza et al., 2018).

Table 1. Observed Cmax at PND 15 (Mortuza et al., 2018).
A summary of the data is also reported in the dose concordance Table 11 (see also Appendix C. **Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)**)

Deltamethrin (mg/kg)	Plasma C_{max} at PND15 (ng/mL)	Brain C_{max} at PND15 (ng/g)	Liver C_{max} at PND15 (ng/g)
0.10	21.0	3.1	26.6
0.25	128.2	10.7	56.9
0.50	221.2	16.3	267.3

3 Rationale for performing the IATA and approach used

3.1. Rational for performing the IATA

This IATA case study has been structured to test the hypothesis that the inclusion of mechanistic information in the postulated AOP network would enhance the certainty for the DNT hazard identification and characterisation of deltamethrin. The mechanistic understanding provided by the *in vitro* evidence is therefore expected to contribute to the WoE for DNT toxicity and to assess a biological plausible, mechanistic link for associations found in HOS. The IATA workflow is presented in Figure 2. Therefore, this IATA is intended to address the problem formulation through an evidence-based approach for which a pre-established protocol was developed covering the steps up to the evidence appraisal (Step 1 in Figure 2, see details in Appendix A: Protocol of the IATA). The protocol describes the strategy for the systematic literature review, including the screening for relevance, the data extraction and the critical appraisal of evidence retrieved in the scientific literature from *in vivo*, *in vitro* and HOS studies (Step 2 in Fig.2. details of the studies retrieved, included and excluded are reported in Appendix B2.1 Deltamethrin DNT. Included studies in vitro). Only *in vivo* and *in vitro* studies from the literature ranked with low risk of bias were considered for further analysis whereas all HOS were considered. The AOP conceptual framework was therefore applied to integrate the evidence obtained from the systematic literature review (Step 3 in Figure 2) and identify data gaps. All data obtained from the systematic review were mapped in specific endpoints and endpoint categories and the evidence was classified as MIEs, KEs or AOs based on their categorisation as molecular, cellular, organ, organism or population responses. In the following step the results of testing in the DNT-IVB (Masjosthusmann et al., 2020) were included as mechanistic evidence and were mapped into the AOP endpoints network in addition to the evidence retrieved from the systematic literature review (Step 4 in Figure 2).

The AOP conceptual framework was therefore applied to integrate the evidence obtained from various lines of evidence by means of a systematic literature review, and the experimental outcome from the DNT-IVB to provide a structured contextualisation of the MIEs and KEs leading to the AO. The AO discussed in this IATA case study is specifically referring to the outcome identified in the *in vivo* animal studies while information from HOS will be used in the WoE analysis.

Figure 2. IATA workflow

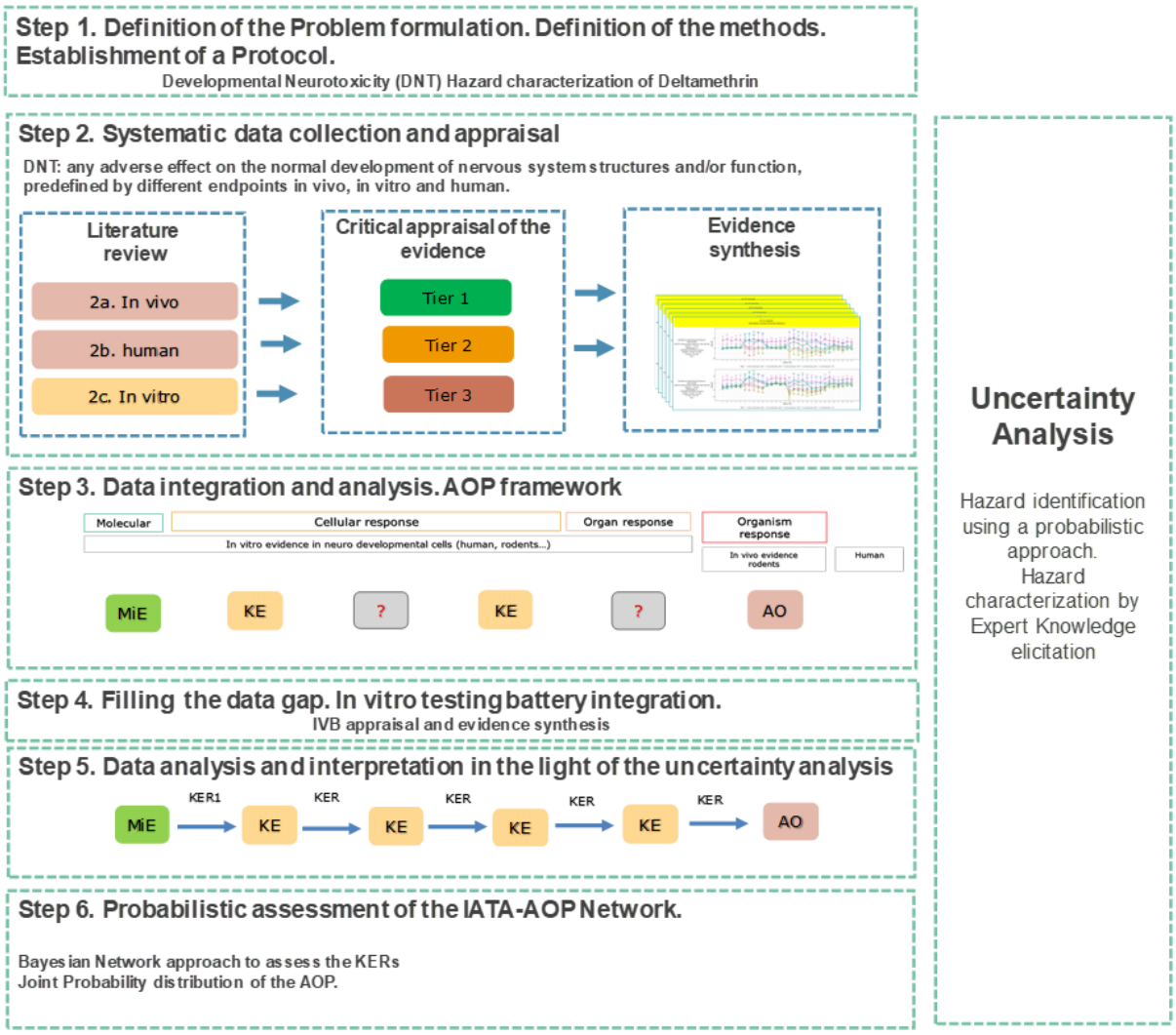
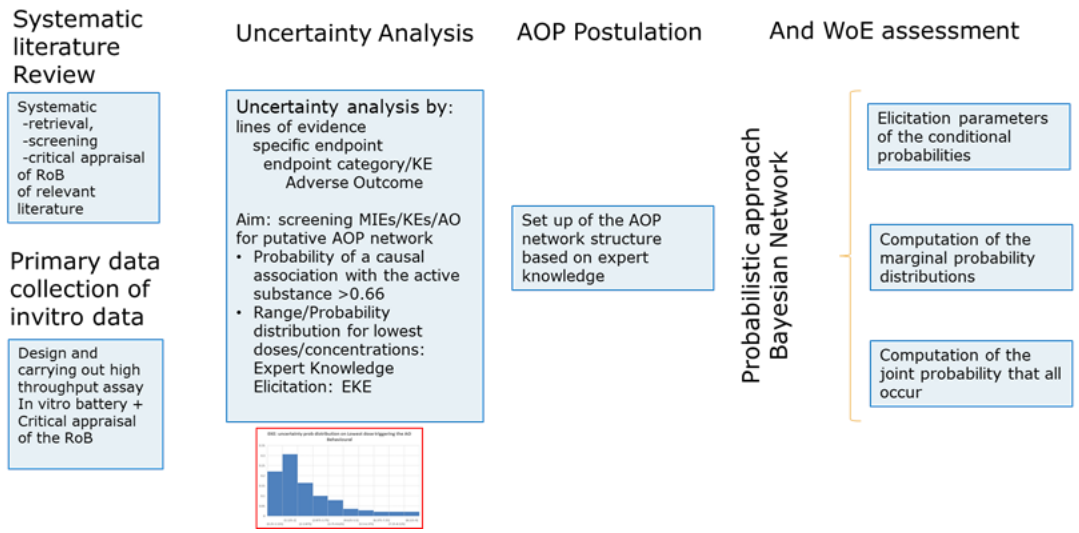


Figure 3. Uncertainty analysis workflow



3.2 The uncertainty analysis and expert knowledge elicitation

The uncertainty analysis and the expert knowledge elicitation (EKE) methodologies are reported in Appendix B1: Statistical analysis report (statistical report). According to EFSA recommendations, uncertainty should be addressed as part of the scientific assessment questions using quantitative approaches whenever possible (EFSA, 2018; EFSA Scientific Committee, 2018).

An uncertainty analysis was therefore performed for each line of evidence and hierarchical level (specific endpoints and categories) to support conclusions on the hazard identification and characterisation questions as it is presented in Figure 3. The final purpose was to screen the evidence and identify molecular initiating events (MIEs), key events (KEs) and adverse outcomes (AOs) to be included in the putative AOP network. The uncertainty analysis was performed in two steps (Steps 1 and 2 to identify and characterize the hazard respectively) for each line of evidence (*in vitro*, *in vivo* and HOS) and the MIEs, KEs and AO to be included in the postulated AOP were selected. .

In Step 1, the uncertainty analysis for the hazard identification was conducted by addressing the questions shown in Table 2**.** The final judgement on the causal association of the endpoint with deltamethrin exposure and its uncertainty was expressed in terms of bounded probability (above/below 0.66 because this means double probability that an adverse effect occurs with respect to it does not occur).

In Step 2 the hazard characterisation and the related uncertainty analysis was conducted by providing answers to the questions shown in Table 3. In the cases for which data were obtained from different studies the expert knowledge elicitation (EKE) approach, as recommended by the corresponding EFSA guidance (EFSA, 2014), was used to estimate the range or the full uncertainty distribution of the lowest concentrations/doses judged to activate the MIEs, KEs and AO of the postulated AOP. First, the working group experts were requested to provide an individual estimate of a range or a full probability distribution. When the uncertainty at individual level was elicited in the form probability distribution, the Roulette method was used to elicit their knowledge (EFSA, 2014). Then a consensus range or probability distribution was achieved based on discussion among the experts (see Appendix B1: Statistical analysis report for further details). Thus, only MIEs, KEs and AOs with an estimated probability of at least 66% were considered for the AOP network postulation (see details in **Appendices**

B and C). An exception was made for the endpoint 'neural crest cell migration' that was positive at high concentrations that could not be aligned in terms of dose concordance. In line with the AOP conceptual framework handbook and AOP development guidance (OECD, 2016, 2017, 2018a), KERs were assessed for their biological plausibility, empirical support and essentiality of the KE (Step 6 in Fig. 2). A probabilistic approach using Bayesian network modelling was used to quantify the strength of KERs by means of: (1) elicitation of parameters of the conditional probabilities, (2) computation of the marginal probability distributions, and (3) computation of the joint probability that all KEs occur. The outcome of the conditional probabilities was used to quantify the strength of the relationship between KEs. The marginal probability distribution was used to predict the most probable status (activation/not activation, occurrence/not occurrence) of the KEs/AO when exposed to deltamethrin. The outcome of the joint probabilities' computation was used to quantify certainty that all events occur concurrently (see Figure 3).

Table 2. Assessment questions for the uncertainty analysis for hazard identification, answered by each working group expert individually, discussed and all working group experts agreed with the overall conclusion (see Appendix B1: Statistical analysis report, Statistical Report for further details)

Line of evidence	Question 1. Hazard identification	Expression of the uncertainty (probability)
***In vitro* experimental studies**	Does exposure to deltamethrin trigger the specific endpoint/KE as measured in acute and developmental protocol (wash-out yes/no) (assuming a monotonic concentration–response relationship) in *in vitro* studies (EFSA DNT-IVB and open literature) carried out in human and/or rat and/or mouse neuro cells in development?	**Bounded probability** No: Prob < 0.66 Yes: Prob ≥0.66
***In vivo* experimental studies**	Does exposure to deltamethrin affect this specific endpoint/endpoint category/adverse outcome in a dose–response relationship in **experimental animal studies** exposed during pregnancy and/or postnatal until weaning (maximum up to 21 days postnatal for rats and mice)?	**Bounded probability** No: Prob < 0.66 Yes: Prob ≥ 0.66
Human observational studies	What is the probability that an association between human individual exposure to deltamethrin in uterus (mothers might have been exposed via dietary and non-dietary sources) and the specific endpoint/adverse outcome occurs?	Approximate probability(*): [0-10) % [10-33) % [33-50) % [50-66) % [66-100] % (*) A round or squared parenthesis indicates that the extreme is excluded or included, respectively

Table 3. Assessment questions for the uncertainty analysis for hazard characterisation, answered by each working group expert individually, and then the probability distribution was integrated, discussed and all working group experts agreed collectively with the overall conclusion (see Appendix B1: Statistical analysis report, Statistical Report for further details)

Line of evidence	Question 2. Hazard characterisation	Expression of the uncertainty (expert knowledge elicitation)
***In vitro* experimental studies**	What is the lowest concentration at which the exposure to deltamethrin triggers the MIE/KE (assuming a monotonic concentration–response relationship)?	Range (uniform distribution) or full probability distribution

***In vivo* experimental studies**	What is the lowest dose at which deltamethrin causes the AO in a dose–response relationship in rodents exposed during pregnancy and/or postnatal until weaning?	(using the Roulette method) for the lowest concentration/dose

Conditional probability distributions. A conditional probability is the probability of each of the possible statuses of a downstream event given each possible status (or combination of statuses) of the connected upstream event(s) (i.e. the conditioning event(s).

Marginal probabilities. A marginal probability distribution describes the probabilities associated to each possible status of a KE/variable (e.g. activated/not activated, occurrence/not occurrence) irrespective of the status of all the others. This probability distribution can be used to infer the most probable status of a KE/AO.

Joint probability: the joint probability distribution of a set/network of KEs describes the probability of each of the possible combinations of the status of the KEs in the network. A natural choice for a joint distribution representing a set of binary variables (i.e. variables assuming only two possible values such as active/not active) is a multinomial distribution. Since the number of combinations dramatically increases when the number of KEs raises, so does the number of distributional parameters. As an illustrative example, for a network with 10 KEs each of which entails only two statuses (active/not active), the number of combinations is 1023. Consequently, the probability attached to each combination is rarely extremely high unless the evidence is supporting it with high certainty.

No documented AOP exists for 'Voltage Gate Sodium Channel Binding during development leading to behavioural effects'. To postulate and develop this AOP network, a stressor-based approach using deltamethrin was therefore implemented (See **Appendices B** and **C**). Although AOPs are agnostic in their nature, this was considered acceptable due to the chemical specific problem formulation and available knowledge for the pyrethroid chemical class (see Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)). The postulated AOP network is described in Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP) and a graphic representation is shown in Figure 5.

The postulated AOP network is the critical element of this IATA case study supporting the chemical assessment and will inform on:

1) the mechanistic data gap that can be fulfilled by the DNT-IVB (KE4 and 5);
2) the relevant residual gaps (including biological knowledge) in the overall pathway;
3) data gaps in the dose concordance analysis;
4) uncertainties on the experimental conditions;
5) the quantitative WoE for guiding the DNT hazard characterisation.

4 Data gathering and application of IATA

4.1. Step 2 Data gathering. Literature searches and risk of bias

4.1.1. Literature searches

The literature searches were conducted in three electronic bibliographic databases (PubMed, Web of Science, TOXNET) and three resources indexing PhD theses (DART, EBSCO and PQDR) in July 2020, and updated on 23 of November 2020 using an information specialist. Search strings are described in the protocol (Appendix A: Protocol of the IATA). Terms for the exposure were combined with relevant terms for DNT outcomes (human and *in vivo* studies) or methods (*in vitro* studies) and a specific search string was designed to identify studies applying high-throughput methods to evaluate potential DNT without terms of exposure. The DNT outcomes were predefined by a series of toxicological *in vivo* and *in vitro* endpoints and measurements in HOS and categorised in endpoint categories translated into keywords for the searches (see Appendix A: Protocol of the IATA Section 2.1.3, see Table 4).

Two independent reviewers screened the literature identified through the searches; 3776 unique references were identified after removing duplicates (see PRISMA Chart, Figure 4). The evidence was clustered as *in vivo* (containing *in vivo* experimental studies), *in vitro* (containing *in vitro* mechanistic studies and behavioural studies in zebrafish up to 120 hours post fertilisation) or human (containing HOS). The title and abstract screening left 291 relevant articles, of primary research studies, that underwent a full-text review, of those, 165 were classified as in vitro, 74 as human evidence and 116 as in vivo. For in vivo, 99 were excluded and 17 publications were included providing relevant data on 58 endpoints. For human, 65 were excluded and eight publications were included providing relevant data on 12 endpoints. For in vitro, 134 were excluded and 31 publications were included on 64 endpoints (see full list of references included and excluded and reasons for exclusion, Appendix B2.1 Deltamethrin DNT. Included studies in vitro).

Table 4. Preliminary list of endpoints laid down during the protocol development

Developmental neurotoxicity predefined endpoints	Endpoints categories	Specific endpoints
In vivo experimental studies	Neuropathology endpoints	Brain weight Quantitative morphometric evaluation (linear measurement, areal measurements, brain morphometric landmarks, brain regions measured, stereology)

		Qualitative neuropathology examination (diagnostic criteria, severity score criteria, standard and special stain used, period) Neuroimaging (quantitative, e.g. MRI)
	Behavioural endpoints	Behavioural ontogeny (functional observation battery e.g. righting reflex, negative geotaxis, motor activity)… Motor activity Auditory startle response Learning and memory: *Letter Mazes M, Y, E (Position, Discrimination: latency to escape, errors, trials to criterion).* *Morris Water Maze (Spatial Learning: latency to escape over trials, path length to locate hidden platform, search parameters during retention probe trials).* *Passive Avoidance (Associative Learning: latency, trials to criterion).* *Biel Water Maze (Sequential learning: latency, errors)* *Cincinnati Maze (Sequential/Egocentric Learning: latency, errors)* *Social behaviour (qualitative or quantitative, tbc test method)* *Elevated maze* Swim test Tactile startle Conditioned freezing Amphetamine challenge MK801 challenge
	Clinical chemistry endpoints	Hormone levels Cholinesterase activity
	Endpoint category 'other'	Ophthalmological evaluation Neurotransmitter levels

		Long-term potentiation
In vitro experimental studies	Proliferation endpoints	
	Apoptosis endpoints	
	Differentiation	Neurogenesis Gliogenesis Oligodendrocyte differentiation Astrocytic differentiation
	Migration endpoints	Neuronal migration Radial migration. Glial migration
	Growth/maturation	Neurite outgrowth Neuronal morphology Synaptogenesis Neuronal cell types
	Network formation/function	Microelectrode array (MEA)
	Cytotoxicity/viability	MTT assay LDH level Neutral red accumulation
	Channels/transporters	Sodium Calcium Chloride Potassium
	Proteins	Synaptophysin SNAP25 Synaptobrevin MAP2
	Receptors/Neurotransmitters	GABA NMDA 5-HT
	Enzymatic activity	Calcineurin Calmodulin Dephosphorylation

	Microglia activation	TNF alpha
	Oxidative stress	ROS production Nrf2 expression/translocation Mitochondrial membrane integrity
	Cell organelles integrity	Nuclear integrity Lysosomal integrity Mitochondrial membrane integrity
	Neurophysiology/patch clamp	Membrane excitability
	Genomic	
	Behavioural endpoints (in zebrafish)	Thigmotaxis Locomotor activity Spasms Swimming activity
	Pathology (in zebrafish)	Cranio-morphological effects Curvature of the body axis Quantitative morphometric examination (body area, head area, head-body angle) Qualitative morphometric evaluation
Human observational studies	Intellectual disability	
	Communication disorders	
	Autism spectrum disorders (ASD)	
	Attention-deficit/hyperactivity disorder (ADHD)	
	Motor disorders	
	Specific learning disorders	

4.1.2. Risk of Bias (RoB)

The risk of bias (RoB) of a given study in relation to a specific outcome (and related endpoints) refers to the risk of systematic errors in the design, conduct or analysis that result in a mistaken estimation of the true effect of the exposure on the outcome.

Critical appraisal tools were laid down upfront and are described in the protocol (Appendix A: Protocol of the IATA). They was applied for each study included in the assessment (including the *in vitro* battery outsourced by EFSA, Masjosthusmann et al., 2020) using a customised version of the OHAT/NTP RoB assessment tool. This tool was selected because provides a parallel approach to the evaluation of RoB across evidence streams (i.e. human, animal and mechanistic studies), based on common terms and categories for RoB rating. Table 5 shows the different questions and domains appraised for *in vivo, in vitro* and human lines of evidence and the three key questions selected for this specific assessment.

RoB in eligible studies on rodents (experimental toxicological studies) and humans (observational studies) was appraised by endpoints using tailored versions of the OHAT-NTP RoB tool (NTP, 2015). For the *in vitro* studies, a non-validated tool developed by OHAT-NTP for a specific project (PFOA and PFOS Monograph (NTP, 2016)) was adapted. Studies were classified as being at low (Tier 1), moderate (Tier 2) or high (Tier 3) RoB for each of the endpoints they measured. RoB tiers were derived weighing the appraisal from the individual RoB domains, some of which were identified as key. The outcome of the RoB appraisal and descriptive forest plots are presented in Appendix B3.1 Outcome of the ROB deltamethrin for *in vivo, in vitro* and human lines of evidence.

RoB was appraised by endpoint in the cases that it was considered that the different endpoints measured in a study used different methodology and therefore had different RoB. RoB for HOS was appraised by answering 7 questions for 11 endpoints, with all endpoints being categorised as Tier 3. RoB for *in vivo* studies was appraised by answering 9 questions for 55 endpoints, with seven endpoints being categorised in Tier 1, five in Tier 2 and 43 in Tier 3. RoB for *in vitro* studies was appraised by answering 10 questions for 64 endpoints, with 31 endpoints being categorised in Tier 1 and 31 in Tier 3 (summary of the studies and results of the RoB are presented in Appendix B3.1 Outcome of the ROB deltamethrin for *in vivo, in vitro* and human).

Figure 4. PRISMA chart systematic literature review result of the screening for relevance

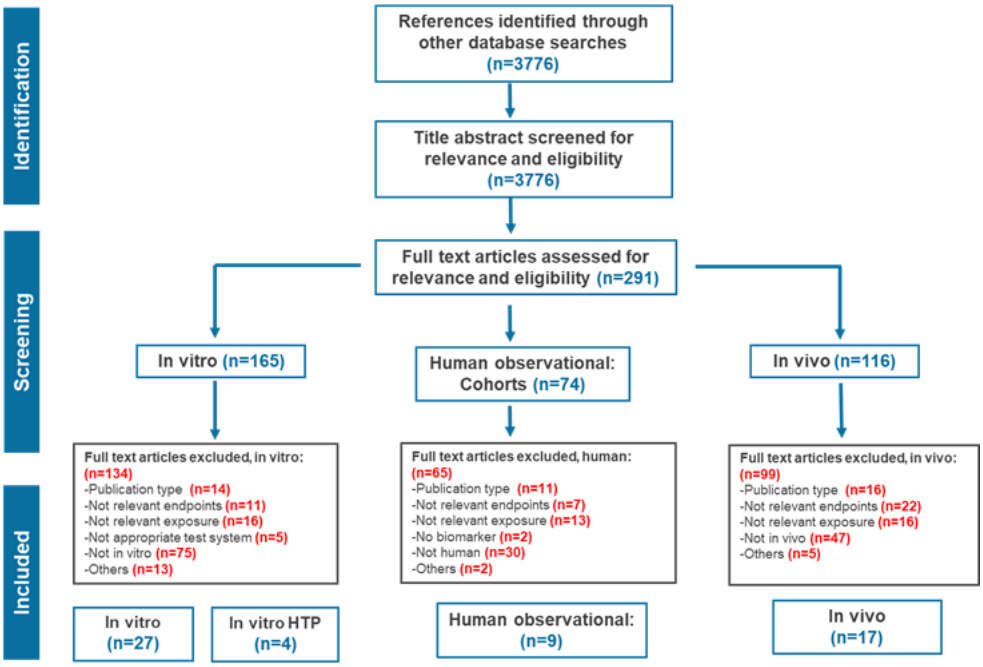


Table 5. Critical appraisal tool questions for the RoB analysis and key questions selected for each line of evidence (see Annex A, Protocol for further details)

Selection Bias	**Human**	**In vivo**	**In vitro**
1. Was administered dose or exposure level adequately randomized?	-	**Key Q**	Yes
2. Was allocation to study groups adequately concealed?	-	Yes	-
3. Did selection of study participants result in appropriate comparison groups	Yes	-	Yes
Confounding Bias			
4. Did the study design or analysis account for important confounding and modifying variables?	**Key Q**	-	-
Performance Bias			
5. Were experimental conditions identical across study groups?	-	Yes	Yes
6. Were the research personnel blinded to the study group during the study?	-	Yes	Yes
Attrition/Exclusion Bias			
7. Were outcome data complete without attrition or exclusion from analysis?	Yes	Yes	Yes
Detection Bias			
8. Can we be confident in the exposure characterisation?	**Key Q**	**Key Q**	**Key Q**
9. Can we be confident in the outcome assessment?	**Key Q**	**Key Q**	**Key Q**
Selective Reporting Bias			
10. Were all measured outcomes reported?	Yes	Yes	Yes
Other Sources of Bias			
11. Were there other potential threats to internal validity?	Statistics	Systemic tox	**Cytoxic**
12. Were there other potential threats to internal validity?			N replic

4.2. Step 3. Systematic review data and uncertainty analysis

The workflow shown in Figure 2 illustrates how the three lines of evidence (*in vitro*, *in vivo*, HOS) were extracted, appraised, weighted and integrated and by this mean assessing whether inclusion of results generated from the IVB would strengthen the conclusion of the hazard identification and characterisation of DNT liability of deltamethrin.

All the identified sources of inconsistencies and uncertainties were listed in tables, which are presented in Appendix B5.1. Uncertainty analysis tables for deltamethrin for *in vivo*, *in vitro* and human lines of evidence and assessed following the uncertainty analysis (UA) Workflow shown in Figure 3.

The Working Group experts (composed by some 7–12 experts) then provided their final judgement on the causal association of the experimental endpoints with deltamethrin and of the statistical association for observational studies, expressed in terms of approximate probability (0–10 %; 10–33%; 33–50%; 50–66%; above 66%). The results of these discussions were summarised in a series of tables presented Appendix B5.1. Uncertainty analysis tables for deltamethrin) and cross-referenced in the summary Table 7**.** Table 3 **and** Table 4 shows the list of the methodologies and publications that were selected for the AOP postulation after this UA process for *in vivo* and *in vitro* evidence respectively.

A threshold of 66% (twice as possible as not) was used as the minimum subjective probability leading to the conclusion of a causal association. Only MIEs, KEs and AOs with an estimated probability of at least 66% in the UA were selected for the AOP postulation based on the robustness of their empirical support (see **Appendices B1 and C**).

Table 6. Summary of Materials and Methods and RoB of the studies providing evidence of MiE, KE and AO of the postulated AOP after the UA for in vivo evidence

Publication	Endpoint categories, specific endpoints and measurements (for the results, see graph report in Appendix B4.1 Graph report In vivo and in vitro deltamethrin)	Study characteristics	Exposure characteristics	RoB tier
RefID 2121. Deltamethrin exposure daily from postnatal day 3–20 in Sprague-Dawley rats causes long-term cognitive and behavioural deficits. Pitzer et al., 2019	**Learning and memory** Freezing behaviour: Cued; Post-conditioned stimulus; Pre-conditioned stimulus. MWM: Cued; Reversal; Shift; Acquisition Latency, path efficiency and speed; CWM: Errors; Latency. Freezing behaviour: Contextual **Startle acoustic and tactile**: Peak amplitude **Neurotransmitters:** Norepinephrine	Sprague-Dawley rats CD IGS strain 001 Charles River Males and Females Non-standard housing conditions Not GLP, no guideline study Public funding source Group size: 45–55 For neurotransmitters 17 (8 males and seven females/group)	From PND 3–20 By gavage in 5 ml of corn oil 0 mg/kg bw day: 55 rats (25 males, 30 females) 0.25 mg/kg bw day: 51 (26 males and 25 females) 0.5 mg/kg bw day: 52 (24 males and 28 females) 1 mg/kg bw day: 45 (20 males and 25 females)	**Tier 2**
RefID 3201. A Developmental Neurotoxicity Screening Study with Technical Grade Deltamethrin in Wistar Rats. OECD 426	**Learning and memory** M-WM: Trials to criterion. Learning phase (Average Errors, duration); Retention phase (Average Errors and duration); Passive Avoidance performance (Latency and trial to criterion) **Startle acoustic and tactile:** Auditory Startle Reflex: Latency to Peak Habituation; Latency to Peak; Peak Amplitude Habituation; Peak Amplitude	Wistar rats HAN CRL: WI – Charles River Males and Females Standard housing condition GLP, OECD 426 compiling Private funding source Group size: 16 rats/sex/dose	From GD 6 to PND 21 by feed to the dams 0 mg/kg bw per day; 1.64 mg/kg bw per day; 6.78 mg/kg bw per day 16.1 mg/kg bw per day.	**Tier 1**
RefID 1116. Prenatal Deltamethrin Exposure-Induced Cognitive Impairment in Offspring Is Ameliorated by Memantine Through NMDAR/BDNF	**Learning and memory** MWM: escape latency; swimming distance; swimming speed; time spent in the target quadrant **Growth factor** BDNF: CA1 region of hippocampus; hippocampus **Proteins** GluN1; GluN2A; GluN2B;	Sprague-Dawley rats (unknown strain) Kunming Laboratory Animal Center Males and Females Standard housing conditions Not GLp, no guideline study Public funding source Group size: 12 (1 M/1F from each litter)	From GD 1 to PND 0 By gavage in corn oil (volume unknown) to the dams. 0 mg/kg bw day 0.54 mg/kg bw day 1.35 mg/kg bw day 2.7 mg/kg bw day 9 mg/kg bw day	**Tier 1**

Signalling in Hippocampus Zhang, C et al., 2018	pCREB/CREB; PTrkB/TrkB			

Table 7. Summary of Materials and Methods and RoB of the studies providing evidence of MiE, KE and AO of the postulated AOP after the UA for in vitro evidence

Publication	Endpoint categories, specific endpoints and measurements (for the results, see graph report in Appendix B4.1 Graph report In vivo and in vitro deltamethrin)	Study characteristics	Exposure characteristics	RoB tier
RefID 2452. Influence of nanomolar deltamethrin on the hallmarks of primary cultured cortical neuronal network and the role of ryanodine receptors. Zheng et al., 2019	Channels/transporters Calcium Influx; Frequency Receptors Ryanodine [3H]Ry binding to RyRs in cortex	Primary mouse cortical cells PND 0–1 RyR homogenate from 3–6 months old Public funding source 3,4 biological replicates	Duration 25 min and 7 Day (Ca) 3h (RyR) Concentration: 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1 µM Chronic Single	Tier 1
	Channels/transporters Ryanodine Mean close time (Tc); Mean open time (T0); Open Probability (P0) Neuronal Network formation (MEA) MBR (mean burst rate) MFR (Mean Firing Rate)	Primary mouse cortical cells PND 0-) Animal medium Public funding source Number of biological replicate: 3	Duration 15 min. Concentration: 0, 0.01, 0.03 µM. Single treatment Microelectrode arrays (MEA) chronic treatment 7 days	Tier 1
RefID 2938. Mechanisms of pyrethroid insecticide-induced stimulation of calcium influx in neocortical neurons. Cao et al., 2011	Channels/transporters Calcium Influx;	Mouse cortical primary cells embryo D16 Animal medium Public funding source Biological replicate: 3	Duration 13 min Concentration: 0, 0.3, 1, 3, 10, 30, 50 µM Single treatment	Tier 1
RefID 3048. Pyrethroid insecticides directly activate microglia through interaction with voltage-gated sodium channels. Hossain et al., 2017	Channels/transporters Sodium Intracellular Influx; Microglia activation TNF alpha	Mouse primary BV2 cells and Primary Microglia cells Postnatal (PND 1–2) Animal medium Public funding source Number of biological replicate: 3	Duration 48 hrs Concentration: 0, 1, 5 µM Treatment frequency: Single	Tier 2
RefID926. Multiparametric characterisation of neuronal network activity for in vitro agrochemical neurotoxicity assessment. Alloisio et al., 2015	Network formation (MEA) % Spikes in Bursts Burst duration MBR (Mean Burst Rate) MISIB (Mean Interspike Interval In Burst) MFR (Mean Firing Rate)	Rat cortical primary cells fetal day 18 Chemically defined medium Public funding source Number of biological replicate: 9	Duration 20 min Concentration: 0, 0.0001, 0.001, 0.01, 0.1, 1 µM, 10 and 100 µM. Single treatment	Tier 1
RefID2842. In vitro evaluation of pyrethroid-	Network formation (MEA) % Spikes in Bursts Burst duration	Rat cortical Primary cells fetal day 18	Duration 30 min Concentration: 0, 0.01, 0.1, 1, 10 and 100 µM.	Tier 1

mediated changes on neuronal burst parameters using microelectrode arrays. Prakhyaet al., 2016	Intervals between bursts MBR (Mean Burst Rate) MISIB (Mean Interspike Interval In Burst)	Chemically defined medium Public funding source Number of biological replicate: 9	Single treatment	
RefID 30344475. Developmental Neurotoxicants Disrupt Activity in Cortical Networks on Microelectrode Arrays: Results of Screening 86 Compounds During Neural Network Formation. Frank, C et al., 2017	Network formation (MEA) % Spikes in Bursts Burst duration Interspike Interval Intervals between bursts MBR (Mean Burst Rate) MISIB (Mean Interspike Interval In Burst) MFR (Mean Firing Rate)	Rat cortical primary cells PN 0–24 hrs Chemically defined medium Public funding source Number of biological replicate: 3	Duration 12 day. Concentration: 0, 0.03, 0.1, 0.3, 1, 3, 10 and 30 µM Chronic treatment	Tier 1
RefID 2959. A multi-laboratory evaluation of microelectrode array-based measurements of neural network activity for acute neurotoxicity testing. Vassallo, A., et al., 2017	Network formation (MEA) Burst duration MFIB (Mean frequency intra burst) MFR (Mean Firing Rate)	Rat cortical primary cells fetal gestation and PND (Rat E18; 0–24 h; mice E14–15) Animal medium and Chemically defined medium = Biological replicate: 3, 4, 7	Duration 10 min Concentration: 0, 0.000001, 0.0001, 0.0003, 0.001 µM; 0.01, 0.1, 0.3, 1, 10 and 100 µM Single treatment	Tier 1
RefID3052. Pyrethroid modulation of spontaneous neuronal excitability and neurotransmission in hippocampal neurons in culture. Meyer, D. A., Carter, J. M., Johnstone, A. F., Shafer, T. J., 2008	Network formation (MEA) Burst duration Interspike Interval MBR (Mean Burst Rate) MFR (Mean Firing Rate) Neurophysiology/patch clamp Membrane excitability: Burst duration); Events/Burst; sEPSC Interevent interval	Rat hippocampal pyramidal neurons PND 2–4 Animal medium Public funding source Number of biological replicate: 4.	Exposure duration 10 min; Concentration: 0 µM; 0.01 µM; 0.1 µM; 1 µM; 10 µM Treatment frequency: Single	Tier 1

Appendix B4.1 Graph report In vivo and in vitro deltamethrin shows the evidence table of all the HOS retrieved and appraised. Table 8 shows a summary of the HOS retrieved. As a result of the RoB and UA, it was decided that the HOS would not be included in the AOP but discussed separately and used as supporting evidence in this IATA due to: (1) all the HOS were appraised as Tier 3 (high RoB), (2) the EKE for a causal association between in utero exposure to deltamethrin and the group of human endpoints measured resulted in a probability of less than 33%; (3) uncertainty in the neurodevelopmental outcomes measured in HOS made the comparison to the AO in the animal study difficult. Details of the UA for HOS are included in Table 9 and Appendix B5.1. Uncertainty analysis tables for deltamethrin.

Table 8. Main features of the nine human observational studies identified in the systematic literature search

Study	Primary exposure	Co-exposure	Confounders	Outcome	Instrument	Children's age
Ref. 129 **Viel et al., 2017** **(n=287)**	3-PBA, 4-F-3-PBA *cis*-DCCA trans-DCCA *cis*-DBCA	OPs (SDAPs) Pb (home dust)	Socio-demographic Lifestyle Environmental factors Clinical information (pregnancy and birth)	Behaviour, other (prosocial behaviour, internalising disorders, externalising disorders)	Strengths and Difficulties Questionnaire (SDQ)	6 years
Ref. 433 **Xue et al., 2013** **(n=497)**	3-PBA *cis*-Cl2CA trans-Cl2CA		Socio-demographic Environmental factors Clinical information (pregnancy and children)	Cognitive impairment	Development Screen Test (DST) scale: – mental development index (MI) – development quotient (DQ)	1 year
Ref. 480 Fluegge et al., (2016) **(n=140)**	3-PBA trans-DCCA	OPs metabolites (TCPy, IMPy)	Socio-demographic Lifestyle Clinical information (pregnancy and infant)	Cognitive impairment (MDI) Impaired psychomotor development (PDI)	Bayley Scales of Infant Development-II (BSID-II): MDI and PDI	3 months
Ref. 876 Dalsager et al., 2019 **(n=948)**	3-PBA trans-DCCA	OPs (TCPy)	Socio-demographic Lifestyle Clinical information (pregnancy and infant)	Attention-deficit/hyperactivity disorder (ADHD)	Child Behaviour Check List for ages 1.5–5 years (CBCL: 1½-5)	1.7–4.1 years
Ref. 1117 Eskenazi et al., 2018 **(n=432)**	3-PBA *cis*-DBCA *cis*-DCCA trans-DCCA	*o,p'*-DDT *p,p'*-DDT *o,p'*-DDE *p,p'*-DDE Pb (children blood)	Socio-demographic Lifestyle Clinical information (pregnancy and children) Occupational and residential history	Cognitive impairment Impaired psychomotor development Communication disorders	Bayley Scales of Infant Development, 3rd edition (BSID-III). Subtests: cognitive, language (receptive and expressive), motor (fine and gross), Social-Emotional	1 year 2 years

Ref. 1118 Furlong et al., 2017 **(n=162)**	3-PBA, trans-DCCA, *cis*-DCCA	OPs metabolites (DEDP, DEP, DETP, DMDP, DMP, DMTP)	Socio-demographic Lifestyle Environmental factors Children data (age, Preterm birth)	Cognitive impairment Behaviour (behavioural regulation index, internalising composite)	Behavioural Assessment System for Children (BASC) Behaviour Rating Inventory of Executive Function (BRIEF)	4–5 years 6 years 7–9 years
Ref. 1152 **Viel et al., 2015** **(n=205)**	3-PBA *cis*-DBCA 4-F-3-PBA, *cis*-DCCA, trans-DCCA	OPs metabolites (six DAPs) Pb (floor dust)	Socio-demographic Lifestyle Environmental factors Exclusion criteria (for mothers and children)	Cognitive impairment	Wechsler Intelligence Scale for Children (WISC-IV). Domains: verbal comprehension, working memory	6 years
Ref. 1432 Watkins et al., 2016 **(n=187)**	3-PBA	Pb (maternal blood)	Socio-demographic Exclusion characteristics for mothers and children	Cognitive impairment Impaired psychomotor development	Bayley Scales for Infant Development—Spanish version (BSID-IIS): MDI, PDI	2 years 3 years
Barkoski et al., 2020 **(n=201)**	3-PBA, trans-DCCA	TCPy	Socio-demographic Maternal features	Autism spectrum disorder (ASD) and non-typical development (non-TD)	Mullen Scales of Early Learning (MSEL) Autism Diagnostic Observation Scale (ADOS)	3 years

Table 9. RoB analysis of the human observational studies using the OHAT/NTP tool (NTP, 2015)

Ref. ID	Q1_Select	Q2_Confnd	Q3_Attr	Q4_Expos	Q5_Outcom	Q6_SelectvRep	Q7_Stats	TIER
129	DLRoB	PHRoB	DLRoB	PHRoB	PLRoB	DLRoB	PLRoB	3*
433	DLRoB	DHRoB	DLRoB	DHRoB	DLRoB	PLRoB	NR	3
433	DLRoB	DHRoB	DLRoB	DHRoB	DLRoB	PLRoB	PHRoB	3
480	DLRoB	DHRoB	PLRoB	DHRoB	PLRoB	PLRoB	DLRoB	3
480	DLRoB	DHRoB	PLRoB	DHRoB	PLRoB	PLRoB	DLRoB	3
876	DLRoB	PLRoB	PLRoB	DHRoB	PLRoB	PLRoB	DLRoB	3
1117	PLRoB	PLRoB	DLRoB	PHRoB	DLRoB	PLRoB	DLRoB	3
1118	DLRoB	PLRoB	PHRoB	DHRoB	PLRoB	PLRoB	PLRoB	3
1152	DLRoB	DLRoB	PHRoB	PHRoB	DLRoB	DLRoB	PLRoB	3
1432	DLRoB	PLRoB	PLRoB	DHRoB	PLRoB	PLRoB	PLRoB	3
1432	DLRoB	PLRoB	PLRoB	DHRoB	PLRoB	PLRoB	PLRoB	3
3034541	DLRoB	PLRoB	PLRoB	PHRoB	DLRoB	DLRoB	DLRoB	3

DLRoB: Definitively low RoB; PLRoB: Probably Low RoB; PHRoB: Probably high RoB; DH: Definitively

high RoB. *indicates that studies classified as DHRoB or PHRoB for critical questions will be considered as Tier 3 (for further details see Appendix A: Protocol of the IATA)

4.3 Step 4. Filling the data gap. *In vitro* testing battery integration. IVB appraisal and evidence synthesis

In vitro methods used for gathering data follow the OECD GD (guidance document) 211 for describing non-guideline *in vitro* test methods. However, OECD GD 211 pays little attention to the description of the test system (i.e. cell culture) and the steps leading to it being established. Therefore, OECD GD 211 was modified to adapt it to the common needs of test developers and regulators by developing an annotated toxicity test method template (ToxTemp; Krebs et al., 2019). ToxTemp for the individual test methods contributing to this IATA case study can be found in Masjosthusmann et al. (2020), Appendices C–G.

For the IVB studies, the adapted RoB Critical appraisal tool used for the *in vitro* tests was used and laid down upfront as described in the protocol (Appendix A: Protocol of the IATA). Studies were classified as being of low (Tier 1), moderate (Tier 2) or high (Tier 3) RoB for each of the endpoints measured. RoB tiers were derived weighing the appraisal of individual RoB domains some of which were identified as key. The outcome of the RoB appraisal and descriptive forest plots are presented in Appendix B3.1 Outcome of the ROB deltamethrin for *in vivo, in vitro* and human lines of evidence.

4.3.1. Test systems and test methods of DNT-IVB

Briefly, test methods are based on test systems geared to assess key neurodevelopmental processes that are needed for brain development and occur in a concerted, spatiotemporal fashion *in vivo*. Seven test systems, i.e. proliferating primary fetal human neural progenitor cells (hNPC; test method NPC1), differentiating hNPC (test method NPC2–5), hiPSC-derived neural crest cells (NCC, test method UKN2), Lund human mesencephalic (LUHMES) cells (UKN4), hiPSC-derived peripheral neurons (UKN5), primary rat cortical mixed cultures (rat Neuronal Network Function-rNNF) and the NeuCyte SynFire kit consisting of human induced pluripotent stem cells (hiPSC)-derived excitatory and inhibitory neurons and human primary astrocytes (human Neuronal Network Function-hNNF) build the cellular bases of the *in vitro* test methods mimicking certain neurodevelopmental processes that occur *in vivo* over time. Therefore, the assays measures compounds' effects on the developmental change of a key neurodevelopmental process over time. Such processes (and corresponding test methods) are neural progenitor cell proliferation (NPC1); migration of a) NCC (UKN2), b) radial glia (NPC2a), c) neurons (NPC2b), d) oligodendrocytes (NPC2c); neuronal differentiation (NPC3); neurite outgrowth/morphology of a) dopaminergic (UKN4), b) peripheral (UKN5) and c) young cortical (NPC4) neurons; oligodendrocyte differentiation (NPC5) as well as rat and human neuronal network formation (rNNF and hNNF, respectively). Endpoint evaluations of these DNT test methods were accompanied by viability and/or cytotoxicity analyses leading to the generation of 23 individual DNT-specific (neurodevelopmental process) and unspecific (viability/cytotoxicity) assessments. At the time, except for the hNNF assay, these test methods had high enough readiness to be used for a screening approach (Bal-Price et al., 2018).

Details of the test methods and test systems used to assess deltamethrin in the DNT-IVB and the results can be found in the External Scientific Report (Majosthusmann et al., 2020). The IVB was appraised with the Critical Appraisal Tool used for the *in vitro* evidence and classified as Tier 1. The outcome of the RoB appraisal is presented in Appendix B3.3 Outcome of the ROB IVB (In vitro battery).

In the following paragraphs, a summary of the test methods are laid out:

1) The test method NPC1 assesses NPC proliferation in a 3D neurosphere format. Therefore, individual neurospheres of defined sizes are grown in suspension culture in single wells of a 96-well plate. Spheres are treated with respective compounds for 72 hours. For the last 16 hours of proliferation, BrdU is added to the cultures and BrdU incorporation into the DNA is measured by BrdU antibody binding using luminescence. At the same time mitochondrial reductase as a possible marker of cell viability is measured by the Alamar blue assay and lactate dehydrogenase (LDH) release into the medium, an indicator for cytotoxicity, is quantified.
2) The UKN2 test method measures NCC migration into an area devoid of cells. Therefore, hiPSC-derived NCC are seeded into 96-well plates containing a stopper in the centre of each well. After stopper removal, cells are allowed for 24 hours to migrate into the cell-free area. Area covered due to cell migration is quantified by high content image analysis (HCA). In parallel, cell viability is assessed by measuring calcein-AM staining by HCA.
3) The NPC2–5 test methods run in a multiplexed fashion. Here, migrated cells are stained for Hoechst (nuclei), TUBB3 (neurons) and O4 (oligodendrocytes) simultaneously. For all NPC2–5 methods, LDH is measured after 72 and 120 hours and the Alamar blue assay is performed after 120 hours. The NPC2 migration assays consist of the three test methods, NPC2a, b and c. Each method assesses migration of an individual cell type in the neurosphere migration area. The predominant cell type determining total migration distance of the neurospheres is radial glia cells. Therefore, NPC2a measures total migration distance by phase contrast images after 72 hours and by high content imaging (HCI) of Hoechst stained nuclei after 120 hours. Simultaneously, migration distances of stained neurons (see 4., NPC3) and oligodendrocytes (see 6., NPC5) are assessed within NPC2b and c, respectively. Here, the mean distance of all neurons (NPC2b) or oligodendrocytes (NPC2c) from the edge of the sphere core to the position of each neuron is measured and plotted as a ratio of radial glia migration (NPC2a). This approach allows the evaluation of the cell type-specificity of a migratory effect.
4) Neuronal differentiation is quantified within the NPC3 test method. The number of TUBB3-positive cells is determined by immunocytochemical staining, HCA and annotation by a convolutional neuronal network (CNN) running on Keras implemented in Python 3. Neuronal differentiation is given as the percentage of TUBB3-positive cells of all Hoechst-positive nuclei in the migration area after 120 h of differentiation.
5) Young, central neurons identified with the CNN in test method NPC3 now undergo morphological evaluation with the NPC4 test method. Therefore, neurons are skeletonised with a self-written algorithm (Schmuck et al., 2017) and neurite length (µm) and neurite area (pixel) are determined for each individual neuron.
6) Oligodendrocyte differentiation is assessed with the NPC5 assay. The number of O4-positive cells is determined by immunocytochemical staining, HCA and annotation by a CNN running on Keras implemented in Python 3. Oligodendrocyte differentiation is given as the percentage of O4-positive cells of all Hoechst-positive nuclei in the migration area after 120 h of differentiation.
7) Neurite area of central, dopaminergic neurons is measured in LUHMES cells by the UKN4 test method 24 hours after start of compound treatment. Cells are stained with calcein-AM and H-33342 and HCA is performed. An automated algorithm is used to identify neurite area and counting double-positive cells for calcein-AM and H-33342 assesses viability.
8) Neurite area of peripheral, hiPSC-derived neurons is measured by the UKN5 test method 24 hours after start of compound treatment. Cells are stained with calcein-AM and H-33342 and HCA is performed. An automated algorithm is used to identify neurite area and counting double-positive cells for calcein-AM and H-33342 assesses viability.
9) The rNNF assay measures network formation based on primary rat cortical cells by exposing the culture to compounds 2h after plating on microelectrode arrays (MEAs). Cells are treated again with compounds at day 5 and 9, whereas recordings are collected on day 2, 5, 7, 9 and

12. After the last measurement at day 12, viability is assessed. Data are analysed from the area under the curve across the measurements at different points.

10) Network formation and function based on human pre-differentiated hiPSC-based excitatory and inhibitory neurons as well as primary astrocytes (SynFire Kit, NeuCyte), is assessed with the hNNF assay. The cells are plated on MEAs and exposed to compounds after 7 days of differentiation. Recordings are performed every week on days 7, 14, 21, 28 and 35. To minimise possible acute effects of compounds in the measurements, in contrast with the rNNF assay, a washout is performed 1 day before each recording day. Additionally, cytotoxicity is assessed after 3 days of compound exposure on days 10, 17, 24 and 35. Data are analysed from the area under the curve across the measurements at different points.

For the storage and management of all testing data a database was set up at the Ruhr University Bochum, Germany. It operates on Post-greSQL and is designed to store raw and meta data for subsequent data processing. To enable assay-specific data analysis, a data evaluation tool was developed in R. It uses the data.table package for data management, as well as the drc and bmd package for curve fitting and plotting. Briefly, concentration-response data were normalised to the median solvent control and re-normalised to the starting point of the curve, the R software (drc package) was used to calculate the optimal curve fit for each experiment by running several non-linear models and using the Akaike´s information criteria to assess its performance, and the benchmark concentrations (BMCs) according to individual assays' predefined benchmark responses (BMRs), as well as upper and lower confidence limits were calculated by parametric bootstrapping in combination with median resampling (200-times). BMCs were used for assay-specific decision trees for compound classification into 'DNT-specific hit', 'unspecific hit', 'borderline hit' or 'no hit'. A 'DNT-specific hit' is defined by an alteration of a DNT-specific endpoint that can be distinguished from general cytotoxicity, while an 'unspecific hit' does not allow this distinction and therefore the change in DNT endpoint is due to general cytotoxicity. A 'borderline hit' cannot clearly be classified in either a 'DNT-specific' or 'unspecific hit' and 'no hit' does not produce a BMC value (for more details on classification see Masjosthusmann et al., 2020).

4.3.2. Results summary

A summary of the results of the DNT IVB for deltamethrin is given in Table 10 and the Benchmark Concentrations (BMCs) resulting in DNT-specific or unspecific hits for deltamethrin is given in Table 8. Deltamethrin caused a DNT-specific and concentration-dependent decrease in rat and human neuronal network formation (r/hNNF), oligodendrocyte differentiation (NPC5) and NCC migration (UKN2) with rNNF and NPC5 as the most sensitive endpoints (BMC50 0.5 µM and BMC30 0.6 µM, respectively). All other endpoints not listed in the table did not result in BMCs and were therefore no hits. Concentration-response curves underlying these BMCs including the concentration–response curves in Section 11.2 of Masjosthusmann et al. (2020).

In addition, the table also includes data that was not generated as part of the EFSA report (Majosthusmann et al., 2020):

- Benchmark concentration (BMC) values for neurite maturation and synaptogenesis (rat cortical neurons 2) were derived from Harrill et al. (2018).
- Data for deltamethrin assessed in the rat neuronal network formation (rNNF) assay was taken from Frank et al. (2017) and flufenacet data from Shafer et al. (2019).
- hNNF values originate from unpublished data generated at IUF.

Table 10. Summary of BMC across the positive assays of the DNT testing battery.
Specific hits according to respective BMC are marked in red. Numbers are given in µM

	Deltamethrin
BMC_{25} migration (UKN2)	**18.4**^{s}
BMC_{25} neurite area (UKN5)	112.8^{ns}
BMC_{10} migration radial cell (NPC2)	16.3^{ns}
BMC_{30} neurite length (NPC4)	14.9^{ns}
BMC_{30} neurite area (NPC4)	15.9^{ns}
BMC_{30} oligodendrocyte differentiation (NPC5)	**0.6**^{s}
BMC_{50} rat neuronal network formation (rNNF)	**0.5**^{s}
BMC_{50} human neuronal network formation (hNNF)	**4.1**^{s}
BMC_{30} neurite maturation (rat cortical neurons 2)	9.8^{ns}
BMC_{30} synaptogenesis (rat cortical neurons 2)	8.6^{ns}

s = specific hits (the BMC for the effect is separated from the BMC for cytotoxicity/cell viability).
ns = non-specific hits (the BMC for the effect is not separated from the BMC for cytotoxicity/cell viability).
Specific hits according to respective compound are marked in bold.
Units for the numerical values given in the table correspond to µM.
*No Benchmark Response (BMR) at concentration higher than 100 µM in the absence of cytotoxicity. The BMR is a value of effect size and is defined as an effect size that is higher than the general variability of the measured endpoint and is therefore determined based on the variability of the respective endpoint. For UKN 2 and 5 the BMR is set at 20% (BMR20). If the parameter is not affected at the BMR20 the compound is classified as a 'no hit'.
**No Benchmark Response (BMR) at concentration higher than 20 µM in the absence of cytotoxicity. For NPC 2, 4 and 5 the BMR is defined as at least 1.5× the standard deviation (SD) (between experiment variation) as BMR10 for migration distance radial glia 72 h and 120 h, cytotoxicity 72 h and 120 h and BMR30 for all other endpoints.

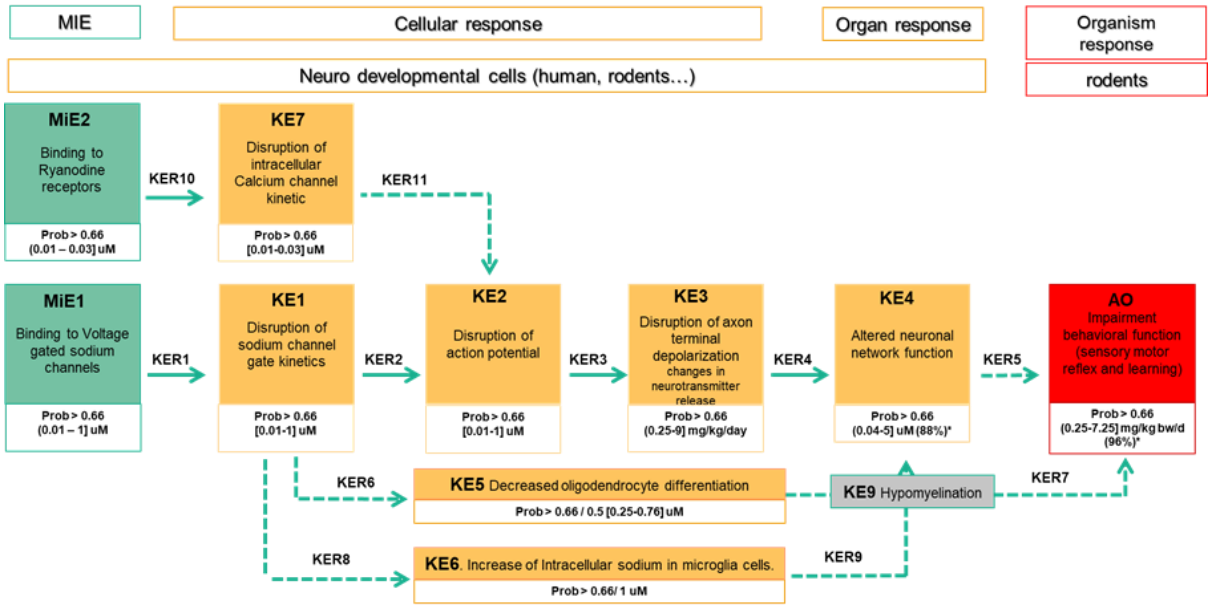
4.4. Step 5. Data analysis and interpretation, AOP postulation

The outcome of the systematic review, complemented by the DNT-IVB (Masjosthusmann et al., 2020) culminated in the postulation of an AOP network which is summarised in Figure 5 and Table 7, which included 2 MiEs, 8 KEs and 1 AO (See Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP) with all the details). However, KE8 was not considered in the probabilistic calculation and in the AOP postulation.

In the subsequent step of the UA, EKE was used to estimate the range of the deltamethrin lowest concentrations/doses judged to activate the MIEs, KEs and AO. Table 11 shows a summary of the MIEs, KEs and AO kept for the AOP postulated and its UA, including the EKE results. Further details are reported in Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP). The outcome of the EKE is reported in the Appendix B1: Statistical analysis report for KE/AO requiring specification of a full probability distribution. The graphic description includes adjacent (solid line) and not adjacent (dash line) KERs. For adjacent KERs the level of certainty is considered higher than for the non-adjacent KERs. KERs were assessed for their biological plausibility and empirical support and essentiality of the KE following a Bayesian network approach by assessing the conditional probabilities (see also 5.5, Step 6, see Appendix B1: Statistical analysis report). Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP) contains the technical notes of the KERs assessment using the Bayesian network and the Table 12 present the assays available for

the measurement of the MIEs, KEs and AO in the network. Table 13 shows the result of the elicited probability during the Bayesian Network analysis.

Figure 5. Postulated AOP network, graphic description



*Results of the expert knowledge elicitation, EKE.
Dashed lines indicate non-adjacent KEs for which biological plausibility and/or empirical support is less certain. A round or squared parenthesis indicates that the extreme is excluded or included, respectively (see also Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP))

Summary of the KE description and dose and temporal concordance in the light of its uncertainty.

IATA OECD IATA Case Studies Project			
		Gather existing and generated information (for a review and detailed information see Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP))	Weight of evidence
Step 3. Data integration into an AOP framework		Step 2. Systematic literature review and appraisal of existing information Step 4. Filling the data gaps of the AOP with the generated information in the in vitro battery	Step 5. Data and uncertainty analysis. Dose/concentration at which deltamethrin is disrupting this KE (UA and EKE)
MIE1	Binding to Voltage Gate Sodium Channels (VGSCs)	In mammals, VGSCs are expressed in the adult and developing brain. Although best studied in neurons, VGSCs are also expressed in developing oligodendrocytes and microglia cells. Sodium channels consist of a highly processed α subunit, which is approximately 260 kDa, associated with auxiliary β subunits of 33–39 kDa. Nine different sodium channels have been identified using electrophysiological recording, biochemical purification and cloning. VGSCs show complex regional and temporal ontogeny in mammals (see Table C.1, MiE in Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)). In general, embryonically expressed forms of VGSCs are replaced by expression of adult forms as neurodevelopment proceeds. This complex ontogeny of VGSCs confounds any simple linkage of VGSCs to adverse outcomes and represents an uncertainty in the development of this AOP. This MiE1 in this AOP specifically refers to chemically induced disruption of VGSCs during brain development. There is currently extensive documentation and broad acceptance of the mechanistic understanding of the function of VGSCs and their interaction with type II pyrethroids such as deltamethrin. Concentration-dependent changes in VGSC kinetics of primary cultures from rat developing brain cortex after exposure to deltamethrin have been retrieved in the systematic literature review and its endpoints appraised as Tier 1 (Meyer et al., 2008).	Binding to VGSC was assessed in the UA as being affected by deltamethrin with a probability higher of 66%. Immediately observed when measured in vitro up to 9 minutes recording (Meyer et al., 2008). Range of Concentrations agreed by EKE: [0.01–1] µM (see UA table, Appendix B5.1. Uncertainty analysis tables for deltamethrin)
MIE2	Binding to ryanodine receptors	Ryanodine receptors (RyR) are the largest known ion channels (homotetramers with a total molecular mass of about 2 MDa). RyR1, RyR2 and RyR3 are intracellular calcium channels, which are classified as 'skeletal muscle', 'heart' and 'brain' types, respectively, although RyR 1 and 2 are also found in brain. RyR are transmembrane channels residing in the endoplasmatic reticulum membranes responsible for the release of Ca^{2+} from intracellular stores therefore regulating intracellular calcium concentrations in the adult and developing brain. Depending on the neuronal localisation, RyR-dependent Ca^{2+}-signalling mediates synaptic plasticity, neurotransmitter release, growth cone activity, kinase and phosphatase activities or gene expression. Sensitisation of RyR in the developing brain alters synaptic connectivity leading to neurobehavioral perturbations. This MIE2 in this AOP specifically refers to chemically induced disruption of RyRs during brain development. Empirical evidence using [3H] ryanodine has shown that deltamethrin at µM concentrations binds to RyR in rat developmental brain from 3 hours to 7 days *in vitro*. This evidence has been retrieved in the systematic literature review and appraised as Tier 1 endpoints.	Binding to RyR has been assessed in the UA as being affected by deltamethrin with a probability higher of 66%. Deltamethrin at concentrations above 3 µM increased the maximum increment of 239.5% of control ryanodine binding in brain homogenate. Deltamethrin at 30 nM altered gating kinetics of RyR1 channels, increasing mean open time, decreasing mean closed time, and therefore enhancing overall open probability (Zheng et al., 2019; see

			UA table, Appendix B5.1. Uncertainty analysis tables for deltamethrin)
KE1	Disruption of VGSC gate kinetics	VGSCs exist in one of three states: Deactivated (closed): at rest, the m gate is closed and does not let sodium ions go through. Activated (open): when a current passes through and changes the voltage difference across a membrane, the channel will activate and the m gate will open. Inactivated (closed) – as the neuron depolarises, the h gate swings shut and blocks sodium ions from entering the cell. Slowed VGSC activation leads to a decrease in peak Na^+ current. By slowing VGSC inactivation and deactivation leads to a prolonged VGSC open time. The longer channel open time results in more Na^+ entering the cell and this leads to hyperexcitability, membrane depolarisation, increase in firing rate and conduction block. Prolongation of the channel opening time for a short period cause repetitive firing of action potential (repetitive discharge). However, if the channel is hold opened for a sufficient long period, the membrane potential eventually becomes depolarised to the point at which generation of action potentials is not possible (depolarisation dependent block). Modification of a small percentage of VGSCs can increase Na^+ current substantially. Evidence retrieved, appraised as Tier 1 in the RoB, indicated concentration-dependent changes in VGSC kinetics of primary cultures from rat developing brain cortex followed by an effect on the neuronal electrical activity when using patch clamp for measuring it (Meyer et al., 2008).	Disruption of sodium channel gate kinetics was assessed in the UA as being affected by deltamethrin with a probability higher than 66%. It was immediately observed when measured *in vitro* up to 9 minutes recording. Range of Concentrations agreed by EKE: [0.01–1] μM (see UA table, **Appendix B5.1. Uncertainty analysis tables for deltamethrin**)
KE2	Disruption of action potential	An action potential is defined as a sudden, fast, transitory and propagating change of the resting membrane potential. It is a cycle of membrane depolarisation, hyperpolarisation and return to the resting value that only neurons and muscle cells are capable of generating. It causes positively charged ions to flow into the cell body, these ions pass through channels that open when a specific neurotransmitter binds to its receptor, leading to channel opening. To measure action potential, the patch clamp or the intracellular recording (impale a sharp electrode into the cell cytosol) technique are generally used. Na^+ enters the postsynaptic cell and causes the postsynaptic membrane to depolarise. This depolarisation is called an excitatory postsynaptic potential (EPSP) and makes the postsynaptic neuron more likely to fire an action potential. Cao et al. (2011) demonstrated that voltage-gated calcium channels (VGCCs) responses of a neuronal network to pyrethroids with an increase of intracellular calcium concentration and these responses are secondary to activation of VGSCs. Evidence retrieved from the systematic review and appraised as Tier 1 showed changes in neuronal excitability in glutamatergic networks following treatment to deltamethrin and permethrin on neuronal activity in hippocampal neuronal cultures using patch clamp and microelectrode array (MEA) recordings (Meyer et al., 2008).	Disruption of action potential was assessed in the UA as being affected by deltamethrin with a probability higher than 66%. It was immediately observed when measured in vitro up to 9 minutes recording. Range of concentrations agreed by EKE: [0.01–1] μM (see UA table, **Appendix B5.1. Uncertainty analysis tables for deltamethrin**)
KE3	Disruption of axon terminal depolarisation; changes in neurotransmitter release	Binding of neurotransmitters to their receptors triggers the following chain of events: (1) generation of small depolarising potentials that opens VGSC (2), sodium ions enter into the cell and when depolarisation reaches a certain threshold an action potentials is produced and propagated through the axon until reaching the presynaptic terminal where open calcium channels sensitive to voltage changes (3) that triggers release of neurotransmitters into the synaptic cleft (4). The neurotransmitter diffuses across the synaptic cleft and binds to receptor proteins on	Disruption of axon terminal depolarisation; changes in neurotransmitter release was assessed as being affected by deltamethrin with a probability higher than 66%.

		the postsynaptic membrane (see Table C.7 in Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)). Evidence retrieved from the systematic review showed the effect of pyrethroids on neurotransmitter release *in vivo* and *in vitro* following treatment to deltamethrin. The distinct abilities of pyrethroids to elevate brain-derived neurotrophic factor (BDNF) mRNA expression are consistent with the demonstration of a range of pyrethroid efficacies in the stimulation of calcium influx. *In vivo*, deltamethrin has been reported to increase BDNF in the cortex and hippocampus (Imamura et al., 2006; Cao et al., 2011), and both deltamethrin and permethrin alter transcription profiles of activity-dependent genes in the cortex including c-fos, Egr1, and Camk1g (Harrill et al., 2008; Cao et al., 2011). Neurotransmitter release was also evaluated *in vivo* using western blotting quantification (1) after treatment with deltamethrin from PND 2 up to PND 28 (effect measured on PND 130), showing a decrease in norepinephrine (Pitzer et al., 2019) and (2) after treatment of the dams until delivery with measurement in pups on 21 PND showing decrease in BDNF (CA1/hippocampus), decrease of phosphorylation of proteins (pCREB/CREB PTrkB/TrkB) and decrease of GluN1 GluN2A and GluN2B (Zhang et al., 2018).	Doses/concentrations agreed by EKE: Decrease norepinephrine from 0.5 mg/kg/day (*in vivo*; Pitzer et al., 2019) Decrease in BDNF (CA1/hippocampus) and decrease of phosphorylation of proteins (pCREB/CREB PTrkB/TrkB) from (2.7 to 9 mg/kg bw) (*In vivo*; Zhang et al., 2018): Decrease of GluN1 GluN2A and GluN2B *In vitro* [0.01–1] µM (Meyer et al., 2008; see UA table, **Appendix B5.1. Uncertainty analysis tables for deltamethrin**)
KE4	Altered neuronal network formation	To encode information, neurons are organised in a complex network of synapses connecting excitable cells (i.e. neurons and certain glia cells). The neural network system encodes information in two principal ways: by establishing an electrical code within neurons and, second, in establishing a wiring code based on neurotransmitters. Plasticity in these signalling modes is responsible for learning, memory, development, and behavioural complexity (Liebeskind et al., 2017) The developing brain differs from the mature brain in neuronal network formation (NNF). It shows a slow maturation and a transient passage from spontaneous, long-duration action potentials to synaptically triggered, short-duration action potentials and is characterised by hyperexcitability, which is related to the increased number of local circuit recurrent excitatory synapses and the lack of γ-amino-butyric acid A receptor ($GABA_A$)-mediated inhibitory function that appears much later. Glutamatergic neurotransmission is dominant at early stages of development and NMDA receptor-mediated synaptic currents are far more times longer during development than those in maturation, allowing more calcium to enter the neurons. Neural network formation and function happens in all brain regions, but it appears to onset at different time points of development (reviewed in Erecinska et al., 2004). Evidence that deltamethrin affects neuronal network formation was retrieved from the systematic literature review and from the IVB. Deltamethrin caused altered NNF of primary rat cortical as well as hiPSC-derived human neurons recorded by MEA *in vitro*. Effective concentrations ranged o from 0.45 to 11.2 µM deltamethrin with the 97% of probability distribution in the EKE at 0.04–5 mM, depending on the test system and test method applied (Alloisio et al., 2015; Krishnan and Prakhya, 2016; Vassallo et al., 2017; Frank et al., 2017; Shafer et al., 2019; Masjosthusmann et al., 2020).	Altered neuronal network formation was assessed as being affected by deltamethrin with a probability higher than 66% after acute (after 21 to 35 days *in vitro* from few minutes to 35 minutes) and chronic exposure (up to 28 DIV on 12 to 35 DIV) Concentrations EKE: 0.04–5 µM (96%) 0.04 µm, corresponding to around 20 ng of deltamethrin per gram of brain. See UA Table BB (**Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)**)

KE5	Decreased oligodendrocyte (OL) differentiation	OLs are responsible for axon myelination facilitating rapid saltatory conduction of action potentials within the central nervous system. They are generated from OL progenitor cells (OPCs) following highly regulated, sequential steps of proliferation, migration and differentiation. OLs are generated by neuroepithelial cells of the ventricular zones and by radial glial cells that, under the influence of transcription factors such as Olig1, Olig2, Nkx2.2, and Sox10, give rise to committed OPCs (see Figure C. 13, Figure C. 14 and Figure C. 15 in Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)). OPCs can then terminally differentiate into postmitotic, pre-myelinating oligodendrocytes (pre-OLs) which will mature and myelinate nearby receptive axons (Emery, 2010). No evidence was retrieved from the literature for this KE and the generation and inclusion of the additional information from the IVB was needed to include this evidence in the AOP Network.	Decreased OL differentiation was assessed as being affected by deltamethrin with a probability higher than 66%. Concentrations from IVB: 0.6 µM (BMC_{30}) 0.45 µm, corresponding to 217 ng of deltamethrin for gram of brain (see below) (see UA table, **Appendix B5.1. Uncertainty analysis tables for deltamethrin**)
KE6	Increase in intracellular sodium in microglia	Microglia as resident macrophages are the immune cells of the CNS. It is highly likely that similar to rodents human microglia derive from primitive yolk sac macrophages. In humans, they colonise the cerebrum between the 4th and 24th gestational weeks anteceding neurogenesis, neuronal migration, and many other neurodevelopmental processes (Menassa and Gomez-Nicola, 2018). Microglia exert a plethora of functions including innate immunity and neuroprotection, synaptic pruning and phagocytosis of cellular debris. They are also suggested to be indirectly involved in pathologies such as schizophrenia or neurodegeneration due to their neuroinflammatory responses, i.e. the release of pro-inflammatory cytokines. Evidence has been retrieved in the systematic review and appraised in Tier 2 that pyrethroids (e.g. deltamethrin and permethrin) significantly reduced the activity of microglia (e.g. phagocytosis) and attenuated the release of pro-inflammatory cytokines. An increase of around 50% in sodium influx in microglia cell was observed from 1 µM deltamethrin and of around 30% of intracellular Na accumulation (Hossain et al., 2017).	Increase in intracellular sodium concentration in microglia was assessed as being affected by deltamethrin with a probability higher than 66%. Concentrations elicited: 1 µM (see UA table, **Appendix B5.1. Uncertainty analysis tables for deltamethrin**)
KE7	Disruption of intracellular Ca $[Ca^{2+}]i$ channel kinetics	Calcium regulation is a critical process in neurons, which have developed extensive and intricate calcium signalling pathways. Cytosolic $[Ca^{2+}]i$ rises are the result of an influx across the plasma membrane by voltage-gated Ca^{2+} channels (VGCCs), ionotropic glutamate receptors (N-methyl-d-aspartic acid receptors, NMDARs; and alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors, AMPARs), and the release from the endoplasmic reticulum (ER) through the inositol 1,4,5-trisphosphate (IP3R) and the ryanodine (RyR) receptors. The ER is a calcium store within the cell with a typical intraluminal calcium concentration of about 0.1–0.5 mM (cytosolic calcium concentration around 100 nM, extracellular concentration approx. 2 mM). An increase in cytoplasmatic calcium concentration induces opening of RyRs which will in a positive feedback release more calcium from the ER. As cytosolic $[Ca^{2+}]i$ rises, RyR are inactivated preventing a cytosolic calcium overload. Altered activities of RyRs channel will therefore influence i.e. calcium dynamics, patterns of synchronous calcium oscillations (SCOs), and electrical spike activity (ESA) functions.	Disruption of intracellular Ca channel kinetics was assessed as being affected by deltamethrin with a probability higher than 66%. Concentration agreed by EKE: 37 nM (see UA table, **Appendix B5.1. Uncertainty analysis tables for deltamethrin**

AO	Impairment behavioural function (sensory motor reflex and associative learning)	The AO is defined as a detrimental effect on behaviour specifically related to sensory motor and cognitive functions. Learning can be defined as the process by which new information is acquired to establish knowledge by systematic study or by trial and error. There are many types of learning including simple and complex ones. Associative learning is based on making associations between different events. In associative learning, a subject learns the relationship among two different stimuli or between the stimulus and the subject's behaviour. The startle response is an unconditional reflex, characterised by the rapid contraction of facial and skeletal muscles, elucidated by a sudden and intense startling stimulus. Startle response has a well-documented physiological pathway. The US EPA and OECD DNT Guidelines (OCSPP 870.6300 or OECD 426) both require testing of learning and memory advising to use the following tests: passive avoidance, delayed-matching-to-position for the adult rat and for the infant rat, olfactory conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze, T-maze and acquisition and retention of schedule-verified behaviour. Two relevant studies were retrieved in the systematic review and one from the regulatory TG study (see summary of the studies in Table 3) containing endpoints appraised as Tier 1 and Tier 2 and underwent the uncertainty analysis. Two of these three studies provided evidence on the AO for deltamethrin (see details in Section 4.4.1) and biological relevance was based on expert judgements taking into consideration also the uncertainties summarised in Section 4.4.1 –Table 10.	Impairment behavioural function was assessed as being affected by deltamethrin with a probability higher than 66%. Doses EKE: 0.25–7.25 mg/kg bw day (96%) Doses of 0.25–2 mg/kg/day the brain concentration corresponds to 16.4 to 131,2 ng of deltamethrin for gram of brain (see **Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)**)

KE4 and KE5 represent the data gap filled in Step 4. From the data of the IVB.

Table 11. available for the measurement of the MIEs, KEs and AO.
First line of the column represents the main AOP of the network.

Key event	MIE1	KE1	KE2	KE3	KE4	AO
Assay	Voltage or patch clamp (indirect). Radio ligand binding	Patch clamp recording	Patch clamp	Western blotting Micro dialysis Modulation of post synaptic evoked response (indirect) Synapto-pHluorins	Micro electrode arrays in rat primary cell and hiPSC-derived network	Behavioural studies OECD TG 426
					KE5	
Assay					Primary human neural progenitor cells (NPC)	
				KE6		
Assay				Intracellular sodium measurement Cell morphology Cytokines release		
	MIE2	**KE7**				
Assay	Radioligand binding	Synchronous calcium oscillation Microelectrode array				

Table 12. Probability of the MIEs, KEs, and AO to occur conditioned to the occurrence of downstream KEs in the AOP Network (for details see KER table in Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)) Colours indicate the conditional probability assessed during the Bayessian Network analysis (see Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP) for further details).

Event	Conditioning events	Conditional probability
MIE1	Deltamethrin	
	Yes	**0.99**
MIE2	Deltamethrin	
	Yes	**0.7**
KE1	MIE1	
	Yes	**0.99**
	No	**0.01**
KE5	KE1	
	Yes	**0.66**
	No	**0.5**

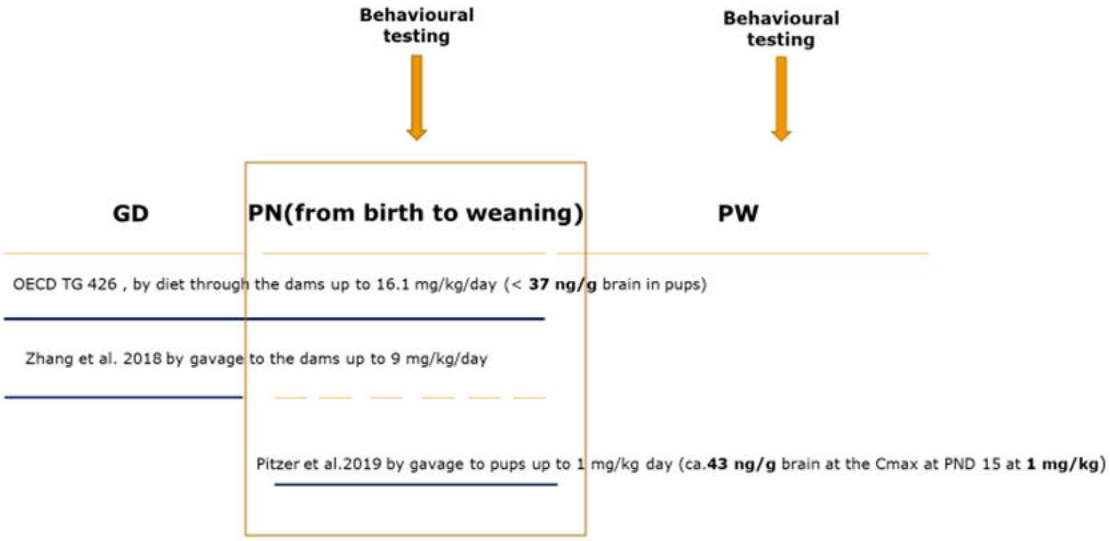
KE6	KE1	
	Yes	0.66
	No	0.5
KE7	MIE2	
	Yes	0.66
	No	0.66
KE2	KE1 and KE7	
	Yes–Yes	0.8
	Yes–No	0.8
	No–Yes	0.5
	No–No	0.01
KE3	KE2	
	Yes	0.95
	No	0.05
KE4	KE3 and KE6	
	Yes–Yes	0.66
	Yes–No	0.66
	No–Yes	0.2
	No–No	0.05
AO1	KE4 and KE5	
	Yes–Yes	0.66
	Yes–No	0.55
	No–Yes	0.55
	No–No	0.2

4.4.1. Adverse outcome

In the postulated AOP network the AO (see Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)) is defined as an effect relevant for regulatory decisions. The postulated AOP network is using evidence retrieved from the systematic literature review and from the DNT guideline study (OECD TG 426) conducted with deltamethrin, resulting in three studies being used for the description of the AO. This section contains a short summary of the study design used in the three studies, its results and the uncertainty analysis to get a better understanding on the dose and temporal concordance of the postulated AOP.

A summary of the treatment scheduling for the three studies is reported in Figure 6.

Figure 6. Treatment scheduling for the in vivo assays used in the postulated AOP and conducted with deltamethrin (GD: gestation days PN: postnatal; PW: postweaning)



Box in the figure indicates thelactation period. The blue bar indicates the dosing period (during gestation and/or lactation). For the TG 426, 30 ng/g brain in pups (from range finding study, where with 20 mg/kg bw exposure resulted in 37 ng/g brain). For Pitzer et al., 2019 (PND 3-20) he linear extrapolation, which results in 31 ng/g brain, is about 75% less of 42 ng/g brain. This is considered a relevant difference and data from the dose-range finding were considered more uncertain for the extrapolation.

Further details are given in Appendix B4.1 Graph report In vivo and in vitro deltamethrin for all the graphs including the data, Appendix B5.1. Uncertainty analysis tables for deltamethrin for the uncertainty analysis and Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP) for the results analysis and discussion. **Table 10** presents a summary table of the AO evidence. While the OECD TG 426 did not show any DNT effect, behavioural changes were reported in studies found in the open literature (Pitzer et al., 2019; Zhang et al., 2018). However, several methodological inconsistencies were observed among the three study designs that were in detail analysed in the UA and are presented in Table 14.

Table 13. Summary table of evidence for Type II pyrethroid DLM on altered behavioural function in rats

Reference	Testing	Endpoints affected within NOAEL-LOAEL range
Pitzer et al. 2019: Sprague-Dawley rat pups exposure by gavage from PND 3 to 20 at 0.25, 0.5, 1 mg/kg bw day	Acoustic and tactile startle response, peak amplitude and pre-pulse inhibition	↑ Peak amplitude in males only (Traditionally both male and female rats have been used in measures of startle reflex magnitude and plasticity. However, the effects of rat gender on this variable has not been studied systematically (Geyer and Swerdlow. 2001). NOAEL/LOAEL = 0.25/0.5 mg/kg bw d No effect on pre-puls inhibition
TG 426 OECD (2007) Wistar rat dam exposure by diet from GD6 to LD21 at 1.8, 7, 16 mg/kg bw d	Acoustic startle response, peak amplitude, latency to peak and habituation for both	No statistically significant and biologically relevant effect
Pitzer et al. 2019: Sprague-Dawley rat pups exposure by gavage from	Cincinnati Water Maze Test	↑ acquisition latency and related increase in number of errors to find the exit in a labyrinth in males pups gavage NOAEL/LOAEL= 0.5 / 1 mg/kg bw d

PND 3 to 20 at 0.25, 0.5, 1 mg/kg bw day	Water Morris Maze Conditioned Freezing Test	no statistically significant and biologically relevant effect
Zhang et al. 2018 Sprague-Dawley rat dam exposure by gavage from GD0 to delivery at 0.54, 1.35, 2.7, 9 mg/kg bw day	Water Morris Maze	↑ escape latency and swimming distance to find the hidden platform (effect in acquisition phase) ↓ time spent in searching the removed platform in the correct target quadrant (effect in the retention phase) NOAEL/LOAEL= 2.7 / 9 mg/kg bw d
TG 426 OECD (2007) Wistar rat dam exposure by diet from GD6 to LD21 at 1.8, 7, 16 mg/kg bw d	letter M Water Maze	no statistically significant and biologically relevant effect between dams dietary levels of 0.25 and 1 mg/kg bw d

A series of uncertainties were also identified in the AO evidence (OECD TG 426; Pitzer et al., 2019; Zhang et al., 2018) and are summarised below. See also Section 5.2.2 in Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP) for more details.

Table 14. Selected uncertainties identified in the *in vivo* studies

Uncertainty	Uncertainty number	Description
Measured behaviours and the underlying brain structure	U1	There is of uncertainty in the biological relationship between the measured behaviours and underlying brain structures and physiology for many neurological functions. One exception is spatial-based cognitive behaviours that are known to involve the hippocampus and related structures.
Methodological differences between available studies	U2	Different behavioural endpoints were measured, and different trains were used in the 3 studies. Differences in exposure paradigms between studies represent another uncertainty. One study used only prenatal exposure that probably limits the postnatal pup brain concentrations of deltamethrin. Another study used only postnatal exposure by gavage and therefore excluded any fetal exposure. In rodent species it is known that there is differential development of the brain prenatally and postnatally, that involves different brain structures and substructures, and that exposures only during the prenatal or postnatal stages can differentially impact behavioural outcomes.
Systemic toxicity	U3	Substantially lower weight gain during the postnatal period especially in the pre-weaning phase can be a confound factor. Therefore, there is uncertainty, whether the DNT effects observed at the overall LOAEL in the Pitzer et al. (2019) study could also be secondary to systemic toxicity.
Lack of Historical control data	U4	Typical effect sizes and typical standard deviations for each assay are other uncertainties since historical control data are not reported in the studies providing the overall LOAEL (Pitzer et al., 2019; Zheng et al., 2018).
Statistically significant effects not biologically relevant	U5	Some statistically significant effects in the Pitzer et al. (2019) study were disregarded as biologically not relevant, due to low effect size and unclear dose-response relationships (e.g. locomotor activity, MWM/memory, learning and memory). The uncertainty of the biological significance of small changes in data from neurobehavioral endpoints data can be difficult to interpret without positive and historical control data.
Lack of toxicokinetics data	U6	The lack of TK data for animals for all three studies makes comparison based on administered dose between studies difficult. Based on other available data (Mortuza et al., 2018;

		TG426 preliminary study), the highest concentrations in pup brain may have been about 43 ng/g for Pitzer et al. (2019) and TG426. Estimates of brain concentrations in the Zhang et al. (2018) study are currently not available or calculable, but the top dose effects are likely to correspond to concentrations in dam's plasma from few ng/g to several hundred ng/g. Anyway, for prenatal exposures, it is likely that the Zhang et al. (2018) study had higher concentrations in the embryo/fetus due to gavage administration to the dams as opposed to diet (TG426) and there was no prenatal exposure at all in Pitzer et al. (2019) study.
Sex-specific effects	U7	Sex-specific effects were observed in Pitzer et al. (2019) study. For the startle response the overall LOEAL corresponded to the mid dose, but there is unexplained inconsistency between male and female control responses (a slightly larger voltage amplitude in females compared with males, despite the female's weight being 2/3 of males; inconsistent also with other publications). The CWM data provide the next highest LOAEL, but effects were observed in males only and a mechanism of this gender-specific effect is unknown; it is important to note that the body weight effect was greater in males compared with females.
Different study power	U8	The study power varied widely across studies, making comparisons difficult. For example, the Pitzer et al. (2019) study used between 44–55 subjects per dose, making the overall power for detecting an effect larger compared with in all other studies. The other two studies employed 12–20 subjects per group.
Probability for false positives and false negatives	U9	The familywise probability for false positives (multiple testing error) and the probability for false negatives (potentially due to lower power in the negative TG 426 study) was not accounted for, but some information can be provided: A) potential for inflated alpha-error: In total more than 38 endpoint measurements by the three studies; in total more than 225 p-values measured; within the two studies providing significant results, i.e. Pitzer et al. (2019) and Zhang et al. (2018), ~ 66 and 16 p-values measured, 15 and 3 p-values = 0.05, 4 and 3 biologically relevant. However, in TG426, ~ 156 p-values were provided, with only 2 p-values <0.05 (but the effect was not in the biologically relevant direction). B) Potential for false negative results in TG 426 study appears to be low: From ~156 p-values provided, 16 were >0.05 with effect sizes =10% AND a probability of false negative >20% AND effect size in the biologically relevant direction. However, for none of these 16 a biologically relevant dose-response relationship was observed.

For assessing the hazard characterisation, the uncertainty affecting the body of evidence was expressed quantitatively using either ranges of probabilities (under the assumption that the uncertainty was limited and a uniform distribution could be assumed) or full probability distributions as suggested in the EFSA guidance on uncertainty analysis (EFSA 2018; EFSA Scientific Committee, 2018).

The estimates were derived using a semi-formal EKE for the AO impairment behavioural function a full probability distribution was elicited of 7 experts using the Roulette method (EFSA, 2014; O'Hagan, 2019). The individual uncertainty distributions.

4.4.2. Dose concordance

A dose concordance table was provided for the path of adjacent KEs, including the AO.

Table 15. Dose concordance table for the main AOP

	MIE	KE1	KE2	KE3	KE4	AO
	Binding to VGSC	Disruption of sodium channel gate kinetics	Disruption of action potential	Disruption of axon terminal depolarisation; changes in neurotransmitter release	Altered neuronal network function	Impairment behavioural function (sensory motor reflex and learning)
Concentration/dose	[0.01–1] µM [1]	[0.01–1] µM [1]	[0.01–1] µM [1]	*In vitro* [0.01–1] µM [2] *In vivo* [0.25–9] mg/kg [3]	0.04–5 µM (0.04 µM, corresponds to 19.3 ng of deltamethrin per gram of brain, 5 µM corresponds to 2,4 µg of deltamethrin per gram of brain) [4]	0.25–7.25 mg/kg bw/day oral gavage doses of 0.25–1 mg/kg/day in pups at PND 15 by gavage (single dose) [5] correspond to a brain concentration of 10.7 to 42.8 ng/g of brain assuming linearity.

1 Range of Concentrations agreed by EKE: [0.01–1] µM (see UA table, Appendix B5.1. Uncertainty analysis tables for deltamethrin)
2 Doses/concentrations agreed by EKE: Decrease norepinephrine from 0.25 mg/kg/day (*in vivo*; Pitzer et al., 2019)
Decrease in BDNF (CA1/hippocampus) and decrease of phosphorylation of proteins (pCREB/CREB PTrkB/TrkB) from (2.7 to 9 mg/kg bw) (*In vivo*; Zhang et al., 2018):
3 Decrease of GluN1 GluN2A and GluN2B *In vitro* [0.01–1] µM (Meyer et al., 2008; see UA table, Appendix B5.1. Uncertainty analysis tables for deltamethrin)
4 Concentrations EKE: 0.04–5 µM (96%)
5 Doses EKE: 0.25–7.25 mg/kg bw day (96%)

4.5. Step 6. Probabilistic assessment of the IATA-AOP for the quantification of the WoE

A Bayesian network analysis was used for the assessment and probabilistic quantification of the biological plausibility, the essentiality and empirical evidence of the KERs. The outcome of the Bayesian network analysis was used for the overall assessment of the postulated AOP network and for the probabilistic quantification of the WoE. Details are included in **Appendices B** and **C**.

The latter is a probabilistic graphical model that allows quantification of the uncertainty in the KERs via conditional probabilities (see Table 16 for definitions) that express the subjective belief that a MIE/KE/AO would occur conditionally to the status of the upstream KEs. Resorting to the BN allows: (1) to quantify the global dependency structure among MIEs, KEs and AOs described in the AOP network

(joint probability, see Table 16 for definitions); (2) to assess the probability that each MIE, KE and AO would occur when exposure to the stressor occurs (marginal probability, see Table 4 for definitions); and (3) to perform scenario analyses assessing the impact of new evidence, such as an individual MIE/KE occurring/not occurring with certainty, on the probability of the other KEs/AO in the network to occur.

The outcome of the conditional probabilities was therefore used to quantify the strength of the relationship between KEs and the associated uncertainty. The marginal probability distribution was used to predict the most probable status (activation/not activation, occurrence/not occurrence) of the KEs/AO when exposed to the substance. The outcome of the joint probabilities computation was used to quantify the certainty that all events occur concurrently (see Appendices B1 and C).

The incorporation of statistical or probabilistic relationships into the AOP was used to develop a probabilistic quantitative WoE for the overall assessment of the AOP.

Table 16. Probabilities associated to the Bayesian network assessment of the AOP (see Appendices B1 and C Section 1.4, for further details)

Probability	Definition	Elicitation
Conditional	The probability of each of the possible statuses of a downstream event given each possible status (or combination of statuses) of the linked upstream event(s) in the network (i.e. the conditioning events).	Provided as expert judgement based on the biological plausibility, essentiality and empirical evidence assessment of the KERs
Marginal	The probabilities associated to each possible state of a KE/variable (e.g. activated/not activated, occurrence/not occurrence) irrespective of the state of all the others.	Calculated based on the conditional probabilities (see Appendix B1: Statistical analysis report)
Joint	The probability of each of the possible combinations of the status of the KEs in the network.	Calculated based on the conditional probabilities (see Appendix B1: Statistical analysis report)

4.5.1. Bayesian network analysis and quantitative assessment of the WoE

Assessment of computed joint probability.

The probabilities for the individual KEs to occur under the condition that the upstream KEs were activated i.e. node (a node represents a random variable in a Bayesian network model) (in this case the MIE, KEs and AO; see Table 12), were used to estimate the joint probability that all events (MIEs, KEs, AO) in the network are activated/occur. This probability is 7 %. The correct interpretation of this figure needs consideration on the number of nodes in the network and the corresponding average probability per node. It is noted that the joint probability for all KEs and the AO to be activated within a network depends also on the number of nodes within the network. Therefore, an interpretation of this probability of 6.5% is neither meaningful in absolute terms, nor by comparison with absolute probabilities for other AOPs or AOP-networks. A summary table is included below in Table 17 displaying these individual probabilities (**see detailed information in section** 5.6.4. Joint Probability: Quantitative estimate for mechanistic knowledge within the putative AOP network **in Appendix AOP**). Considering that it is biologically plausible that effects on neuronal network function will affect behaviour, it is therefore useful to analyse the sequence of adjacent KEs (MIE1-KEs 1-2-3-4- AO) without the *in vivo* rodent AO. This results in an average probability of 0.87 for the KEs being activated within this sequence of adjacent KEs. Therefore, the mechanistic knowledge supports that deltamethrin affect VGSC which ultimately

leads to altered neuronal network function (KE4). This information addresses the question on the usefulness of the DNT-IVB and in the specifics of the NNF assays to fill a relevant mechanistic gap. In addition, the joint probability analysis, while quantitively informing mechanistic contribution, helps to support the overall conclusion on the AO based on the 0.76 probability averaged over all KEs (see Table 17). The non-adjacent KERs, for which limited information is available, including the most upstream KERs leading to the AO, are the items limiting the total strength of the pathway (0.66 probability for AO to occur when upstream KEs are activated).

Table 17. Joint probability for all KEs and the AO being activated by number of nodes in the network/AOP string and per-node average conditional probably of the downstream KEs to occur given the activation of the connected upstream KE(s)

	AOP_network	AOP1: MEI1-KEs1-2-3-4-AO	AOP2: MEI2-KEs7-2-3-4-AO	AOP3: MIE1-KEs1-5-AO	AOP4: MIE1-KEs1-6-4-AO	MIE1-KEs1-2-3-4
Achieved probability	0.07	0.32	0.15	0.40	0.24	0.49
Average probability per node	0.76	0.83	0.73	0.79	0.75	0.87
No. nodes	10	6	6	4	5	5

Assessment of computed marginal probability

The integration of conditional probabilities over all KERs using the Bayesian network approach provides marginal probabilities for the AO to be activated/ not activated. Based on the structure of the postulated AOP, the marginal probability for the AO identifies the 'occurrence' as more probable than not, irrespective of the status of the other MIEs/KEs.

Table 18 displays the marginal probabilities for all the MIEs/KEs/AO in the network (activated/not activated, occurrence/not occurrence) irrespective of the status of all the others. All the KEs have a probability to be activated greater than 0.5, leading to the conclusion that their activation is more probable than not when exposure to deltamethrin occurs.

The marginal probabilities illustrate how uncertainty propagates across the pathway. Upstream KEs (i.e. MIEs as triggered by exposure to the stressor – deltamethrin) generally have a greater marginal probability to be activated whereas for downstream KEs which are expected to represent a more complex biological level (i.e. organ and organism response) the marginal probability tends to approach the maximum uncertainty (i.e. 0.5).

Table 18. Marginal probabilities for all the MIEs/KEs/AO on the network

		Probability	
MIE/KE/AO		**To be activated**	**To be not activated**
MIE1	Binding to VGSC	0.99	0.01
MIE2	Binding to ryanodine receptors	0.7	0.3
KE1	Disruption of sodium channel gate kinetics	0.98	0.02
KE2	Disruption of action potential generation; membrane depolarisation	0.79	0.21

KE3	Disruption of axon terminal depolarisation; changes in neurotransmitter release	0.76	0.24
KE4	Altered neuronal network function	0.54	0.46
KE5	Decreased oligodendrocyte differentiation	0.66	0.34
KE6	Increase in intracellular sodium in microglia	0.66	0.34
KE7	Disruption of intracellular Ca channel kinetics	0.66	0.34
AO	Impairment behavioural function	0.55	0.45

The assessment of the marginal probabilities complemented the assessment of joint probabilities.

The Bayesian network approach allows also to perform scenario analyses to identify which MIEs/KEs most influence the marginal probability of AO to occur. MIEs/KEs can be ranked for their impact and this ranking could be used to support which tests are most useful within an IATA. For the putative AOP network outlined here, the strongest impact is from the most downstream KEs, i.e. KE4 (altered neuronal network function), followed by KE5 (decreased oligodendrocyte differentiation) and both assays are included in the DNT-IVB.

4.6. Human observational studies, IATA contextualisation

For the purposes of the present case study, i.e. consideration of the evidence for deltamethrin being a developmental neurotoxicant using an IATA approach, only birth cohorts were considered as suitable observational studies to be included in the evidence base. The main benefit of using observational studies in human health risk assessment is that they are concerned with an AO of interest in a population of interest and are a reflection of 'real life' in terms of including heterogenous populations who have been exposed to relevant concentrations of a stressor by relevant routes of exposure. However, there are several factors limiting their use in the regulatory risk assessment, including 1) the presence of confounders and effect-modifying factors, and 2) their inability to provide a realistic assessment of exposure. According to the selection criteria specified in the protocol (see Appendix A: Protocol of the IATA, nine studies were identified for deltamethrin. Table 19 presents a summary of the associations observed in the human observational studies in the light of the uncertainties identified. They were assessed for RoB, in total, 3/9 studies were judged to have probably or definitively high RoB, while for exposure assessment, all studies had probably or definitively high RoB. Although only studies assessing exposure using biomonitoring were included, none of the studies measured deltamethrin in blood, but were specific or non-specific metabolites in urine. This does not allow an accurate estimate of exposure to deltamethrin based on the uncertainty in kinetics, the uncertainty on the correct timing of urine samples collection and the presence of the metabolites preformed in environmental media. Overall, the nine studies retrieved from the systematic literature search were categorised as Tier 3 (high RoB), mainly due to the inadequate assessment of confounding, selection bias and suboptimal measurements of exposure with non-specific biomarkers, which are limitation inherent to pesticide epidemiological studies. This tier resulted from the application of an algorithm of OHAT/NTP to the seven questions addressing the RoB.

For most endpoints and studies, the level of probability for a causal association was rated 0–10%. Only for the endpoint category communication, addressed in a single study (Eskenazi et al., 2018), the probability level was rated 10–33% for a causal association. No higher probability could be awarded principally because of remaining uncertainties over the appropriateness of the time-point for urine sampling and lack of assessment of relevant co-exposure to other developmental neurotoxicants.

Measurement of the non-specific 3-PBA metabolite does not allow differentiation between exposure to more or less toxic pyrethroids. In addition, the presence of 3-PBA in maternal urine samples during gestation does not represent direct evidence of exposure to parent pyrethroid compounds. The metabolite 3-PBA has a longer half-life compared with the parent compound, it is persistent and

refractory to degradation in natural environment and so can accumulate in the environment ultimately reaching agricultural products. Since the measurement of 3-PBA in urine reflects exposure to both parent compounds and the non-toxic 3-PBA metabolite preformed in the environment, this exposure metric can result in misclassification of past exposures. Therefore, results should be interpreted with caution (Burns and Pastoor, 2018).

Despite all these limitations, the available epidemiological evidence linking prenatal exposure to deltamethrin with neurodevelopmental disorders (NDDs) is supported by experimental data from animal models of some neurodevelopmental outcomes and is consistent with the biology of NDDs. This observation is also supported by the work carried out in the context of this project. The available evidence structured in the AOP conceptual framework indicates that a plausible mechanistic link between exposure to deltamethrin and NDDs exist. However, the current (low) doses of exposure during pregnancy may not be enough for triggering the whole process leading to NDDs and additional co-exposure to other chemicals, environmental factors or maternal lifestyles may be needed, apart from the role of specific genetic mutations in sodium channels. However, it can be anticipated that the higher the exposure to deltamethrin the less the need for additional factors to trigger NDDs.

Table 19. Summary of the associations observed in the human observational studies in the light of the uncertainties identified

Endpoint categories	N studies	N analyses	Metabolites	Age range	Associations detected (one line per study)
Cognitive impairment	8	14	3-PBA, *cis*-DBCA, total Permethrins[1]	3 months to 6 years	Inverse association with MDI (3 months) for 3-PBA
					Inverse association with MDI (1 year) for total permethrins
Impaired psychomotor development	3	15	3-PBA, *cis*-DBCA	3 months to 3 years	Inverse (♀) and direct (♂) association with gross motor (2 years) for 3-PBA 3-PBA (↑ motor composite, 2-y ♂) *cis*-DBCA (↓motor composite, ↓gross motor, 2-y ♀)
Communication	1	14	3-PBA, *cis*-DBCA	1–2 years	Inverse association with expressive communication (2 years, ♀ for 3-PBA/♀ and all children for *cis*-DBCA)
					Inverse association with language composite (2 years, ♀ for 3-PBA/♀ and all children for *cis*-DBCA) Inverse association with language composite (1 year, ♀ for *cis*-DBCA)
Behavioural	3	14	3-PBA, *cis*-DBCA	4–9 years	Inverse association with Internalising Composite (4–5, 6, 7–9 year for 3-PBA)
					Inverse association with behavioural regulation index (4–5, 6, 7–9 year for 3-PBA)
					Inverse association with Social-Emotional (1 year, ♂ for 3-PBA) [2]
ADHD	1	1	3-PBA	2–4 years	Direct association with ADHD and ADHD >90th-ile (2–4 years)

5 Discussion and Conclusion

The overall purpose of this IATA was to address the question: How certain are we that the pesticide active substance deltamethrin is a developmental neurotoxicant in humans based on the data collected, appraised, synthesised and integrated using an operational protocol and in line with the IATA framework?

This was carried out using a transparent and trackable evidence-based approach with a probabilistic quantification of the WoE. A key step of the IATA iterative process was the inclusion of the DNT-IVB in the AOP-informed IATA process.

To ensure the transparency and reliability of the assessment for decision making, an extensive uncertainty analysis was performed, covering from the methods' development to the limitations of the conclusions drawn. An extensive analysis of various lines of evidence, to obtain data to be utilised for developing a stressor-specific AOP was conducted. An AOP network using deltamethrin as a model chemical stressor was postulated to inform the IATA because a pre-existent AOP was not available. The KEs included in the putative AOP network were identified by assessing the probability that a causal association between exposure to deltamethrin and the activation/occurrence of the different KEs would occur in the various lines of evidence (i.e. humans, rodents, *in vitro* test systems). To support this judgement, an UA was performed assessing sets of predefined uncertainty domains tailored to each line of evidence (see Appendix B1: Statistical analysis report). KEs for which the probability of a causal association was judged by expert consensus to be 66% or above were included in an initial list that was then used to postulate the resulting AOP network. The threshold of 66% was considered sufficiently conservative since it means that it is judged at least twice as probable that deltamethrin is activating the KEs than not activating the KEs. A range/probability distribution expressing the uncertainty on the lowest concentration/dose triggering the causal relationship was also elicited and associated with each MIE/KE/AO. This analysis was used for the assessment of the dose concordance in the postulated AOP network.

The strength of the KERs was quantified using a Bayesian network analysis by means of conditional probability tables, i.e. probabilities expressing the certainty that a downstream KE would activate/not activate given upstream KE(s) activation/no activation and their combinations (if more than one upstream KE exists for the KER). The conditional probabilities were elicited using expert knowledge taking into consideration the available evidence in terms of biological plausibility, essentiality and empirical evidence. The conditional distribution of a dichotomous variable (e.g. yes/no), i.e. probability of occurring and probability of not occurring depending on other variables (as happening in a network of KEs) can be very different from the distribution of the unconditioned variable (marginal distribution for the definition of the KE).

The Bayesian network approach allows decomposition of the KEs joint probability distribution using conditional probabilities. This allows to quantify to what extent the KEs and thus the DNT-IVB, informs the WoE approach. The joint probability of the concurrent activation of all the MIEs/KEs/AO in the AOP network enables an estimation of the overall level of certainty of the AOP developed for deltamethrin. The Bayesian network can also be used to assess selected scenarios (selected combinations of the activation/no activation of KEs) to predict the probability of the AO to occur (higher or lower than 50%) and to perform influence analyses identifying those KEs that have a greater influence on AO occurrence.

The analysis of the computed joint probability, when considering the number of nodes and the average probability per node in the interpretative process, supports the strength of the mechanistic understanding (MIE1-KEs1–2-3–4), indicating that the postulated sequence of adjacent KEs has the highest joint probability to occur (MIE1-KEs1–2-3–4-AO). The analysis also indicates which KERs are weaker, i.e. the association in terms of biological plausibility, empirical support and essentiality are more uncertain. The analysis of the computed marginal probabilities (which identifies the 'occurrence' as more probable than not irrespective of the status of the other MIEs/KEs) indicated that the instance of activation of each KE, including the AO was more probable than not and that the KE4 was the most impactful. The analysis also showed that KE5, and its testing, has a relevant impact although without adjacent KER. The probable reason for this is that toxicological evidence for the KER on inhibition of deltamethrin-induced KE5 (oligodendrocyte differentiation) is sparse, therefore accompanied by a large uncertainty. This is due to a lack of data on the effects of pesticides and other contaminants on this cell type. However, biological plausibility of this KE for a human AO is large as there is a plethora of clinical and experimental data supporting the high susceptibility especially of oligodendrocyte progenitor cells towards many stressors.

Overall, the Bayesian network analysis and the quantification of the WoE for the overall assessment of the postulated AOP network resulted in an acceptable level of certainties for the purposes of the assessment. The joint probability of all KEs to occur for the pathway of adjacent KEs was 32%. The conditional probability of downstream KEs to be activated when the upstream KEs being activated, averaged on the number of nodes (n=6), was 0.83 with a marginal probability for all the KEs in the AOP string being always above 0.50. The analysis of the marginal probabilities allows us to classify the triggering of the MIE by the stressor according to the state of each KE and the outcome. The marginal probabilities being always above 0.50 indicates that is more probable than not that the MIEs, KEs and AO are activated/occurring. This is also in line with the EKE performed on the available data for the postulation of the AOP network. The Bayesian network analysis also supports the critical impact of the mechanistic understanding in the overall assessment of the WoE and the relevance of measuring KEs, in particular KE4 and 5 that are included in the DNT-IVB.

The critical gap driving most of the uncertainty in the last two KERs is the lack of empirical data on the correlative and/or causal relationships between deltamethrin-induced disturbed neuronal network function (KE4) and/or hypomyelination (as a result of KE5) and altered behavioural function (AO). The inconsistencies reported for the deltamethrin AO are likely to reflect the difference in the protocols and exposure conditions and the recognised uncertainties associated with the current *in vivo* DNT models. By developing a PBPK model on deltamethrin and performing reverse dosimetry it was found that the lowest concentration with 66% probability of affecting the KE4 (decrease in neuronal network function), nominally 0.04 µM in *in vitro* test systems, corresponded to 19 ng of deltamethrin/gram brain. This is comparable to the *in vivo* effect dose-range of 0.25–1 mg/kg directly administered by gavage to pups as a single dose at PND 15, which corresponded to 10.7 to 42.8 ng/g of brain assuming linearity. This is in line with the dose concordance paradigm in the AOP conceptual framework (OECD, 2016) for which higher doses/concentration or longer exposure time are needed to elicit KEs when moving downstream through the pathway. It is therefore possible that at least part of the inconsistencies observed in the AO are due to the dose regimen used in the studies and effective exposure at the target site was only achieved in the study conducted by direct dosing of pups.

This IATA also includes evidence from HOS. Although HOS from the systematic review were rated as high RoB, they were retained as supporting evidence as there is a recognition of their intrinsic limitations but also of their relevance by representing a real-life scenario. For most endpoints and studies, the level of probability for a causal association was rated 0–10% and only for the endpoint category communication the probability level was rated 10–33% in a single study. However, the postulated AOP network supports that a plausible mechanistic link between exposure to deltamethrin and neurodevelopmental outcomes exists and the current (low) doses of exposure during pregnancy may

not be enough for triggering the whole process leading to a human AO. With this qualification, the outcome of the HOS, although with a limited weight, would still be included in the process of hazard characterisation.

Conclusion

Overall, the case study shows the applicability of the DNT-IVB in a hazard characterisation context when *in vivo* DNT data exist with equivocal results.

The evidence available and the approach taken in this IATA case study (evidence-based postulation of an AOP network informing IATA through a probabilistic quantification of the WoE), could achieve an acceptable level of certainty in DNT hazard identification and characterisation of deltamethrin which was the purpose of the assessment. The analysis could be used to draw regulatory conclusions.

Furthermore, the case study illustrates the usefulness of a postulated AOP network and probabilistic quantification of weight of evidence to improve regulatory decision making. The overall process and the *in vitro* data also increased the ability of interpreting the HOS by providing a plausible mechanistic link to a human-relevant AO, therefore supporting the contextualisation of these studies in the future risk assessment process.

The Bayesian network analysis could quantify the impact of measuring mechanistic KEs at cellular level highlighting the additional value of the DNT-IVB.

Without the *in vitro* data and just with just the human epidemiology and the rodent *in vivo* evidence available, it would have been too uncertain to conclude that DNT may result from DM exposure. This will also depend on biological modifiers which currently cannot be tested in any system, e.g. combination of epigenetic and genetic background, socio-economic status, diet, life style, stress and co-exposure including environmental contaminants and drugs or maternal infection and viruses. Therefore, for this IATA, DNT is considered as a molecular/cellular/organ/organism burden that is not necessarily detected (measurable) as a fully obvious, severe organism level adversity. Thus, the effects at the cellular KE are critical to the overall conclusion.

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Appendix A: Protocol of the IATA

Please refer to the separate publication for full Appendix A.

ENV/CBC/MONO(2022)24/ANN1

Appendix B1: Statistical analysis report

Please refer to the separate publication for full Appendix B1.

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Appendix B3.1 Outcome of the ROB deltamethrin

Critical Appraisal table – *In vitro*

Channels/transporters: Calcium

Zheng J, Yu Y, Feng W, LI J, Liu J, Zhang C, Dong Y, Pessah IN, Cao Z, 2019 – RefID: 2452

Study characteristics and test system	Specific endpoint	Calcium: Influx; Frequency
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3; 4
Exposure	Exposure duration:	25 minutes; 7 Days
	Concentration:	0 μM; 0.003 μM; 0.01 μM; 0.03 μM; 0.1 μM; 0.3 μM; 1 μM
	Treatment frequency:	Chronic; Single
Measurement	Measurement time:	6 Day/s; 7 Day/s; 8 Day/s; 10 Day/s; 12 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely based on the information reported in the methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. At least 3 independent cultures were used, as reported in the methods section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or media. Source was ChemService and purity was 99%. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard methods were used but no blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity not reported, the highest concentration has been reported to be cytotoxic in other papers. In addition, IC50 values are below cytotoxic levels.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N/group is only 3 or 4 which is usually acceptable for in vitro studies, but it is low for most of all other types of studies.

Ihara D, Fukuchi M, Honma D, Takasaki I, Ishikawa M, Tabuchi A, Tsuda M, 2012 – RefID: 2623

Study characteristics and test system		Specific endpoint	Calcium – [Influx]
		Test system:	Primary cells
		Species:	Rat
		Origin of the test system:	Cortical
		Stage of development of the primary cells:	Post-natal
		Medium:	Animal medium
		Funding source:	Public
		Number of biological replicate:	3
Exposure		Exposure duration:	6 hours
		Concentration:	0 μM; 1 μM
		Treatment frequency:	Single
Measurement		Measurement time:	0 sec.; 50 sec.; 100 sec.; 150 sec.; 200 sec.; 240 sec.
	Risk of bias appraisal		Tier: 3

Bias domain		Question	Score	Judgement
Randomisation		Q1: was administered dose or exposure level adequately randomised?	PLRoB	Source is considered homogeneous. However, it is not reported how many animals were used.
Allocation concealment		Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups		Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely based on the information reported in the methods section.
Blind research personnel		Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely that lack of blinding for cell cultures study would bias result.
Attrition or exclusion		Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine. The figure presents photomicrographs for 10 min and 1 hr with only n = 1. There is no information in the methods section and on the total number of cultures used for any endpoint.

Exposure characterisation		Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out on the source, stock or media solutions. Source was technical-grade deltamethrin purchased from Wako Pure Chemicals (Tokyo, Japan). Only one concentration and one control were tested for morphology work.
Outcome assessment		Q7: can we be confident in the OUTCOME assessment?	PHRoB	Methods are well established with suitable time points. No blinding, and subjective assessments without any quantitative results, n = 1.
Selective outcome report		Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity		Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity was assessed, viability was not changed for 10 µM. Viability was increased for 0.1 and 1 µM.
Replicates and Repetitions		W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PHRoB	n = 1 with subjective assessments of outcome.

Cao Z, Shafer TJ, Murray TF, 2011 – RefID: 2938

Study characteristics and test system	Specific endpoint	Calcium – [Influx]
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Embryo
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	13 minutes
	Concentration:	0 µM; 0.3 µM; 1 µM; 10 µM; 100 µM; 3 µM; 30 µM; 50 µM
	Treatment frequency:	Single
Measurement	Measurement time:	13 min; 100 sec.; 200 sec.; 250 sec.; 400 sec.; 600 sec.; 800 sec.

Risk of bias appraisal	Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported, but in vitro assay setup is unlikely to cause bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. All conditions are similar.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact bias.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not directly reported, but not likely to impact bias. All the experiments reported in Figure 2 were carried out 3 times.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out. There were solubility limits for all concentrations. However, highest dose is within known limits. Chemicals used were of technical grade (deltamethrin 98.9%).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The study was well conducted with established methods. Times used were adequate. 8 concentrations were tested, and all conditions were similar. The lack of blinding is unlikely to bias results.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	In Figure 1 and Figure 3 only n = 1 are presented when there were three replicates. All experiments described in the methods section are reported in the results section.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity was not reported, but effects were reported at concentrations (e.g. IC50 3.75 µM) that is well below the known cytotoxicity.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	No. Three independent experiments were conducted.

Channels/transporters: Ryanodine

Zheng J, Yu Y, Feng W, Li J, Liu J, Zhang C, Dong Y, Pessah IN, Cao Z, 2019 – RefID: 2452

Study characteristics and test system	Specific endpoint	Ryanodine: Mean close time (Tc); Mean open time (T0); Open Probability (P0)
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	15 minutes
	Concentration:	0 μM; 0.01 μM; 0.03 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–9 nr
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely based on the information in the methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely to impact automated procedures or growth of cultures.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. At least 3 independent cultures were used, as reported in the methods section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or media. Source was ChemService and purity was 99%. Exposure, culture, times etc. are all considered adequate. Three groups were tested (control and two deltamethrin).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard methods used but no blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity not reported, the highest concentration has been reported to be cytotoxic in other papers. In addition, IC50 values are below cytotoxic levels.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N/group is only 3 which is usually acceptable for in vitro studies, but it is low for most of all other types of studies.

Channels/transporters: Sodium

Hossain MM, Liu J, Richardson JR, 2017 – RefID: 3048

Study characteristics and test system	Specific endpoint	Sodium – intracellular influx
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	BV2 cells; Primary Microglia cells
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public

	Number of biological replicate:	3
Exposure	Exposure duration:	48 hours
	Concentration:	0 μM; 1 μM; 5 μM
	Treatment frequency:	Single
Measurement	Measurement time:	24 PDN; 48 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported, but the procedure indicated that the primary cells were collected from the pups, suspended and mixed. The mixed pool of cells derived from different animals seeded.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not all details were reported, but the information is considered sufficient for inferring a probably low risk of biases.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but lack of adequate blinding during the study would not appreciably bias results.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported but can be inferred from the evaluation of the tabulated results.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	The purity was not reported. Only one concentration was used for this endpoint.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding was not reported but it is considered of not having impact because of the automated recording.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Measures outcomes were reported in line with Materials and methods and Abstracts. A formal protocol is lacking.

Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Method described and reported. Cytotoxicity was measured and the assay/method used was described in the Materials and methods section
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Yes, replicates were carried out. Direct evidence that sufficient number of replicates were used.

Genomic: Transcriptional alterations

Christen V, Rusconi M, Crettaz P, Fent K, 2017 – RefID: 357

Study characteristics and test system	Specific endpoint	Transcriptional alterations: Camk2a; Camk2b; Gap-43; nf-h; tubulin-alpha; tubulin-beta
	Test system:	Cell line
	Species:	Rat
	Origin of the test system:	Adrenal gland pheochromocytomas
	Stage of development of the primary cells:	
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	5 Day
	Concentration:	0 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	5 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	PC-12 cells were obtained from Sigma-Aldrich (Buchs, Switzerland) and it is considered a homogeneous stock. As reported in Materials and methods section of the publication.

Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. As reported in Materials and methods section of the publication (Sections 2.2 and 2.3)
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. As reported in Figure C. 13 of the publication.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	Purity is >99% (source Sigma) prepared in DMSO. No analytical work was carried out. The duration of exposure was suitable for the test system and investigated endpoints positive control. Contamination is not reported. Only two concentrations were tested. However, it is considered enough for this transcriptional alterations studies based on the threshold set up and on the consistency of the results.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The methods used are well established, but the cell line used is not gold standard. Measurements were carried out by a machine.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. All the outcomes in Materials and methods section are reported in the results section. However, no protocol is available.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity was not measured in the same study, but the concentration used are well-known to be non-cytotoxic.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Three independent experiments as reported in Figure C. 13 of the publication.

Imamura L, Yasuda M, Kuramitsu K, Hara D, Tabuchi A, Tsuda M, 2006 – RefID: 2622

Study characteristics and test system	Specific endpoint	Transcriptional alterations: Bdnf eIII-V mRNA; Bdnf eIII-V mRNA (with TTX); Bdnf eIII-V mRNA (without TTX); Bdnf pIII activity
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Embryo
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	3 hours
	Concentration:	0 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM
	Treatment frequency:	Multiple; Single
Measurement	Measurement time:	0 PDN; 1 PDN; 3 PDN; 6 PDN; 12 PDN; 24 PDN; 48 PDN; 72 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely they were based on the information in methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely to impact in vitro cultures measurements.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Hard to determine – no n/group are reported in methods section. Figures reported have mean and SD from three experiments.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out on stock, source or media. Source was technical-grade deltamethrin purchased from Wako Pure Chemicals (Tokyo, Japan). Only one dose and a control were tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard methods. No blinding, but it is assumed to have no impact on bias
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity not measured, but all concentrations were below the normal reported cytotoxicity levels
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PHRoB	Not clear. The only information available is 'The columns represent the mean and SD from three independent experiments'. Nothing is reported about the numbers in each experiment which could be only one.

Ihara D, Fukuchi M, Honma D, Takasaki I, Ishikawa M, Tabuchi A, Tsuda M, 2012 – RefID: 2623

Study characteristics and test system	Specific endpoint	Transcriptional alterations: Bdnf eIV–IX mRNA; Bdnf pIV activity
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3

Exposure	Exposure duration:	6 hours
	Concentration:	0 μM; 1 μM
	Treatment frequency:	Single
Measurement	Measurement time:	0 PDN; 3 PDN; 6 PDN; 12 PDN; 24 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	It is considered a homogeneous source, but not reported how many animals were used.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but methods descriptions suggest the conditions were identical.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely that lack of blinding for cell cultures study would not appreciably bias the results.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Hard to determine. There is no information in the methods section except for a sentence in the statistics section 'All data were expressed as the mean (plus/minus) SEM for a number of separate experiments performed, as indicated in the corresponding figures.' Also, there is no information on the total number of cultures used for any endpoint.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out on the source, stock or media solutions. The source was technical-grade deltamethrin purchased from Wako Pure Chemicals (Tokyo, Japan). Only one concentration and a control for gene expression endpoints were used.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods. Time points were suitable. No blinding, but it is unlikely to have an impact.

Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity was assessed. Viability was not changed for 10 µM. Viability was increased for 0.1 and 1 µM.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PHRoB	Figure legend reports n = 3–5. Methods section makes no mention of n/group. Also, it is unclear where the 3–5 replicates were obtained from (Was it 3–5 replicates from one cell culture or 3–5 independent replicates from 3–5 mice based cultures?)

Growth/Maturation: Neurite outgrowth

Christen V, Rusconi M, Crettaz P, Fent K, 2017 – RefID: 357

Study characteristics and test system	Specific endpoint	Neurite outgrowth – Neurite length
	Test system:	Cell line
	Species:	Rat
	Origin of the test system:	Adrenal gland pheochromocytomas
	Stage of development of the primary cells:	
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	5 Day
	Concentration:	0 µM; 1 µM; 10 µM; 100 µM
	Treatment frequency:	Single
Measurement	Measurement time:	5 Day/s
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	PC-12 cells were obtained from Sigma-Aldrich (Buchs, Switzerland) and it is considered a homogeneous stock. However, the differentiation of the cells in the laboratory was conducted without randomisation
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable. Lack of adequate allocation concealment would not appreciably bias results for In vitro studies.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. As reported in Materials and methods section of the publication.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported, but inferred from, Fig. 3 in the publication.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	Purity is >99% (source Sigma) prepared in DMSO. No analytical work was carried out. The duration of exposure was suitable for the test system and investigated endpoints positive control. Contamination is not reported. Only 3 concentrations were tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The methods used are well established, but the cell line is not gold standard. There is better available test system for evaluation of DNT (outgrowth). Only 3 concentrations were tested. Blinded: To prevent false results, the pictures were only labelled with numbers so that the person, performing the analysis, was not influenced before Image J analysis. All experiments were performed.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. All the outcomes in Materials and methods section are reported in the results section. However, no protocol is available.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not reported but low concentrations are well known to be non-cytotoxic.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	For positive, negative control and the different concentrations of the test substance, three pictures per well and three wells in total were randomly taken under the microscope (resulting in 9 pictures). Neurite lengths were analysed using ImageJ software.

Ihara D, Fukuchi M, Katakai M, Shinoda Y, Katoh-Semba R, Furuichi T, Ishikawa M, Tabuchi A, Tsuda M, 2017 – RefID: 1865

Study characteristics and test system	Specific endpoint	Neurite outgrowth – Complexity; Neurite length
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Embryo
	Medium:	NA
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	6 hours
	Concentration:	0 μM; 1 μM
	Treatment frequency:	Single
Measurement	Measurement time:	0 PDN; 3 PDN; 6 PDN; 12 PDN; 24 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported, but it is likely based on methods descriptions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is not likely to alter bias.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Difficult to determine. Methods states that 'All studies were independently repeated at least 3 times (n = 3–5)', but no samples sizes were reported in results.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out. No reporting of purity of deltamethrin, source 'Wako Pure Chemicals. Only one concentration of deltamethrin was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Methods are well established and work carefully described and reported. Assessors were blinded.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity was not reported but the concentration of deltamethrin was 1 µM, well below cytotoxicity levels.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	No. There were 3–5 independent replicates.

Zheng J, Yu Y, Feng W, Li J, Liu J, Zhang C, Dong Y, Pessah IN, Cao Z, 2019 – RefID: 2452

Study characteristics and test system	Specific endpoint	Neuronal Morphology: Axon Length; Dendritic Complexity – no. of crossing (Radius 30); Dendritic Complexity – no. of crossing (Radius 60)
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public

	Number of biological replicate:	3
Exposure	Exposure duration:	48 hours; 7 Day
	Concentration:	0 μM; 0.003 μM; 0.01 μM; 0.03 μM; 0.1 μM; 0.3 μM; 1 μM
	Treatment frequency:	Chronic
Measurement	Measurement time:	2 Day/s; 7 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely based on the information reported in the methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely to impact automated procedures or growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. At least 3 independent cultures were used, as reported in the methods section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or media. Source was ChemService and purity was 99%. Exposure, culture, times etc. are all considered adequate. Three groups were tested (control and two deltamethrin).

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard methods were used but no blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Unclear. Methods report that 'Each experiment was repeated on at least two independent cultures, with at least three wells in each culture'. However, n/group in Figure 6 ranges from 701–941 with no explanation of the large difference in group sizes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity was not reported. The highest concentration has been reported to be cytotoxic in other papers. IC50 values are below cytotoxic levels. Axons and dendrites were not differentiated with specific stains/antibodies – differentiated instead by length. Therefore, no conclusions about differential effects of deltamethrin on these two endpoints can be made.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N/group is 3. This number is usually acceptable for in vitro studies but is low for most all other types of studies.

Ihara D, Fukuchi M, Honma D, Takasaki I, Ishikawa M, Tabuchi A, Tsuda M, 2012 – RefID: 2623

Study characteristics and test system	Specific endpoint	Neurite outgrowth: Complexity (100 μm distance from the body); Complexity (120 μm distance from the body); Complexity (140 μm distance from the body); Complexity (160 μm distance from the body); Complexity (180 μm distance from the body); Complexity (20 μm distance from the body); Complexity (200 μm distance from the body); Complexity (40 μm distance from the body); Complexity (60 μm distance from the body); Complexity (80 μm distance from the body); Neurite length
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical

	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	6 hours
	Concentration:	0 μM; 1 μM
	Treatment frequency:	Single
Measurement	Measurement time:	0 sec.; 50 sec.; 100 sec.; 150 sec.; 200 sec.; 240 sec.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Source is considered homogeneous. However, it is not reported how many animals were used.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely based on the information reported in the methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely that lack of blinding for cell cultures study would bias result.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine. The figure presents photomicrographs for 10 min and 1 hr with only n = 1. There is no information in the methods section and on the total number of cultures used for any endpoint.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out on the source, stock or media solutions. Source was technical-grade deltamethrin purchased from Wako Pure Chemicals (Tokyo, Japan). Only one concentration and one control were tested for morphology work.

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Methods are well established with suitable time points. No blinding, and subjective assessments without any quantitative results, n = 1.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity was assessed, viability was not changed for 10 µM. Viability was increased for 0.1 and 1 µM.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PHRoB	n = 1 with subjective assessments of outcome.

Harrill JA, Freudenrich T, Wallace K, Ball K, Shafer TJ, Mundy WR, 2018 – RefID: 30344476

Study characteristics and test system	Specific endpoint	Neuronal Morphology: Dendrites Maturation; Total neurite length per neuron; Synaptogenesis – Puncta per total dendrite length
	Test system:	Cell lines differentiated from stem cells; Primary cells
	Species:	Human; Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	1
Exposure	Exposure duration:	48 hours; 5 Day
	Concentration:	0 µM; 0.001 µM; 0.003 µM; 0.01 µM; 0.03 µM; 0.1 µM; 0.3 µM; 1 µM; 10 µM; 3 µM; 30 µM
	Treatment frequency:	Chronic; Single
Measurement	Measurement time:	12 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	The study was performed using a uniform standardised cell system. All plates were treated equally and a positive and negative control included in each plate. The system is highly automated
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	All the experimental conditions were identical across the study groups. The process is fully automated.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	The process is fully automated.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	For all the assays, complete concentration-response data were generated within the plate. All the responses were uniformly analysed through the same methodology.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Commercial source was reported. The concentrations were prepared and distributed automatically. All the relevant parameters for definition of exposure were properly described.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	The methods for the assessment of the outcome is described in detail. The method is giving an automatic read-out.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. Information were detailed in supplementary data and in the summary tables available in the paper.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Yes, even if the method is not described in details.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Usage of replicates is described in the paper.

Growth/Maturation: Synaptogenesis

Grosse G, Thiele T, Heuckendorf E, Schopp E, Merder S, Pickert G, Ahnert-Hilger G, 2002 – RefID: 1794

Study characteristics and test system	Specific endpoint	Synaptogenesis – no. of neurons
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	–9 nr; 12 Day
	Concentration:	0 nM; 2 nM; 20 nM; 200 nM; 2000 nM; 60 nM
	Treatment frequency:	Multiple; Single
Measurement	Measurement time:	5 Day/s; 18 Day/s
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported, but concurrent control was used.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported, but it is highly likely based on methods descriptions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but lack of blinding would not appreciably bias the results.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Only one concentration and a control were tested.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	Only two concentrations were reported.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Methods used were acceptable. Assessed at the same time point after exposure in all study groups. The conditions for cultivation and exposure were appropriate. No blinding, and subjective assessments.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	The measured outcome was reported but not for all doses.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Excess cytotoxicity was reported for concentration greater than 20 nM (20nM = 14%, 60 nM = 45%), but there were some effects on some synaptic measures at 2 and 20 nM.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	NR	No information on the number of replicates used.

Microglia activation: TNF alpha

Hossain MM, Liu J, Richardson JR, 2017 – RefID: 3048

Study characteristics and test system	Specific endpoint	TNF alpha
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	BV2 cells; Primary Microglia cells
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	48 hours

	Concentration:	0 μM; 0.5 μM; 1 μM; 5 μM
	Treatment frequency:	Single
Measurement	Measurement time:	24 PDN; 48 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported. The procedure indicated that the primary cells were collected from the pups, suspended and mixed. The mixed pool of cells derived from different animals seeded.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not all details were reported but considered sufficient for inferring a probably low risk of biases.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but lack of adequate blinding during the study would not appreciably bias results.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported but can be inferred from the evaluation of the tabulated results.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	Purity was not reported. Only one concentration was used for this endpoint.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding not reported. It is considered not impacting the outcome because the procedure used was automated.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Measures outcomes were reported in line with Materials and methods and Abstracts. A formal protocol is lacking.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	method described and reported Cytotoxicity was measured and the assay/method used was described in the Materials and methods section

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	yes, replicates were carried out Direct evidence that sufficient number of replicates were used.

Microglia activation: visual inspection (microscopy)

Hossain MM, Liu J, Richardson JR, 2017 – RefID: 3048

Study characteristics and test system	Specific endpoint	Visual inspection (microscopy)
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	BV2 cells; Primary Microglia cells
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	48 hours
	Concentration:	5 μM
	Treatment frequency:	Single
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported. The procedure indicated that the primary cells were collected from the pups, suspended and mixed. The mixed pool of cells derived from different animals seeded.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not all details were reported but considered sufficient for inferring a probably low risk of biases.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but lack of adequate blinding during the study would not appreciably bias results.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Not reported, only one showed in the figure of the publications.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	Purity was not reported. Only one concentration was used for this endpoint.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding not reported.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Measures outcomes were reported in line with Materials and methods and Abstracts. A formal protocol is lacking.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Method described and reported Cytotoxicity was measured and the assay/method used was described in the Materials and methods section
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Yes, replicates were carried out Direct evidence that sufficient number of replicates were used.

Network formation/function (MEA): % Spikes in Bursts

Alloisio S, Nobile M, Novellino A, 2015 – RefID: 926

Study characteristics and test system	Specific endpoint	% Spikes in Bursts
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical

	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	9
Exposure	Exposure duration:	20 minutes
	Concentration:	0 μM; 0.0001 μM; 0.001 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	21 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Unknown number of GD18 rats were used. However, this is a standard method, unlikely to impact bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not explicitly stated, but it is likely since 96 well plates were used. There is no certainty of the number of plates and if all plates were generated at the same time.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but with automated methods and cell-based assay it is not likely to impact bias.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. Everything was reported with the exception of the number of wells that did not met the inclusion criteria. This is not likely to impact bias.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on test substance and dosing solutions. Purity reported as 95–99% depending on the chemical (source Sigma).

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods. Time point were suitable and the same for all groups. The culture conditions were appropriate. No blinded.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. However, there was no reporting on the number of well that were excluded due to not meeting inclusion criteria 'Only channels with >2 bursts/min were included in the analysis (active electrodes).'
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity is not reported. Some concentrations of deltamethrin are above those reported to cause cytotoxicity (e.g. 100 μM). However, significant effects on MBR were reported for concentrations well below those reported as cytotoxic by other papers.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N=9 or 10 per group. However, it is unclear if these are replicates or repetitions. It is possible to have generated all data on one plate.

Mohana Krishnan B, Prakhya BM, 2016 – RefID: 2842

Study characteristics and test system	Specific endpoint	% Spikes in Bursts
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	30 minutes
	Concentration:	0 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	24 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the system was considered homogeneous (from the details reported in M&M in terms of cells pooling and automated read-out of the test).
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Experimental conditions were well reported and described in Materials and methods.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is deemed that lack of adequate blinding during the study would not appreciably bias results. Most of the test is automated and the duration is short.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported but can be inferred. All cells are pulled together and automatically counted at the time of plating.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	It is reported, except for the contamination check.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Blinding was not reported but the recording is automated clearing the impact of lack of blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	The report is in line with the abstract and with the Materials and methods section. However, a formal protocol is not reported.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not carried out but multiple doses, including low doses, were used and an IC50 calculated.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Direct evidence, reported.

Frank CL, Brown JP, Wallace K, Mundy WR, Shafer TJ, 2017 – RefID: 30344475

Study characteristics and test system	Specific endpoint	% Spikes in Bursts
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	
	Treatment frequency:	Chronic
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Cell system and method were uniform and fully automated, with negative and positive control added to each plate.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	All the conditions were well described in the paper and characteristic of the high-throughput methodology.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	The method is fully automated and, although not declared in the paper, it is very likely that the compound is blinded up to the elaboration of the data as described in TOXCAST testing.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Criteria are applied to each step of the protocol across all the 17 assays and for all chemicals in the battery. The high-throughput approach guarantee that all outcomes are treated equally and the same criteria for the assessment are applied.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Commercial source declared and concentrations are prepared and administered automatically.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	The prediction model is well described and in line with the experiments having a similar approach.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Raw data are available as supplement and summary table are presented in the paper.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Two cytotoxicity methods are applied, one metabolic and one directly measuring biomarker of cell death.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	The number of replicates is described and the definition of biological replicate, from which the assessment is performed, is reported.

Network formation/function (MEA): Burst duration

Alloisio S, Nobile M, Novellino A, 2015 – RefID: 926

Study characteristics and test system	Specific endpoint	Burst duration
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	9
Exposure	Exposure duration:	20 minutes

	Concentration:	0 μM; 0.0001 μM; 0.001 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	21 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Unknown number of GD18 rats were used. However, this is a standard method and it is unlikely to impact bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not explicitly stated, but likely to be okay via use of 96 well plates. Unsure of number of plates used and if all plates were generated at the same time
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but with automated methods and cell-based assay this is not likely to impact bias
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. Everything is reported with the exception of the number of wells that did not met inclusion criteria. This is not likely to impact bias.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on test substance and on dosing solutions. Purity reported as 95–99% depending on the chemical (source Sigma).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods. The time points were suitable and same for all groups. The culture conditions were appropriate. No blinded.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. However, there was no reporting on the number of well that were excluded due to not meeting inclusion criteria 'Only channels with >2 bursts/min were included in the analysis (active electrodes).'

Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity is not reported and some concentrations of deltamethrin are above those reported to cause cytotoxicity (e.g. 100 μM). However, significant effects on MBR were reported for concentrations well below those reported as cytotoxic by other papers.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PHRoB	N=9 or 10 per group. It is unclear if these are replicates or repetitions. It is possible to have generated all data on one plate.

Mohana Krishnan B, Prakhya BM, 2016 – RefID: 2842

Study characteristics and test system	Specific endpoint	Burst duration
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	30 minutes
	Concentration:	0 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–99 Day/s; 24 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the system was considered homogeneous (from the details reported in M&M in terms of cells pooling and automated read-out of the test).

Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Well reported in the Materials and methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is deemed that lack of adequate blinding during the study would not appreciably bias results. Most of the test is automated and the duration is short.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported but can be inferred. All cells are pulled together and automatically counted at the time of plating.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	Reported except for contamination check.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Blinding was not reported but the recording is automated clearing the impact of lack of blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Report is in line with the abstract and with the Materials and methods. A formal protocol is not reported.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not carried out but multiple doses, including low doses, were used and an IC50 calculated
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Direct evidence, reported.

Vassallo A, Chiappalone M, De Camargos Lopes R, Scelfo B, Novellino A, Defranchi E, Palosaari T, Weisschu T, Ramirez T, Martinoia S, Johnstone AFM, Mack CM, Landsiedel R, Whelan M, Bal-Price A, Shafer TJ, 2017 – RefID: 2959

Study characteristics	Specific endpoint	Burst duration
	Test system:	Primary cells

and test system	Species:	Mouse; Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation); Post-natal
	Medium:	Animal medium; Chemically defined medium (CDM)
	Funding source:	
	Number of biological replicate:	3; 4; 7
Exposure	Exposure duration:	10 minutes
	Concentration:	0 μM; 0.000001 μM; 0.0001 μM; 0.0003 μM; 0.001 μM; 0.01 μM; 0.1 μM; 0.3 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	15 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	All doses were carried out the same across all laboratories in an ascending sequence. This is not likely to cause bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	They were identical between study groups with a laboratory, but not across different laboratories.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not carried out in a blinded manner. It would be very hard to bias the results when using the automated MEA apparatus.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	No. Some data were not included due to: 1) a requirement that experiments with less than 5 concentrations were excluded -; 2) some experiments with corrupt or missing files were excluded 3) not all cultures were used (e.g. only well differentiated and healthy neuronal cultures were used) ;4) There were specific criteria listed for inclusion based on electrophysiological criteria. These criteria increased the quality of the cultures and did not likely
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	All criteria except solubility. However, concentrations used are known to be soluble at all concentrations tested except at 100µM.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Studies were well conducted and used well established methods etc. Moreover, findings were repeated across 4 laboratories. No blinding,
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	All measured outcomes were reported.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not directly assessed, but references are provided that there is no cytotoxicity at up to 50µM and effective concentrations were lower than that dose for deltamethrin.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	The study was well conducted and used enough replicates and experiments (N=3 to 9 depending on laboratory).

Meyer DA, Carter JM, Johnstone AF, Shafer TJ, 2008 – RefID: 3052

Study characteristics and test system	Specific endpoint	Burst duration
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public

	Number of biological replicate:	4
Exposure	Exposure duration:	10 minutes
	Concentration:	0 μM; 0.1 μM
	Treatment frequency:	Single
Measurement	Measurement time:	12 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the recording was automated.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Direct evidence. It is reported.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported but the experiment was of short duration.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Not reported. However, the condition on which the background peak to peak noise and threshold are well described.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Only one concentration was reported for this endpoint. However, this concentration was selected based on the outcome of the spontaneous inhibition (IC50) of the network activity in the same test system.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding not reported but considered of not affecting the outcome because of the automatic and computerised recording of the measures.

Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes, all reported. A formal protocol is lacking.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not reported but multiple doses were used in the inhibition of spontaneous network activity and a dose–response curve is described. The selected dose is representing the IC50 (in the same test system).
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Replicates were carried out.

Frank CL, Brown JP, Wallace K, Mundy WR, Shafer TJ, 2017 – RefID: 30344475

Study characteristics and test system	Specific endpoint	Burst duration
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	
	Treatment frequency:	Chronic
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Cell system and method were uniform and fully automated, with negative and positive control added to each plate.

Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	All the conditions were well described in the paper and characteristic of the high-throughput methodology.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	The method is fully automated and, although not declared in the paper, it is very likely that the compound is blinded up to the elaboration of the data as described in ToxCast testing.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Criteria are applied to each step of the protocol across all the 17 assays and for all chemicals in the battery. The high-throughput approach guarantee that all outcomes are treated equally and the same criteria for the assessment are applied.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Commercial source declared and concentrations are prepared and administered automatically.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	The prediction model is well described and in line with the experiments having a similar approach.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Raw data are available as supplement and summary table are presented in the paper.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Two cytotoxicity methods are applied, one metabolic and one directly measuring biomarker of cell death.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	The number of replicates is described and the definition of biological replicate, from which the assessment is performed, is reported.

Network formation/function (MEA): Interspike Interval

Meyer DA, Carter JM, Johnstone AF, Shafer TJ, 2008 – RefID: 3052

Study characteristics and test system	Specific endpoint	Interspike Interval
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	4
Exposure	Exposure duration:	10 minutes
	Concentration:	0 μM; 0.1 μM
	Treatment frequency:	Single
Measurement	Measurement time:	12 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the recording was automated.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Direct evidence, it is reported.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported but the experiment was of short duration.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	It is reported how the condition for inclusion should be used.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Only one concentration was reported for this endpoint. However, this concentration was selected based on the outcome of the spontaneous inhibition (IC50) of the network activity in the same test system.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding not reported but considered of not affecting the outcome because of the automatic and computerised recording of the measures.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes, all reported. A formal protocol is lacking.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not reported but multiple doses were used in the inhibition of spontaneous network activity and a dose–response curve is described. The selected dose is representing the IC50 (in the same test system).
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Replicates were carried out.

Frank CL, Brown JP, Wallace K, Mundy WR, Shafer TJ, 2017 – RefID: 30344475

Study characteristics and test system	Specific endpoint	Interspike Interval
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	

	Treatment frequency:	Chronic
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Cell system and method were uniform and fully automated, with negative and positive control added to each plate.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	All the conditions were well described in the paper and characteristic of the high-throughput methodology.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	The method is fully automated and, although not declared in the paper, it is very likely that the compound is blinded up to the elaboration of the data as described in ToxCast testing.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Criteria are applied to each step of the protocol across all the 17 assays and for all chemicals in the battery. The high-throughput approach guarantee that all outcomes are treated equally and the same criteria for the assessment are applied.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Commercial source declared and concentrations are prepared and administered automatically.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	The prediction model is well described and in line with the experiments having a similar approach.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Raw data are available as supplement and summary table are presented in the paper.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Two cytotoxicity methods are applied, one metabolic and one directly measuring biomarker of cell death.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	The number of replicates is described and the definition of biological replicate, from which the assessment is performed, is reported.

Network formation/function (MEA): Intervals between bursts

Mohana Krishnan B, Prakhya BM, 2016 – RefID: 2842

Study characteristics and test system	Specific endpoint	Intervals between bursts
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	30 minutes
	Concentration:	0 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–99 Day/s; 24 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the system was considered homogeneous (from the details reported in M&M in terms of cells pooling and automated read-out of the test)
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Experimental conditions were well reported and described in Materials and methods.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is deemed that lack of adequate blinding during the study would not appreciably bias results. Most of the test is automated and the duration is short.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported but can be inferred. All cells are pulled together and automatically counted at the time of plating.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	It is reported, except for the contamination check.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Blinding was not reported but the recording is automated clearing the impact of lack of blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	The report is in line with the abstract and with the Materials and methods section. However, a formal protocol is not reported.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not carried out but multiple doses, including low doses, were used and an IC50 calculated.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Direct evidence. Replicates reported.

Frank CL, Brown JP, Wallace K, Mundy WR, Shafer TJ, 2017 – RefID: 30344475

Study characteristics and test system	Specific endpoint	Interval between burst
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal

	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	
	Treatment frequency:	Chronic
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Cell system and method were uniform and fully automated, with negative and positive control added to each plate.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	All the conditions were well described in the paper and characteristic of the high-throughput methodology.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	The method is fully automated and, although not declared in the paper, it is very likely that the compound is blinded up to the elaboration of the data as described in ToxCast testing.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Criteria are applied to each step of the protocol across all the 17 assays and for all chemicals in the battery. The high-throughput approach guarantee that all outcomes are treated equally and the same criteria for the assessment are applied.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Commercial source declared and concentrations are prepared and administered automatically.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	The prediction model is well described and in line with the experiments having a similar approach.

Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Raw data are available as supplement and summary table are presented in the paper.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Two cytotoxicity methods are applied, one metabolic and one directly measuring biomarker of cell death.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	The number of replicates is described and the definition of biological replicate, from which the assessment is performed, is reported.

Network formation/function (MEA): MBR (mean burst rate)

Alloisio S, Nobile M, Novellino A, 2015 – RefID: 926

Study characteristics and test system	Specific endpoint	MBR (mean burst rate)
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	9
Exposure	Exposure duration:	20 minutes
	Concentration:	0 μM; 0.0001 μM; 0.001 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	21 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Unknown number of GD18 rats were used. However, this is a standard method, unlikely to impact bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not explicitly stated, but it is likely since 96 well plates were used. There is no certainty of the number of plates and if all plates were generated at the same time.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but with automated methods and cell-based assay it is not likely to impact bias.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. Everything was reported with the exception of the number of wells that did not met the inclusion criteria. This is not likely to impact bias.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on test substance and dosing solutions. Purity reported as 95–99% depending on the chemical (source Sigma).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods. Time point were suitable and the same for all groups. The culture conditions were appropriate. No blinded.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. However, there was no reporting on the number of well that were excluded due to not meeting inclusion criteria 'Only channels with >2 bursts/min were included in the analysis (active electrodes).'
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity is not reported. Some concentrations of deltamethrin are above those reported to cause cytotoxicity (e.g. 100 µM). However, significant effects on MBR were reported for concentrations well below those reported as cytotoxic by other papers.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N=9 or 10 per group. However, it is unclear if these are replicates or repetitions. It is possible to have generated all data on one plate.

Zheng J, Yu Y, Feng W, Li J, Liu J, Zhang C, Dong Y, Pessah IN, Cao Z, 2019 – RefID: 2452

Study characteristics and test system	Specific endpoint	MBR (mean burst rate)
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	7 Day
	Concentration:	0 μM; 0.01 μM; 0.03 μM
	Treatment frequency:	Chronic
Measurement	Measurement time:	5 Day/s; 8 Day/s; 11 Day/s; 14 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely based on the information in the methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely to impact automated procedures or growth of cultures.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. At least 3 independent cultures were used, as reported in the methods section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or media. Source was ChemService and purity was 99%. Exposure, culture, times etc. are all considered adequate. Three groups were tested (control and two deltamethrin).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard methods used but no blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity not reported, the highest concentration has been reported to be cytotoxic in other papers. In addition, IC50 values are below cytotoxic levels.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N/group is only 3 which is usually acceptable for in vitro studies, but it is low for most of all other types of studies.

Mohana Krishnan B, Prakhya BM, 2016 – RefID: 2842

Study characteristics and test system	Specific endpoint	MBR (mean burst rate)
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3

Exposure	Exposure duration:	30 minutes
	Concentration:	0 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–99 Day/s; 24 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the system was considered homogeneous (from the details reported in M&M in terms of cells pooling and automated read-out of the test)
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Experimental conditions were well reported and described in Materials and methods.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is deemed that lack of adequate blinding during the study would not appreciably bias results. Most of the test is automated and the duration is short.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported but can be inferred. All cells are pulled together and automatically counted at the time of plating.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	It is reported, except for the contamination check.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Blinding was not reported but the recording is automated clearing the impact of lack of blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	The report is in line with the abstract and with the Materials and methods section. However, a formal protocol is not reported.

Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not carried out but multiple doses, including low doses, were used and an IC50 calculated.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Direct evidence. reported

Meyer DA, Carter JM, Johnstone AF, Shafer TJ, 2008 – RefID: 3052

Study characteristics and test system	Specific endpoint	MBR
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	4
Exposure	Exposure duration:	10 minutes
	Concentration:	0 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM
	Treatment frequency:	Single
Measurement	Measurement time:	12 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the recording was automated.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not reported but considered not appreciably bias results.

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Direct evidence, reported.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported but the experiment was of short duration.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	It is reported how the condition for inclusion should be used.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Only one concentration was reported for this endpoint. However, this concentration was selected based on the outcome of the spontaneous inhibition (IC50) of the network activity in the same test system.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding not reported but considered of not affecting the outcome because of the automatic and computerised recording of the measures.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes, all reported. A formal protocol is lacking.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not reported but multiple doses were used in the inhibition of spontaneous network activity and a dose–response curve is described. The selected dose is representing the IC50 (in the same test system).
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Replicates were carried out.

Frank CL, Brown JP, Wallace K, Mundy WR, Shafer TJ, 2017 – RefID: 30344475

Study characteristics and test system	Specific endpoint	MBR
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal

	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	
	Treatment frequency:	Chronic
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Cell system and method were uniform and fully automated, with negative and positive control added to each plate.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	All the conditions were well described in the paper and characteristic of the high-throughput methodology.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	The method is fully automated and, although not declared in the paper, it is very likely that the compound is blinded up to the elaboration of the data as described in ToxCast testing.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Criteria are applied to each step of the protocol across all the 17 assays and for all chemicals in the battery. The high-throughput approach guarantee that all outcomes are treated equally and the same criteria for the assessment are applied.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Commercial source declared and concentrations are prepared and administered automatically.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	The prediction model is well described and in line with the experiments having a similar approach.

Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Raw data are available as supplement and summary table are presented in the paper.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Two cytotoxicity methods are applied, one metabolic and one directly measuring biomarker of cell death.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	The number of replicates is described and the definition of biological replicate, from which the assessment is performed, is reported.

Network formation/function (MEA): Mean firing rate in burst (spikes/sec.) (MFIB)

Vassallo A, Chiappalone M, De Camargos Lopes R, Scelfo B, Novellino A, Defranchi E, Palosaari T, Weisschu T, Ramirez T, Martinoia S, Johnstone AFM, Mack CM, Landsiedel R, Whelan M, Bal-Price A, Shafer TJ, 2017 – RefID: 2959

Study characteristics and test system	Specific endpoint	MFIB – Mean frequency intra-burst
	Test system:	Primary cells
	Species:	Mouse; Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation); Post-natal
	Medium:	Animal medium; Chemically defined medium (CDM)
	Funding source:	
	Number of biological replicate:	3; 4; 7
Exposure	Exposure duration:	10 minutes
	Concentration:	0 μM; 0.000001 μM; 0.0001 μM; 0.0003 μM; 0.001 μM; 0.01 μM; 0.1 μM; 0.3 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	15 Day/s
Risk of bias appraisal		Tier: NA

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?		
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?		
Exposure condition across groups	Q3: were experimental conditions identical across study groups?		
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?		
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?		
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?		
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?		
Selective outcome report	Q8: were all measured outcomes reported?		
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY		
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES		

Network formation/function (MEA): Mean Interspike Interval in Burst (MISIB)

Alloisio S, Nobile M, Novellino A, 2015 – RefID: 926

Study characteristics and test system	Specific endpoint	MISIB (Mean interspike interval in burst)
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	9
Exposure	Exposure duration:	20 minutes
	Concentration:	0 μM; 0.0001 μM; 0.001 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	21 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Unknown number of GD18 rats were used. However, this is a standard method, unlikely to impact bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not explicitly stated, but it is likely since 96 well plates were used. There is no certainty of the number of plates and if all plates were generated at the same time.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but with automated methods and cell-based assay it is not likely to impact bias.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. Everything was reported with the exception of the number of wells that did not met the inclusion criteria. This is not likely to impact bias.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on test substance and dosing solutions. Purity reported as 95–99% depending on the chemical (source Sigma).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods. Time point were suitable and the same for all groups. The culture conditions were appropriate. No blinded.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. However, there was no reporting on the number of well that were excluded due to not meeting inclusion criteria 'Only channels with >2 bursts/min were included in the analysis (active electrodes).'
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity is not reported. Some concentrations of deltamethrin are above those reported to cause cytotoxicity (e.g. 100 µM). However, significant effects on MBR were reported for concentrations well below those reported as cytotoxic by other papers.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N=9 or 10 per group. However, it is unclear if these are replicates or repetitions. It is possible to have generated all data on one plate.

Mohana Krishnan B, Prakhya BM, 2016 – RefID: 2842

Study characteristics and test system	Specific endpoint	MISIB (Mean interspike interval in burst)
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	30 minutes

	Concentration:	0 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–99 Day/s; 24 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the system was considered homogeneous (from the details reported in M&M in terms of cells pooling and automated read-out of the test)
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Experimental conditions were well reported and described in Materials and methods.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is deemed that lack of adequate blinding during the study would not appreciably bias results. Most of the test is automated and the duration is short.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported but can be inferred. All cells are pulled together and automatically counted at the time of plating.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	It is reported, except for the contamination check.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Blinding was not reported but the recording is automated clearing the impact of lack of blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	The report is in line with the abstract and with the Materials and methods section. However, a formal protocol is not reported.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not carried out but multiple doses, including low doses, were used and an IC50 calculated.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Direct evidence. reported

Network formation/function (MEA): MFR

Alloisio S, Nobile M, Novellino A, 2015 – RefID: 926

Study characteristics and test system	Specific endpoint	MFR
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	9
Exposure	Exposure duration:	20 minutes
	Concentration:	0 μM; 0.0001 μM; 0.001 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	21 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Unknown number of GD18 rats were used. However, this is a standard method, unlikely to impact bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant.

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not explicitly stated, but it is likely since 96 well plates were used. There is no certainty of the number of plates and if all plates were generated at the same time.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but with automated methods and cell-based assay it is not likely to impact bias.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. Everything was reported with the exception of the number of wells that did not met the inclusion criteria. This is not likely to impact bias.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on test substance and dosing solutions. Purity reported as 95–99% depending on the chemical (source Sigma).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods. Time point were suitable and the same for all groups. The culture conditions were appropriate. No blinded.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. However, there was no reporting on the number of well that were excluded due to not meeting inclusion criteria 'Only channels with >2 bursts/min were included in the analysis (active electrodes).'
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity is not reported. Some concentrations of deltamethrin are above those reported to cause cytotoxicity (e.g. 100 µM). However, significant effects on MBR were reported for concentrations well below those reported as cytotoxic by other papers.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N=9 or 10 per group. However, it is unclear if these are replicates or repetitions. It is possible to have generated all data on one plate.

Zheng J, Yu Y, Feng W, Li J, Liu J, Zhang C, Dong Y, Pessah IN, Cao Z, 2019 – RefID: 2452

Study characteristics and test system	Specific endpoint	MFR
	Test system:	Primary cells
	Species:	Mouse

	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	7 Day
	Concentration:	0 μM; 0.01 μM; 0.03 μM
	Treatment frequency:	Chronic
Measurement	Measurement time:	5 Day/s; 8 Day/s; 11 Day/s; 14 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely based on the information in the methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely to impact automated procedures or growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Yes. At least 3 independent cultures were used, as reported in the methods section.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or media. Source was ChemService and purity was 99%. Exposure, culture, times etc. are all considered adequate. Three groups were tested (control and two deltamethrin).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard methods used but no blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity not reported, the highest concentration has been reported to be cytotoxic in other papers. In addition, IC50 values are below cytotoxic levels.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N/group is only 3 which is usually acceptable for in vitro studies, but it is low for most of all other types of studies.

Vassallo A, Chiappalone M, De Camargos Lopes R, Scelfo B, Novellino A, Defranchi E, Palosaari T, Weisschu T, Ramirez T, Martinoia S, Johnstone AFM, Mack CM, Landsiedel R, Whelan M, Bal-Price A, Shafer TJ, 2017 – RefID: 2959

Study characteristics and test system	Specific endpoint	MFR
	Test system:	Primary cells
	Species:	Mouse; Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Fetal (=gestation); Post-natal
	Medium:	Animal medium; Chemically defined medium (CDM)
	Funding source:	
	Number of biological replicate:	3; 4; 7
Exposure	Exposure duration:	10 minutes

	Concentration:	0 μM; 0.000001 μM; 0.0001 μM; 0.0003 μM; 0.001 μM; 0.01 μM; 0.1 μM; 0.3 μM; 1 μM; 10 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–99 nr; –9 nr; 15 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	All doses were carried out the same across all laboratories in an ascending sequence. This is not likely to cause bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not reported. But allocation bias is not a concern for in vitro studies.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	They were identical between study groups with a laboratory, but not across different laboratories.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not carried out in a blinded manner. It would be very hard to bias the results when using the automated MEA apparatus.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	No. Some data were not included due to: 1) a requirement that experiments with less than 5 concentrations were excluded -; 2) some experiments with corrupt or missing files were excluded.'; 3) not all cultures were used (e.g. only well differentiated and healthy neuronal cultures were used) 4) There were specific criteria listed for inclusion based on electrophysiological criteria.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	All criteria except solubility. However, concentrations used are known to be soluble at all concentrations tested except at 100μM.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Studies were well conducted and used well established methods etc. Moreover, findings were repeated across 4 laboratories.

Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	All measured outcomes were reported.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not directly assessed, but references are provided that there is no cytotoxicity at up to 50µM and effective concentrations were lower than that dose for deltamethrin.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	The study was well conducted and used enough replicates and experiments (N=3 to 9 depending on laboratory).

Meyer DA, Carter JM, Johnstone AF, Shafer TJ, 2008 – RefID: 3052

Study characteristics and test system	Specific endpoint	MFR
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	4
Exposure	Exposure duration:	10 minutes
	Concentration:	0 µM; 0.01 µM; 0.1 µM; 1 µM; 10 µM
	Treatment frequency:	Single
Measurement	Measurement time:	12 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the recording was automated.

Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not reported but considered not appreciably bias results.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Direct evidence, reported.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported but the experiment was of short duration.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	It is reported how the condition for inclusion should be used.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Only one concentration was reported for this endpoint. However, this concentration was selected based on the outcome of the spontaneous inhibition (IC50) of the network activity in the same test system.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding not reported but considered of not affecting the outcome because of the automatic and computerised recording of the measures.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes, all reported. A formal protocol is lacking.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not reported but multiple doses were used in the inhibition of spontaneous network activity and a dose–response curve is described. The selected dose is representing the IC50 (in the same test system).
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Replicates were carried out.

Frank CL, Brown JP, Wallace K, Mundy WR, Shafer TJ, 2017 – RefID: 30344475

Study characteristics and test system	Specific endpoint	MFR
	Test system:	Primary cells
	Species:	Rat

	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	
	Treatment frequency:	Chronic
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Cell system and method were uniform and fully automated, with negative and positive control added to each plate.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	All the conditions were well described in the paper and characteristic of the high-throughput methodology.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	The method is fully automated and, although not declared in the paper, it is very likely that the compound is blinded up to the elaboration of the data as described in ToxCast testing.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Criteria are applied to each step of the protocol across all the 17 assays and for all chemicals in the battery. The high-throughput approach guarantee that all outcomes are treated equally and the same criteria for the assessment are applied.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Commercial source declared and concentrations are prepared and administered automatically.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	The prediction model is well described and in line with the experiments having a similar approach.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Raw data are available as supplement and summary table are presented in the paper.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Two cytotoxicity methods are applied, one metabolic and one directly measuring biomarker of cell death.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	The number of replicates is described and the definition of biological replicate, from which the assessment is performed, is reported.

Neurophysiology/patch clamp: Membrane excitability

Rekling JC, Theophilidis G, 1995 – RefID: 2718

Study characteristics and test system	Specific endpoint	Membrane excitability: Burst duration; decrementing phase duration; Interval between spikes; peak amplitude
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	
	Stage of development of the primary cells:	Post-natal
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	4
Exposure	Exposure duration:	–99 nr; 20 minutes
	Concentration:	0 nM; 100 nM
	Treatment frequency:	Single

Measurement	Measurement time:	–99 nr; 0 min; 5 min; 16 min
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Each slice served as its own control.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Each slice served as its own control. However, it is unknown whether conditions were similar between controls.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported. It is likely that a lack of adequate blinding during the study would not appreciably bias results.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	There is no information on the total number of mice or slices used. n = 1 for all the results figures.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out on source purity, stock or media concentrations. It is likely that the purity is of technical grade from the manufacturer Roussel-Uclaf. Only one concertation and a control were tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The methods used were well established. Time and culture conditions were adequate. No blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	NR	Cytotoxicity was not measured in the slices.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DHRoB	Yes. Only n = 1 for all the results. No replicates or repetitions.

Ruigt GS, Neyt HC, Van der Zalm JM, Van den Bercken J, 1987 – RefID: 2861

Study characteristics and test system	Specific endpoint	Membrane excitability: (td); (ts); (tt)
	Test system:	Cell line
	Species:	Mouse
	Origin of the test system:	
	Stage of development of the primary cells:	
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	–99
Exposure	Exposure duration:	–99 nr
	Concentration:	0 μM; 100 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–99 nr;
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	The randomisation sequence is not reported. However, the type of response expected for this kind of studies (yes/no) indicates that the impact of lack of randomisation would not overall representing a bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not reported but considered not to influence the outcome of the study.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Experimental conditions were well described in the Materials and methods section.

Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported but considering the short duration of the test it was considered of probably low risk of bias (PLRoB).
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	It is not clear how many replicates were carried out.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	The concentration used can only be inferred.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The method is considered acceptable but not fulfilling the higher requirements.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. Details are reported
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity was not performed
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	NR	Not possible to infer if replicates were carried out.

Ogata N, Vogel SM, Narahashi T, 1988 – RefID: 2930

Study characteristics and test system	Specific endpoint	Membrane excitability
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Dorsal root ganglia
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	–99

Exposure	Exposure duration:	–99 nr
	Concentration:	0.01 mM
	Treatment frequency:	Single
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	One single exposure applied. The read-out of the study was recorded by a machine
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not reported but the read-out was automated
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Sufficiently reported.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	All data listed in the Materials and methods section and in the abstract were reported without the necessity of inferring. However, no raw data available.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	Only the single used concentration is reported.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The outcome of the assessment was well reported, but blinding is not reported.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Results are in line with the abstract and with the Materials and methods section.

Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not reported, but all the cells responded to the electrical stimulus.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	5 experiments were conducted

Meyer DA, Shafer TJ, 2006 – RefID: 3002

Study characteristics and test system	Specific endpoint	Membrane excitability: mEPSC; sEPSC; sEPSC – Interevent interval
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	–99
Exposure	Exposure duration:	–99 nr
	Concentration:	0 μM; 1 μM; 10 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–99 nr; 0 min; 2.5 min; 7 min; 50 sec.; 1000 sec.; 1300 sec.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but the system is automated; therefore, it is considered that the influence of blind would be probably low.

Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not reported but considered to not appreciably bias the results.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, well reported.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported. However, it is consider to not appreciably bias the results because of the short duration of the study and automated procedures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	From the analysis of the data it is possible to retrieve that there was no attrition.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	Only one concentration tested
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding was not reported. All the remaining criteria were reported
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Not reported and only representative recording shown.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not reported. The study duration is short, and cells responded to the electrophysiological stimulation at the tested concentration.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	5 Replicates were reported.

Meyer DA, Carter JM, Johnstone AF, Shafer TJ, 2008 – RefID: 3052

Study characteristics and test system	Specific endpoint	Membrane excitability: Burst duration; Events/Burst; sEPSC – Interevent interval
	Test system:	Primary cells

	Species:	Rat
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	–99
Exposure	Exposure duration:	10 minutes
	Concentration:	0 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM
	Treatment frequency:	Single
Measurement	Measurement time:	8 Day/s
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but mixed hippocampal cell cultures were used, and the recording was automated.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not applicable.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Direct evidence, reported
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported but considered of low impact because most of the procedures were automated and the study duration was short, therefore minimising the impact of blinded personnel on handling the cells.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Not reported but inferred from the results.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Many details were reported, the use of multiple concentration is retrieved from the results.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Blinding not reported but considered of not affecting the outcome because of the automatic and computerised recording of the measures.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes, all reported and tabulated. A formal protocol is lacking.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Not reported but multiple concentrations were used, including low concentrations. A dose–response curve was provided.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	NR	Not reported.

Neurotransmitters: GABA

Grosse G, Thiele T, Heuckendorf E, Schopp E, Merder S, Pickert G, Ahnert-Hilger G, 2002 – RefID: 1794

Study characteristics and test system	Specific endpoint	GABA – Basal release
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	0 nM; 20 nM; 200 nM
	Treatment frequency:	Multiple

Measurement	Measurement time:	17 Day/s
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported, but concurrent control was used.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported, but it is highly likely based on methods descriptions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but lack of blinding would not appreciably bias the results.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Only one concentration reported.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	Only two concentrations.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Methods used were acceptable. Assessed at the same time point after exposure in all study groups. The conditions for cultivation and exposure were appropriate. No blinding, and subjective assessments.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	The measured outcome was reported but not for all doses.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Excess cytotoxicity was reported for concentration greater than 20 nM (20nM = 14%, 60 nM = 45%), but there were some effects on some synaptic measures at 2 and 20 nM.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	Only one replicate presented in the figure, but the median and standard deviations were reported for 11 replicates.

Neurotransmitters: Glutamate

Grosse G, Thiele T, Heuckendorf E, Schopp E, Merder S, Pickert G, Ahnert-Hilger G, 2002 – RefID: 1794

Study characteristics and test system	Specific endpoint	Glutamate – Basal release
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	0 nM; 20 nM; 200 nM
	Treatment frequency:	Multiple
Measurement	Measurement time:	17 Day/s
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported, but concurrent control was used.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant.

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported, but it is highly likely based on methods descriptions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but lack of blinding would not appreciably bias the results.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Only one concentration reported
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	Only two concentrations
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Methods used were acceptable. Assessed at the same time point after exposure in all study groups. The conditions for cultivation and exposure were appropriate. No blinding, and subjective assessments.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	The measured outcome was reported but not for all doses.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Excess cytotoxicity was reported for concentration greater than 20 nM (20nM = 14%, 60 nM = 45%), but there were some effects on some synaptic measures at 2 and 20 nM.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	Only one replicate presented in the figure, but the median and standard deviations were reported for 11 replicates.

Proteins: Apoptotic proteins

Wu A, Li L, Liu Y, 2003 – RefID: 2613

Study characteristics and test system	Specific endpoint	Apoptotic proteins: Bax; Bcl-2; p53
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Cortical

	Stage of development of the primary cells:	Post-natal
	Medium:	Chemically defined medium (CDM)
	Funding source:	Public
	Number of biological replicate:	–9
Exposure	Exposure duration:	–9 nr
	Concentration:	0 nM; 100 nM
	Treatment frequency:	Single
Measurement	Measurement time:	0 PDN; 24 PDN; 48 PDN; 72 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported. No information provided on how the cultures were divided for use. Nothing on number of cultures or number of rats used to generate cultures.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported. It is assumed that methods descriptions applied to all cultures.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported but unlikely to impact bias for cell culture experiments.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Not reported. There is information in the paper, either in the methods or results sections, on the number of samples for any endpoint. A search for n = , group, etc. was negative.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	No purity and no source provided. No analytical work was carried out.

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Methods used were standard for cultures and staining. No blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes. All the endpoints mentioned in the methods are reported.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DHRoB	Cytotoxicity was measured. Deltamethrin at concentration of 100 and 300 nM caused from 70–90 % cytotoxicity (depending on concentrations and time points). All data presented on apoptotic proteins was conducted at 100 nM.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	NR	No information on replicates or repetitions.

Proteins: Phosphorylation proteins

Imamura L, Yasuda M, Kuramitsu K, Hara D, Tabuchi A, Tsuda M, 2006 – RefID: 2622

Study characteristics and test system	Specific endpoint	Phosphorylation proteins (BDNF)
	Test system:	Primary cells
	Species:	Rat
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Embryo
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	3 hours
	Concentration:	0 μM; 1 μM
	Treatment frequency:	Single
Measurement	Measurement time:	3 PDN; 24 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely they were based on the information in methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely to impact in vitro cultures measurements.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Hard to determine – no n/group are reported in methods section. Figures present in the publication reported mean and SD from three experiments.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on stock, source or media. Source was technical-grade deltamethrin purchased from Wako Pure Chemicals (Tokyo, Japan).
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard methods. No blinding, but it is assumed to have no impact on bias
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity not measured, but all concentrations were below the normal reported cytotoxicity levels
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PHRoB	Not clear. The only information available is 'The columns represent the mean and SD from three independent experiments'. Nothing is reported about the numbers in each experiment which could be only one.

Proteins: Synaptic Proteins

Grosse G, Thiele T, Heuckendorf E, Schopp E, Merder S, Pickert G, Ahnert-Hilger G, 2002 – RefID: 1794

Study characteristics and test system	Specific endpoint	Synaptic proteins: Kv1.1; Kv1.2; SNAP25; Synaptobrevin; Synaptophysin
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Hippocampal pyramidal neurons
	Stage of development of the primary cells:	Fetal (=gestation)
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	12 Day
	Concentration:	0 nM; 2 nM; 20 nM; 200 nM; 2000 nM
	Treatment frequency:	Multiple
Measurement	Measurement time:	17 Day/s
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported, but concurrent control was used.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported, but it is highly likely based on methods descriptions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but lack of blinding would not appreciably bias the results.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Only one concentration reported
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	Only two concentrations
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Methods used were acceptable. Assessed at the same time point after exposure in all study groups. The conditions for cultivation and exposure were appropriate. No blinding, and subjective assessments.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	The measured outcome was reported but not for all doses.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Excess cytotoxicity was reported for concentration greater than 20 nM (20nM = 14%, 60 nM = 45%), but there were some effects on some synaptic measures at 2 and 20 nM.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	Only one replicate presented in the figure, but the median and standard deviations were reported for 11 replicates.

Receptors: Ryanodine

Zheng J, Yu Y, Feng W, Li J, Liu J, Zhang C, Dong Y, Pessah IN, Cao Z, 2019 – RefID: 2452

Study characteristics and test system	Specific endpoint	Ryanodine – [3H]Ry binding to RyRs in cortex
	Test system:	Primary cells
	Species:	Mouse
	Origin of the test system:	Cortical
	Stage of development of the primary cells:	Post-natal
	Medium:	Animal medium
	Funding source:	Public
	Number of biological replicate:	3

Exposure	Exposure duration:	3 hours
	Concentration:	0 μM; 0.003 μM; 0.01 μM; 0.03 μM; 0.1 μM; 0.3 μM; 1 μM; 10 μM; 3 μM
	Treatment frequency:	Single
Measurement	Measurement time:	–9 nr
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but it is likely based on the information reported in the methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but it is unlikely to impact automated procedures or growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Data only presented in supplemental files.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or media. Source was ChemService and purity was 99%. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard methods were used but no blinding.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.

Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Cytotoxicity was not reported. The highest concentration has been reported to be cytotoxic in other papers. IC50 values are below cytotoxic levels.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	N/group is 3 or 4. This number is usually acceptable for in vitro studies but is low for most all other types of studies.

Critical Appraisal table zebrafish

BehavZF: Locomotor activity ## Awoyemi, O. M., Kumar, N., Schmitt, C., Subbiah, S., Crago, J., 2019 – RefID: 1532

Study characteristics and population	Specific endpoint	Locomotor activity – Average velocity; Locomotor activity – Total distance travelled
	Experiment description:	videorecorded for 50 min with 10 min acclimation followed by two cycles of light/dark for 10 min
	Strain:	Hybrid strain (AB – Females, 5D – males)
	EmbryWell:	24
	Water:	Egg water-60 μg/mL sea salt
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	5–120 hours
	Concentration:	0 μg/L; 0.01 μg/L; 0.1 μg/L; 10 μg/L
Measurement	Measurement time:	120
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported. But likely to not severely impact bias.

Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but methods descriptions state similar culture conditions, same media, etc. were used in the 24-well plate.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	not reported. but automated test equipment likely minimised potential bias
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Unclear. The only description in the methods is 'The ZF embryos 5 hpf (n = 24) were exposed to five treatments of each pyrethroid (0.0, 0.01, 0.1, 10, 1000 mg/L pyrethroid)'. Does this translate to n = 24/group, or 24 total. And the figures do not contain any information on group size. There is a statement about interplate variability – but unclear if this was between treatments.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	Yes. They reported use of 99% deltamethrin AND they ran analytical checks on the media on days 0, 1, 2, 3 and 5.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Not blinded. But, well established automated methods. same time points, etc.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Exceptions are: 1) mortality which was reported to occur in the high dose, but no mention in the results of real data. 2) descriptions of morphological outcomes were reported in the results without data. And no methods reported in the methods section.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	Mortality at high concentration. But this concentration data were excluded from analyses

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PLRoB	Unclear if there was more than one plate was run or what total n/group was.

Kung TS, Richardson JR, Cooper KR, White LA, 2015 – RefID: 1935

Study characteristics and population	Specific endpoint	Locomotor activity – Total distance travelled
	Experiment description:	Swim activity following a transition into darkness of 2-week-old larval. Larvae were allowed to acclimate on the testing apparatus for 1 h in light (400 lux). After 1 h, lights were turned off to stimulate activity and video was recorded.; together with another substance
	Strain:	AB
	EmbryWell:	30
	Water:	Egg water-60 μg/mL sea salt
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	3–72 hours
	Concentration:	0 μg/L; 0.25 μg/L; 0.33 μg/L; 0.50 μg/L
Measurement	Measurement time:	10; 15; 20; 25; 30; 35; 40; 5
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but probably not going to affect bias.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported but methods section provides good descriptions and same media, temp, time, etc.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	not reported, but automated methods make it unlikely to impact bias
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Unclear. Figure reports (n = 31–38). But methods states only that 'Studies were repeated 3 times (from 3 different breeding sets)'. So unclear how many animals were actually exposed.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work carried out on media, source or stock solutions.. Purity (99%) and source (ChemServ) were reported
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	well established automated methods were used, suitable time point for assessment, etc. not blinded
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	yes
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PLRoB	not reported but methods states. 'Embryos were incubated at 25–26°C in darkness and observed daily using a dissecting microscope for mortality and to ensure that there were no developmental abnormalities or signs of acute toxicity.'
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	No. Studies were repeated 3 times (from 3 different breeding sets). For behavioural tests 3 replicate plates were used but stats model did not include plate as a dependent variable.. Three doses and control used.

Liu X, Zhang Q, Li S, Mi P, Chen D, Zhao X, Feng X, 2018 – RefID: 1971

Study characteristics and population	Specific endpoint	Locomotor activity – Rest bouts; Locomotor activity – Rest bouts length; Locomotor activity – Rest total; Locomotor activity – Total activity; Locomotor activity – Waking activity
	Experiment description:	dark condition (biological exp 1); dark condition (biological exp 2); light condition (biological exp 1); light condition (biological exp 2)
	Strain:	AB
	EmbryWell:	30
	Water:	Standard
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	96–108 hours
	Concentration:	0 μg/L; 0.01 μg/L; 0.1 μg/L; 1 μg/L; 10 μg/L; 100 μg/L; 50 μg/L

Measurement	Measurement time:	48
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported, but likely based on methods descriptions
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact automated assessments.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	The methods states that n = 30 per group. The figure dose not report the N/group but only states that 'Each value represents the average of about 30 larvae (Because of the insecticide treatment, larvae had different levels of death.).
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	Analytical purity of the source was confirmed. No analytical work on the stock solutions of the exposure media.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	No blinding
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DHRoB	Also the effect reported as significant in Figure <Author: Please add figure number.>have extremely high variability (e.g. some CVs are greater than 100%). And some means with very small differences (e.g, about 8%). All concentrations were associated with increased mortality (Figure 1)
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PHRoB	The issue of only one exposure well for 30 embryos makes it questionable as to whether they were independent measures. 'The next morning, fertilised embryos were collected after spawning, and then moved to 6-well plates, 30 embryos per well.'

BehavZF: Photomotor Response (EPR – Embryo photomotor response)

Hagstrom D, Truong L, Zhang SQ, Tanguay R, Collins EMS, 2019 – RefID: 30344671

Study characteristics and population	Specific endpoint	Photomotor Response (EPR – Embryo photomotor response); Photomotor Response (LPR – Larval photomotor response)
	Experiment description:	the data were converted to binary data: hit or no hit
	Strain:	Tropical 5D wild-type zebrafish
	EmbryWell:	1
	Water:	nr
	Funding source:	Public
	Number of biological replicate:	36
Exposure	Exposure duration:	6–120 hours
	Concentration:	
Measurement	Measurement time:	120; 24
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	digitally dispensed directly from the 20 mM stocks into the test wells using a Hewlett Packard D300e
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	to be deleted
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Same conditions
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	Automatic process
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Automatic analysis
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	See Supplementary material
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Well established methods
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	No protocol available?
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Mortality checked and considered in the analysis

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	NR	NR replicates for zebrafish

BehavZF: Photomotor Response (LPR – Larval photomotor response)

Hagstrom D, Truong L, Zhang SQ, Tanguay R, Collins EMS, 2019 – RefID: 30344671

Study characteristics and population	Specific endpoint	Photomotor Response (EPR – Embryo photomotor response); Photomotor Response (LPR – Larval photomotor response)
	Experiment description:	the data were converted to binary data: hit or no hit
	Strain:	Tropical 5D wild-type zebrafish
	EmbryWell:	1
	Water:	nr
	Funding source:	Public
	Number of biological replicate:	36
Exposure	Exposure duration:	6–120 hours
	Concentration:	
Measurement	Measurement time:	120; 24
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	digitally dispensed directly from the 20 mM stocks into the test wells using a Hewlett Packard D300e
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	DLRoB	to be deleted
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Same conditions
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	Automatic process
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Automatic analysis
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	See Supplementary material

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Well established methods
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	No protocol available?
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Mortality checked and considered in the analysis
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	NR	NR replicates for zebrafish

BehavZF: Spasms

DeMicco A, Cooper KR, Richardson JR, White LA, 2010 – RefID: 1687

Study characteristics and population	Specific endpoint	Spasms
	Experiment description:	exp. 1 (spasms)
	Strain:	AB
	EmbryWell:	1
	Water:	Standard
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	3–96 hours
	Concentration:	0 μg/L; 1 μg/L; 10 μg/L; 50 μg/L
Measurement	Measurement time:	96
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. Eggs were 'collected and randomly sorted into groups'
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Allocation bias does not exist in zebrafish studies

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported, but indirect evidence that all conditions were the same across groups based on described methods.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Lack of blinding in study not likely to impact bias.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Unknown. The methods state that three replicates of 5–25 fish per dose group. 'Exposure studies were repeated at least three times for each compound tested, with 5 to 25 fish per dose group' But most figures state 20 to 25 fish per dose group. But no explanation for why the group size varied to such a large degree and why the figure legends are different from the methods section text.. Unable to determine whether this was due to exclusions.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DHRoB	There was not analytical work carried out on the stock chemicals nor on the water exposures. DLT solubility in water ranges from 0.2–2.0 μg/L. NO information provided on solubility, and unknown how nn-dimethylformamide (DMF) in stock solutions affects solubility. Sigma reports 'The product is insoluble in water. '
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Subjective method. Time points okay. No blinding.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PHRoB	Effective concentrations caused up to 60% mortality for some endpoints.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	There were adequate numbers of subjects (n = 20–25).

BehavZF: Thigmotaxic

Li M, Liu X, Feng X, 2019 – RefID: 1959

Study characteristics and population	Specific endpoint	Thigmotaxic
	Experiment description:	NA
	Strain:	nr
	EmbryWell:	30
	Water:	nr
	Funding source:	Public
	Number of biological replicate:	3
Exposure	Exposure duration:	0–0 hours
	Concentration:	0 μg/L; 0.1 μg/L; 1 μg/L; 10 μg/L; 25 μg/L; 50 μg/L
Measurement	Measurement time:	72
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported but not likely to impact bias in zebrafish.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Not relevant
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported but likely based on information reported in the methods section.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported but unlikely to impact bias in a zebrafish study
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Unclear. method states n = 30/group. But figure legend reports ‘Experimental repeated 3 times, n = 20–30. Error bars represents the standard error of the mean (SEM).’

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	More than most studies. The purity of the source chemicals was checked. But no check of media concentrations or stock solutions
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Unclear. The method used not standard and was subjective. There was minimal description of the method provided in methods section. No blinding of assessors.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	yes
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	PHRoB	Yes, Thigmotaxis effects seen at high dose. But Morphological effects were found at all dose except the lowest dose. And vascular effects were found at all doses. This confounds the very small effect on thigmotaxis at the lowest dose.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	PHRoB	It appears that all embryos from each dose group are all from the same exposure well. From methods '10 h after fertilisation (hpf), the fertilised embryos were transferred to a 6-well plate, 30 embryos per well.' This suggests that the N=1/group.

BehavZF: Total distance travelled (Photomotor Behaviour)

Dach K, Yaghoobi B, Schmuck MR, Carty DR, Morales KM, Lein PJ, 2019 – RefID: 30344692

Study characteristics and population	Specific endpoint	Total distance travelled (Photomotor Behaviour)
	Experiment description:	Exposure paradigm: 5 min light, 5 min dark, 5 min light, 15 min dark. Endpoint measured at 4 and 5 dpf
	Strain:	Tropical 5D wild-type zebrafish
	EmbryWell:	1
	Water:	System fish water (salt water)
	Funding source:	Public
	Number of biological replicate:	16
Exposure	Exposure duration:	1–5 hours
	Concentration:	0 μM; 0.003 μM; 0.01 μM; 0.03 μM; 0.1 μM; 0.3 μM

Measurement	Measurement time:	120.1; 120.10; 120.11; 120.12; 120.13; 120.14; 120.15; 120.16; 120.17; 120.18; 120.19; 120.2; 120.20; 120.21; 120.22; 120.23; 120.24; 120.25; 120.26; 120.27; 120.28; 120.29; 120.3; 120.30; 120.4; 120.5; 120.6; 120.7; 120.8; 120.9; 96.1; 96.10; 96.11; 96.12; 96.13; 96.14; 96.15; 96.16; 96.17; 96.18; 96.19; 96.2; 96.20; 96.21; 96.22; 96.23; 96.24; 96.25; 96.26; 96.27; 96.28; 96.29; 96.3; 96.30; 96.4; 96.5; 96.6; 96.7; 96.8; 96.9
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Randomisation, automated
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Question to be deleted
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, 96 plate automated
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	DLRoB	Automated process
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	All data analysis, automatic analysis
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	See Material and methods
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Well established method
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Mortality measured in the same study and reported in the paper, see Figure 3 (30 μM).
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	Each experiment included 16 biological replicates.

Critical Appraisal table-In vivo

Behavioural: Impulsive Behaviour

Richardson JR, Taylor MM, Shalat SL, Guillot TS, 3rd Caudle WM, Hossain MM, Mathews TA, Jones SR, Cory-Slechta DA, Miller GW, 2015 – RefID: 361

Study characteristics and population	Specific endpoint	Impulsive Behaviour: FR Resets; Mean Long Wait
	Specie:	Mouse
	Strain:	C57BL/6J
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	8 (4–9 litters/treatment group), (1 mouse/sex per litter/behavioural test)
Exposure	Period of exposure:	From GD 0 to PDN 22
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	6 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly reported, but likely based on methods used.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Unable to ascertain. Insufficient information provided in the Materials and methods of the paper: 3 separate cohorts of animals, representing 9 litres per treatment group, with the litter used as the individual unit of analysis and no more than 1 mouse/sex/litter used for any behavioural test. There is no rationale or explanation for the very unbalanced group sizes. Also, there is no mention of whether there was balance across the '3 cohorts'. The figure in publication, reports 'n = 4–7' – so it is unclear what happened to the n = 9.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, probably technical grade (source ChemService). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods, but no blinding reported.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Some data not reported.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	Not measured or reported if any toxicity resulted from the exposures. It is not likely at the doses used.

Behavioural: Learning and memory

Richardson JR, Taylor MM, Shalat SL, Guillot TS, 3rd Caudle WM, Hossain MM, Mathews TA, Jones SR, Cory-Slechta DA, Miller GW, 2015 – RefID: 361

Study characteristics and population	Specific endpoint	Learning and memory (Y maze): % alternation; Same arm entries
	Specie:	Mouse
	Strain:	C57BL/6J
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	8 (4–9 litters/treatment group), (1 mouse/sex per litter for each behavioural test)
Exposure	Period of exposure:	From GD 0 to PDN 22

	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	6 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly reported, but likely based on methods used.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Unable to ascertain. Insufficient information provided: 3 separate cohorts of animals, representing 9 litters per treatment group, with the litter used as the individual unit of analysis and no more than 1 mouse/sex/litter used for any behavioural test. There is no rationale or explanation for the very unbalanced group sizes. There is no mention of whether there was balance across the '3 cohorts' The figure in publication reports 'n = 7–8' – so unclear what happened to the n = 9.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, probably technical grade (source ChemService). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose was tested.

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods, but no blinding reported.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Some data are missing e.g. maternal toxicity and body weight.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	Not measured or reported if any toxicity resulted from the exposures. It is not likely at the doses used.

Vester AI, Chen M, Marsit CJ, Caudle WM, 2019 – RefID: 961

Study characteristics and population	Specific endpoint	Learning and memory (Y maze): Errors; Same arm entries
	Specie:	Mouse
	Strain:	C57BL/6NCrl
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	–99
Exposure	Period of exposure:	From Pre-Mating day 1 to PDN 21
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	8 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Stated randomisation of dams.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PHRoB	Only partially reported for lighting on/off, no information about T°, humidity, feed type and water source. Unclear whether peanut butter vehicle was given to controls. Same water used for CORT exposures in both groups.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine since there were no reports of the number of litters used. Number of samples reported varies from 7–9 per group depending on the figure.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, but considered as technical grade based on the source (Sigma). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose used.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Automated and well established methods not prone to bias. But no clear statement that assessors were blind to treatment.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Pups body weight and survival were measured but not reported, except for the statement 'However, the exposures themselves did not significantly affect the body weight gain and survival (data not shown). It is unclear if dam body weights were recorded. CORT was administered in water, but there is no mention of dams' water consumption.

Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	No data on toxicity or body weight of pups or dams. Body weight and pup survival were measured but not reported. Dose used is at the high end of the no effect level for these endpoints when oral gavage exposure is used. Unclear about dosing via peanut butter. For statistical analysis males and females were separately tested. Authors said that they stratified by sex instead using a two-way ANOVA. Therefore, those samples violated the independent sample assumption as the litter was considered the smallest independent statistical unit. However, experts disagreed with this reasoning because standard statistical methods are available to test for sex within litter effects.

Zhang C, Xu Q, Xiao X, Li W, Kang Q, Zhang X, Wang T, Li Y, 2018 – RefID: 1116

Study characteristics and population	Specific endpoint	Learning and memory (MWM): escape latency; swimming distance; swimming speed; time spent in the target quadrant
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male and Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	12 (1M/1F from each litter)
Exposure	Period of exposure:	From GD 1 to PDN 0
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.54 mg/kg deltamethrin; 1.35 mg/kg deltamethrin;; 2.7 mg/kg deltamethrin;; 9 mg/kg deltamethrin;
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN; 22 PDN; 23 PDN; 24 PDN; 25 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Yes. Pups were randomly selected from each litter (2 of each sex from 6 litter)
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not reported. But methods support this. Same vehicle and similar housing conditions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. All samples sizes in results are consistent with the methods section. For statistical analysis and graphs, only a summary is reported
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	The analytical work was not carried out. The technical material was obtained from Sigma with a purity > 98%. Exposure timing and duration are adequate. The solubility is adequate. 4 dose groups (3 doses and a control) are adequate for the evaluation of a dose–response.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	No blinding but automated.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No clinical signs or changes in body weight were observed.

Pitzer EM, Sugimoto C, Gudelsky GA, Huff Adams CL, Williams MT, Vorhees CV, 2019 – RefID: 2121

Study characteristics and population	Specific endpoint	Learning and memory Freezing behaviour: Cued (Post-conditioned stimulus); Cued (Pre-conditioned stimulus); Post-conditioned stimulus; Pre-conditioned stimulus; Contextual; MWM: Cued; Reversal; Shift; Acquisition Latency; Acquisition Path efficiency; Acquisition speed; CWM : Errors; Latency;
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Female; Male; Male and Female
	Housing condition:	Non-standard
	Funding source:	Public
	Group size:	CO: 55 total, 25M, 30F; 0.25 mg/kg DLM: 51total, 26M, 25 F; 0.5 mg/kg DLM: 52 total, 24 M, 28 F; 1 mg/kg DLM: 45 total 20 M, 25 F
Exposure	Period of exposure:	From PDN 3 to PDN 20
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.25 mg/kg deltamethrin; 0.5 mg/kg deltamethrin; 1 mg/kg deltamethrin
	Dose frequency:	Day
Measurement	Measurement time:	63 PDN; 64 PDN; 65 PDN; 66 PDN; 67 PDN; 68 PDN; 86 PDN; 106 PDN; 107 PDN; 108 PDN
Risk of bias appraisal		Tier: 2

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. Pups were randomly assigned to 4 treatment groups
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. The study was well conducted and described.

Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	A lot of information on numbers of animals. Checking numbers of animals in graphs they seem to be consistent with methods section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	The test substance was supplied as technical grade from the manufacturer (Bayer Crop Science) with a purity of 99.9%. Concentrations were well below the known solubility in corn oil. Moreover, duration and number of concentrations are adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The study was well conducted and run by a well-regarded laboratory. Blinding was not reported, but most tests were automated and not likely to bias results.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. All data reported in text or figures as group means.
Toxicity	Q9: were there other potential threats to internal validity?	PHRoB	There was no maternal exposure, so no risk of maternal toxicity. However, there were large body weight effects in the two high dose groups, during the preweaning period (up to about 28 and 33% in the mid and high doses at the end of weaning). The effect on body weight persisted through adult ages, but the magnitude was decreased (~10 and 12% by 112 days of age)

CONFIDENTIAL (2006) – RefID: 3201

Study characteristics and population	Specific endpoint	Learning and memory M-WM: Trials to criterion; Learning phase (Average Errors); Learning phase (Duration); Retention phase (Average Errors); Retention phase (Duration); Passive Avoidance performance: Latency: Trials to criterion
	Specie:	Rat
	Strain:	Wistar
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Private
	Group size:	16 rats/sex/dose

Exposure	Period of exposure:	From GD 6 to PDN 21
	Route of administration:	Oral (via feed)
	Vehicle:	nr
	Doses:	0 mg/kg bw per day; 1.64 mg/kg bw per day; 16.1 mg/kg bw per day; 6.78 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	22 PDN; 29 PDN; 60 PDN; 60.1 PDN; 60.2 PDN; 67 PDN; 67.1 PDN; 67.2 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. 'Following at least six days of acclimation, P-generation females were weighed and those with a body weight more or less than 20% of the mean weight [18] were rejected. The remaining females were assigned to the control or an exposure group in sequence, as they were determined to be inseminated. This approach was used to ensure unbiased assignment of the animals to dose groups and that there would be approximately an equal number of litters from each dose group available for testing on a given day'.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Supported by methods description.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. All exceptions were noted, and all data were presented in appendices.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Yes. Extensive analytical work was conducted on the diet and brain concentrations were measured in offspring.

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Yes. Not blinded but the methods are well established, and there is an extensive validation of the data provided. The work was conducted in GLP .
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. Extensive methods and results sections. All data are included in appendices.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	Maternal body weight gain was marginally impacted, but not enough to bias results.

Behavioural: Motor activity

Richardson JR, Taylor MM, Shalat SL, Guillot TS, 3rd Caudle WM, Hossain MM, Mathews TA, Jones SR, Cory-Slechta DA, Miller GW, 2015 – RefID: 361

Study characteristics and population	Specific endpoint	Locomotor activity: Ambulatory count (day 1); Ambulatory count (day 2); Ambulatory count (day 3); Total distance travelled (Persistence); Open field – Total distance travelled
	Specie:	Mouse
	Strain:	C57BL/6J
	Sex:	Female; Male
	Housing condition:	Standard
	Funding source:	Public
	Group size:	7 (4–9 litters/treatment group), (1 mouse/sex per litter/behavioural test)
Exposure	Period of exposure:	From GD 0 to PDN 22
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 0.3 mg/kg deltamethrin; 1 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	–9 nr; 6 Post-natal week; 8 Post-natal week; 10 Post-natal week; 12 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.

Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly reported, but likely based on methods used.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Unable to ascertain. Insufficient information provided: 3 separate cohorts of animals, representing 9 litters per treatment group, with the litter used as the individual unit of analysis and no more than 1 mouse/sex/litter used for any behavioural test. There is no rationale or explanation for the very unbalanced group sizes. Also, there is no mention of whether there was balance across the '3 cohorts' figure in publication reports 'n = 4–7' – so it is unclear what happened to the n = 9.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No purity reported, considered technical grade based on the source (ChemService). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods, but no blinding reported. Methods were automated for motor activity assessments.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Some data are missing e.g. maternal toxicity and body weight.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	Not measured or reported if any toxicity resulted from the exposures. It is not likely at the doses used.

Vester AI, Chen M, Marsit CJ, Caudle WM, 2019 – RefID: 961

Study characteristics and population	Specific endpoint	Locomotor activity (open field) – Ambulatory count; Locomotor activity (Y maze) – Total distance travelled; Motor activity – Marble burying

	Specie:	Mouse
	Strain:	C57BL/6NCrl
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	–99
Exposure	Period of exposure:	From Pre-Mating day 1 to PDN 21
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	8 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Stated randomisation of dams.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PHRoB	Only partially reported for lighting on/off, no information about T°, humidity, feed type and water source. Unclear whether peanut butter vehicle was given to controls. Same water used for CORT exposures in both groups
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine since there were no reports of the number of litters used . Number of samples reported varies from 7–9 per group depending on the figure.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, probably of technical grade (source Sigma). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose was used.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Automated and well established methods not prone to bias. But no clear statement that assessors were blind to treatment.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Pups body weight and survival were measured but not reported. It is unclear if dam body weights were recorded. CORT was administered in water, but there is no mention of dams' water consumption.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	No data on toxicity or body weight of pups or dams. Dose used is at the high end of the no effect level for these endpoints when oral gavage exposure is used. Unclear about dosing via peanut butter.

Eriksson P, Fredriksson A, 1991 – RefID: 1725

Study characteristics and population	Specific endpoint	Motor activity: Locomotor mean (0–20 min); Locomotor mean (20–40 min); Locomotor mean (40–60 min); Rearing mean (0–20 min); Rearing mean (20–40 min); Rearing mean (40–60 min); total activity (0–20 min); total activity (20–40 min); total activity (40–60 min)
	Specie:	Mouse
	Strain:	NMRI
	Sex:	Male
	Housing condition:	Standard
	Funding source:	Public
	Group size:	12
Exposure	Period of exposure:	From PDN 10 to PDN 16
	Route of administration:	Oral (via gavage)
	Vehicle:	peanut oil and egg lecithin
	Doses:	0 mg/kg bw; 0.7 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	4 Post-natal month
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported. But with concurrent control.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly stated, but highly likely due to careful descriptions in the methods section
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	PHRoB	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Hard to determine. In methods section, it was stated that each treatment group is characterised by mice from three to four litters. However, the figure legend reports that each treatment group contained 12 (N=12) mice from 3 different litters N is adequate for behave study. STDs are not reported.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The analytical work was not carried out. Chemical was supplied by manufacturer, so it is likely to be of technical grade. Concurrent control. Solubility is adequate. Only one dose was tested. .
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Yes. Well established methods. Good techniques, No blinding but automated equipment
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No. No changes in body weight or no clinical signs of toxicity.

Gray LEJR, Kavlock RJ, Ostby J, Ferrell J, 1983 – RefID: 1790

Study characteristics and population	Specific endpoint	Motor activity
	Specie:	Mouse
	Strain:	CD 1

	Sex:	Female
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	
Exposure	Period of exposure:	From GD 8 to GD 12
	Route of administration:	Oral (via gavage)
	Vehicle:	nr
	Doses:	10 mg/kg
	Dose frequency:	Day
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported, but concurrent control.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	NR	Not directly reported.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine. No data or figures are presented.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	There is no information about the test chemical. Only one dose was tested.

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	NR	No data are reported. There is only one sentence reporting results 'Three chemicals administered during gestation resulted in hypoactivity in the offspring at 22 d of age but had no effect on later activity levels (chlordecone (Days 8 through 12), 6-aminonicotinamide, and deltamethrin), '
Selective outcome report	Q8: were all measured outcomes reported?	DH RoB	No. Not all outcomes are reported.
Toxicity	Q9: were there other potential threats to internal validity?	NR	Was measured but not reported.

Patro N, Shrivastava M, Tripathi S, Patro IK, 2009 – RefID: 2098

Study characteristics and population	Specific endpoint	Motor activity: Ambulatory time; Distance travelled; Resting time; Stereotypic time
	Specie:	Rat
	Strain:	Wistar
	Sex:	Male and Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	6 pups/group
Exposure	Period of exposure:	From PDN 0 to PDN 7
	Route of administration:	Intraperitoneal
	Vehicle:	DMSO
	Doses:	0 mg/kg bw per day; 0.7 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN; 30 PDN; 90 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions are assumed to be identical since authors did not report differences in housing or husbandry
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Hard to determine. In methods sections it is reported that total number of pups was 42 per group. No report of number of pups in figure legends.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The analytical work was not conducted. Purity reported as 'Pure compound from Sigma'. DMSO used as vehicle and intraperitoneal (i.p.) as route of administration. Kinetics will differ from oral exposures. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Not reported. But the methods are acceptable and same time points were tested. No blinding – but this is not likely to bias the automated measurement of activity.
Selective outcome report	Q8: were all measured outcomes reported?	NR	Not reported for pathology and not all time points are shown.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Not reported. But, in a previous report by these same authors, this dose regimen resulted in 38 to 41% lower body weight and up to 25% decrease in brain weights in pups during preweaning period.

Pitzer EM, Sugimoto C, Gudelsky GA, Huff Adams CL, Williams MT, Vorhees CV, 2019 – RefID: 2121

Study characteristics and population	Specific endpoint	Motor activity – Elevated Zero Maze; Motor activity (Open field) – Total activity
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male and Female
	Housing condition:	Non-standard
	Funding source:	Public

	Group size:	CO: 55 total, 25M, 30F; 0.25 mg/kg DLM: 51total, 26M, 25 F; 0.5 mg/kg DLM: 52 total, 24 M, 28 F; 1 mg/kg DLM: 45 total 20 M, 25 F
Exposure	Period of exposure:	From PDN 3 to PDN 20
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.25 mg/kg deltamethrin; 0.5 mg/kg deltamethrin; 1 mg/kg deltamethrin
	Dose frequency:	Day
Measurement	Measurement time:	60 PDN; 61 PDN
Risk of bias appraisal		Tier: 2

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. Yes. Pups were randomly assigned to 4 treatment groups
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, the study was well conducted and described.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Raw data not available. However, checking numbers of animals in graphs they seem to be consistent with methods' section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	Test substance was supplied as tech grade from the manufacturer (Bayer Crop Science) as 99.9%. Concentrations were well below the known solubility in corn oil. Duration and number of concentrations are adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The study was well conducted and run by a well-regarded laboratory. Blinding was not reported, but most tests were automated and not likely to bias results.

Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. All data reported in text or figures as group means.
Toxicity	Q9: were there other potential threats to internal validity?	PHRoB	There was no maternal exposure, so no risk of maternal toxicity. However, there were large body weight effects in the two high dose groups, during the preweaning period (up to about 28 and 33% in the mid and high doses at the end of weaning). The effect on body weight persisted through adult ages, but the magnitude was decreased (~10 and 12% by 112 days of age)

CONFIDENTIAL (2006) – RefID: 3201

Study characteristics and population	Specific endpoint	Locomotor activity (figure-eight maze) total activity; Motor activity (figure-eight maze) total activity
	Specie:	Rat
	Strain:	Wistar
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Private
	Group size:	16 rats/sex/dose
Exposure	Period of exposure:	From GD 6 to PDN 21
	Route of administration:	Oral (via feed)
	Vehicle:	nr
	Doses:	0 mg/kg bw per day; 1.64 mg/kg bw per day; 16.1 mg/kg bw per day; 6.78 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	13 PDN; 17 PDN; 21 PDN; 60 PDN; 120 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. A protocol for randomisation was applied: 'Following at least six days of acclimation, P-generation females were weighed and those with a body weight more or less than 20% of the mean weight [18] were rejected. The remaining females were assigned to the control or an exposure group in sequence, as they were determined to be inseminated. This approach was used to ensure unbiased assignment of the animals to dose groups and that there would be approximately an equal number of litters from each dose group available for testing on a given day.'
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Supported by methods description.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. All exceptions were noted, and all data were presented in appendices.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Yes. Extensive analytical work was conducted on the diet and brain concentrations were measured in offspring.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Yes. Yes. Not blinded but the methods are well established, and there is an extensive validation of the data provided. The work was conducted in GLP
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. Extensive methods and results sections. All data included in appendices.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	Maternal body weight gain was marginally impacted, but not enough to bias results.

Behavioural: Motor coordination

Kumar K, Patro N, Patro I, 2013 – RefID: 1930

Study characteristics and population	Specific endpoint	Motor coordination (Latency to fall from Rotorod)
	Specie:	Rat
	Strain:	Wistar
	Sex:	Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	6 pups
Exposure	Period of exposure:	From GD 7 to GD 10
	Route of administration:	Intraperitoneal
	Vehicle:	DMSO
	Doses:	0 mg/kg bw per day; 0.75 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN; 30 PDN; 60 PDN; 90 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported but same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were assumed identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Methods and results agree on n = 6.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out but purity reported as 98% from Sigma. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods. Same time points. Not blinding is unlikely to impact bias.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	No clinical signs reported. Decreases in body weight during lactation ranging from 35–41%.

Behavioural: Startle

Pitzer EM, Sugimoto C, Gudelsky GA, Huff Adams CL, Williams MT, Vorhees CV, 2019 – RefID: 2121

Study characteristics and population	Specific endpoint	Startle acoustic and tactile (peak amplitude)
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Female; Male
	Housing condition:	Non-standard
	Funding source:	Public
	Group size:	CO: 55 total, 25M, 30F; 0.25 mg/kg DLM: 51total, 26M, 25 F; 0.5 mg/kg DLM: 52 total, 24 M, 28 F; 1 mg/kg DLM: 45 total 20 M, 25 F
Exposure	Period of exposure:	From PDN 3 to PDN 20
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.25 mg/kg deltamethrin; 0.5 mg/kg deltamethrin; 1 mg/kg deltamethrin
	Dose frequency:	Day
Measurement	Measurement time:	104 PDN
Risk of bias appraisal		Tier: 2

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. Pups were randomly assigned to 4 treatment groups.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. the study was well conducted and described.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Raw data not available. However, checking numbers of animals in graphs they seem to be consistent with methods' section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	Test substance was supplied as technical grade from the manufacturer (Bayer Crop Science) with a purity of 99.9%. Concentrations were well below the known solubility in corn oil. Duration and number of concentrations are adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The study was well conducted study and run by a well-regarded laboratory. Blinding was not reported, but most tests were automated and not likely to bias results.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. All data reported in text or figures as group means.
Toxicity	Q9: were there other potential threats to internal validity?	PHRoB	There was no maternal exposure, so no risk of maternal toxicity. However, there were large body weight effects in the two high dose groups, during the preweaning period (up to about 28 and 33% in the mid and high doses at the end of weaning). The effect on body weight persisted through adult ages, but the magnitude was decreased (~10 and 12% by 112 days of age)

CONFIDENTIAL (2006) – RefID: 3201

Study characteristics and population	Specific endpoint	Startle – Auditory Startle Reflex: Latency to Peak Habituation Latency to Peak Peak Amplitude Habituation; Peak Amplitude
	Specie:	Rat
	Strain:	Wistar
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Private
	Group size:	16 rats/sex/dose
Exposure	Period of exposure:	From GD 6 to PDN 21
	Route of administration:	Oral (via feed)
	Vehicle:	nr
	Doses:	0 mg/kg bw per day; 1.64 mg/kg bw per day; 16.1 mg/kg bw per day; 6.78 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	22 PDN; 22.1 PDN; 22.2 PDN; 22.3 PDN; 22.4 PDN; 22.5 PDN; 60 PDN; 60.1 PDN; 60.2 PDN; 60.3 PDN; 60.4 PDN; 60.5 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. A protocol for randomisation was applied: 'Following at least six days of acclimation, P-generation females were weighed and those with a body weight more or less than 20% of the mean weight [18] were rejected. The remaining females were assigned to the control or an exposure group in sequence, as they were determined to be inseminated. This approach was used to ensure unbiased assignment of the animals to dose groups and that there would be approximately an equal number of litters from each dose group available for testing on a given day.'
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Supported by methods descriptions.

Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. All exceptions were noted, and all data were presented in appendices.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Yes. Extensive analytical work was conducted on the diet. Brain concentrations were measured in offspring.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Yes. Not blinded but Well established methods, extensive validation data provided. GLP work.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. Extensive methods and results sections. All data included in appendices.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	Maternal body weight gain was marginally impacted, but not enough to bias results.

Behavioural: Swimming Behaviour

Pitzer EM, Sugimoto C, Gudelsky GA, Huff Adams CL, Williams MT, Vorhees CV, 2019 – RefID: 2121

Study characteristics and population	Specific endpoint	Swimming Behaviour (Straight channel swimming)
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male and Female
	Housing condition:	Non-standard
	Funding source:	Public
	Group size:	CO: 55 total, 25M, 30F; 0.25 mg/kg DLM: 51total, 26M, 25 F; 0.5 mg/kg DLM: 52 total, 24 M, 28 F; 1 mg/kg DLM: 45 total 20 M, 25 F
Exposure	Period of exposure:	From PDN 3 to PDN 20
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.25 mg/kg deltamethrin; 0.5 mg/kg deltamethrin; 1 mg/kg deltamethrin

	Dose frequency:	Day
Measurement	Measurement time:	62 PDN
Risk of bias appraisal		Tier: 2

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. Pups were randomly assigned to 4 treatment groups.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. The study was well conducted and described.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Raw data not available. However, checking numbers of animals in graphs they seem to be consistent with methods section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	Test substance was supplied as technical grade from the manufacturer (Bayer Crop Science) with a purity of 99.9%. Concentrations were well below the known solubility in corn oil. Duration and number of concentrations are adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The study was well conducted study and run by a well-regarded laboratory. Blinding was not reported, but most tests were automated and not likely to bias results.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. All data reported in text or figures as group means.

Toxicity	Q9: were there other potential threats to internal validity?	PHRoB	There was no maternal exposure, so no risk of maternal toxicity. However, there were large body weight effects in the two high dose groups, during the preweaning period (up to about 28 and 33% in the mid and high doses at the end of weaning). The effect on body weight persisted through adult ages, but the magnitude was decreased (~10 and 12% by 112 days of age)

Neurochemistry: Growth factor

Zhang C, Xu Q, Xiao X, Li W, Kang Q, Zhang X, Wang T, Li Y, 2018 – RefID: 1116

Study characteristics and population	Specific endpoint	Growth factor – BDNF (CA1 region of hippocampus); BDNF (hippocampus)
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male and Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	12 (1M/1F from each litter)
Exposure	Period of exposure:	From GD 1 to PDN 0
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.54 mg/kg deltamethrin; 1.35 mg/kg deltamethrin;; 2.7 mg/kg deltamethrin;; 9 mg/kg deltamethrin;
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Yes. Pups were randomly selected from each litter (2 of each sex from 6 litter)
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not reported. But methods support this. Same vehicle used and similar housing conditions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. All samples sizes in results are consistent with methods section. For statistical analysis and graphs, only a summary is reported
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	The analytical work was not carried out. The technical material was obtained from Sigma with a purity > 98%. Exposure timing and duration are adequate . The solubility is adequate. 4 dose groups (3 doses and a control) are adequate for the evaluation of a dose–response.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	No blinding but automated.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No clinical signs or changes in body weight were observed.

Magby JP, Richardson JR, 2017 – RefID: 1988

Study characteristics and population	Specific endpoint	Growth factor (BDNF) – Cortex; Growth factor (BDNF) – Striatum
	Specie:	Mouse
	Strain:	C57BL/6J
	Sex:	Male and Female
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	5 males
Exposure	Period of exposure:	From GD 6 to PDN 25
	Route of administration:	Oral (via gavage)

	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin;
	Dose frequency:	Every 3 day
Measurement	Measurement time:	10 Post-natal month
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly reported, but same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were assumed identical if authors did not report differences in housing or husbandry
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Results and methods agree on N/group.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The analytical work was not carried out. Purity was 99.5% from ChemService. Only one dose was tested
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Same times and well established methods. Not blinded, but unlikely to impact bias
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No effects on maternal or pup body weights and clinical signs.

Neurochemistry: Neurotransmitters

Richardson JR, Taylor MM, Shalat SL, Guillot TS, 3rd Caudle WM, Hossain MM, Mathews TA, Jones SR, Cory-Slechta DA, Miller GW, 2015 – RefID: 361

Study characteristics and population	Specific endpoint	Neurotransmitters – Dopamine level; Dopamine uptake; DOPAC; Dopamine – nucleus accumbens; Homovanillic acid
	Specie:	Mouse
	Strain:	C57BL/6J
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	–99
Exposure	Period of exposure:	From GD 0 to PDN 22
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 0.3 mg/kg deltamethrin; 1 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	6 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly reported, but likely based on methods used.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Unable to ascertain. Insufficient information provided: 3 separate cohorts of animals, representing 9 litters per treatment group, with the litter used as the individual unit of analysis and no more than 1 mouse/sex/litter used for any behavioural test. There is no rationale or explanation for the very unbalanced group sizes. Also, there is no mention of whether there was balance across the '3 cohorts'. The figure in publication, reports 'n = 4–7 or 5–6' – so it is unclear what happened to the n = 9
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, probably technical grade (source ChemService). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods, but no blinding reported. Methods were automated for motor activity assessments.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Some data are missing e.g. maternal toxicity and body weight.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	Not measured or reported if any toxicity resulted from the exposures. It is not likely at the doses used.

Vester AI, Chen M, Marsit CJ, Caudle WM, 2019 – RefID: 961

Study characteristics and population	Specific endpoint	Neurotransmitters
	Specie:	Mouse
	Strain:	C57BL/6NCrl
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	–99
Exposure	Period of exposure:	From Pre-Mating day 1 to PDN 21
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day

Measurement	Measurement time:	8 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Stated randomisation of dams.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PHRoB	Only partially reported for lighting on/off, no information about T°, humidity, feed type and water source. Unclear whether peanut butter vehicle was given to controls. Same water used for CORT exposures in both groups.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine since there were no reports of the number of litters used. Number of samples reported varies from 5–9 per group depending on the figure.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, probably of technical grade (source Sigma). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose was used.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Quantitative measurement with well established methods. Not blinded but influence of blinding low.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Pups body weight and survival were measured but not reported. It is unclear if dam body weights were recorded.

Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	No data on toxicity or body weight of pups or dams. Body weight and pups survival were measured but not reported. Dose used is at the high end of the no effect level for these endpoints when oral gavage exposure is used. Unclear about dosing via peanut butter. . . Authors said that they stratified by sex instead using a two-way ANOVA. Therefore, those samples violated the independent sample assumption as the litter was considered the smallest independent statistical unit. However, experts disagreed with this reasoning because standard statistical methods are available to test for sex within litter effects

Pitzer EM, Sugimoto C, Gudelsky GA, Huff Adams CL, Williams MT, Vorhees CV, 2019 – RefID: 2121

Study characteristics and population	Specific endpoint	Neurotransmitters (NE)
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male and Female
	Housing condition:	Non-standard
	Funding source:	Public
	Group size:	CO: 17 total, 8 M, 9 F; 0.25 mg/kg DLM: 17 total, 8 M, 9 F; 0.5 mg/kg DLM: 17 total, 8 M, 9 F; 1.0 mg/kg DLM: 14 total, 7 M, 7 F
Exposure	Period of exposure:	From PDN 3 to PDN 20
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.25 mg/kg deltamethrin; 0.5 mg/kg deltamethrin; 1 mg/kg deltamethrin
	Dose frequency:	Day
Measurement	Measurement time:	18 Post-natal week
Risk of bias appraisal		Tier: 2

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. Pups were randomly assigned to 4 treatment groups

Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. The study was well conducted and described.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Raw data not available. However, checking numbers of animals in graphs they seem to be consistent with methods section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisations?	PLRoB	Test substance was supplied as technical grade from the manufacturer (Bayer Crop Science) with a purity of99.9%. Concentrations were well below the known solubility in corn oil. Duration and number of concentrations are adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The study was well conducted study and run by a well-regarded laboratory. Blinding was not reported, but most tests were automated and not likely to bias results.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. All data reported in text or figures as group means.
Toxicity	Q9: were there other potential threats to internal validity?	PHRoB	There was no maternal exposure, so no risk of maternal toxicity. However, there were large body weight effects in the two high dose groups, during the preweaning period (up to about 28 and 33% in the mid and high doses at the end of weaning). The effect on body weight persisted through adult ages, but the magnitude was decreased (~10 and 12% by 112 days of age)

Neurochemistry: Proteins

Zhang C, Xu Q, Xiao X, Li W, Kang Q, Zhang X, Wang T, Li Y, 2018 – RefID: 1116

Study characteristics and population	Specific endpoint	Proteins: GluN1; GluN2A; GluN2B; pCREB/CREB; PTrkB/TrkB
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male and Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	12 (1M/1F from each litter)
Exposure	Period of exposure:	From GD 1 to PDN 0
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.54 mg/kg deltamethrin; 1.35 mg/kg deltamethrin;; 2.7 mg/kg deltamethrin;; 9 mg/kg deltamethrin;
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Yes. Pups were randomly selected from each litter (2 of each sex from 6 litter).
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not reported and methods support this. Same vehicle used and similar housing conditions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. All samples sizes in results are consistent with methods section. For statistical analysis and graphs, only a summary is reported
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	The analytical work was not carried out. The technical material was obtained from Sigma with a purity > 98%. Exposure timing and duration are adequate . The solubility is adequate. 4 dose groups (3 doses and a control) are adequate for the evaluation of a dose–response.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	No blinding but automated.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No clinical signs or changes in body weight were observed.

Neurochemistry: Receptor

Eriksson P, Fredriksson A, 1991 – RefID: 1725

Study characteristics and population	Specific endpoint	Receptor:- Muscarinic (Cortex); Muscarinic (High affinity site); Muscarinic (Hippocampus); Muscarinic (Low affinity site; Muscarinic (Striatum
	Specie:	Mouse
	Strain:	NMRI
	Sex:	Male
	Housing condition:	Standard
	Funding source:	Public
	Group size:	18
Exposure	Period of exposure:	From PDN 10 to PDN 16
	Route of administration:	Oral (via gavage)
	Vehicle:	peanut oil and egg lecithin
	Doses:	0 mg/kg bw; 0.7 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	4 Post-natal month

Risk of bias appraisal	Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Not reported. But with concurrent control.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly stated but highly likely due to careful descriptions in the methods section.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	PHRoB	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Hard to determine. In methods section, it was stated that each treatment group is characterised by mice from three to four litters. However, the figure legend reports that each treatment group contained 12 (N=12) mice from 3 different litters N is adequate for behave study. STDs are not reported
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The analytical work was not carried out. Chemical was supplied by manufacturer, so it is likely to be of technical grade. Concurrent control. Solubility is adequate. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Yes. Well established methods. Good techniques, No blinding but automated equipment.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No. Report states no changes in body weight or no clinical signs of toxicity.

Eriksson P, Nordberg A, 1990 – RefID: 1727

Study characteristics and population	Specific endpoint	Receptor: Muscarinic (Cortex); Muscarinic (High affinity site); Muscarinic (Hippocampus); Muscarinic (Low affinity site); Nicotinic (Cortex); Nicotinic (Hippocampus)
	Specie:	Mouse
	Strain:	NMRI
	Sex:	Male and Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	15
Exposure	Period of exposure:	From PDN 10 to PDN 17
	Route of administration:	Oral (via gavage)
	Vehicle:	peanut oil and egg lecithin
	Doses:	0 mg/kg bw; 0.71 mg/kg bw; 1.2 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	18 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Housing, husbandry and food type were reported. Same vehicle was used in all groups.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	PLRoB	Not reported but is unlikely to impact bias for neurochemical methods.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine. N= 6–18 for various endpoints. No information on initial starting number of litter/treatment.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	The analytical work was not carried out. Chemical was supplied by manufacturer, so it is likely to be of technical grade. Concurrent control. Solubility is adequate. Two doses and a control were tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Yes. Well established methods. Good techniques, no blinding but not it is not likely to impact the neurochemistry endpoints.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. Data presented for all methods described in the methods section.
Toxicity	Q9: were there other potential threats to internal validity?	PHRoB	No body weights or brain weight were reported. However, doses are well below those reported to cause toxicity in previous reports, including those by same authors. Neurotoxicity and choreoathetosis were observed.

Pauluhn J, Schmuck G, 2003 – RefID: 2099

Study characteristics and population	Specific endpoint	Receptor – Muscarinic (Cortex)
	Specie:	Mouse
	Strain:	NMRI
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Mixed
	Group size:	8 pups per group
Exposure	Period of exposure:	From PDN 10 to PDN 16
	Route of administration:	Oral (via gavage)
	Vehicle:	peanut oil and egg lecithin
	Doses:	0 mg/kg bw; 0.7 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	17 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Yes. Animals were randomly assigned to groups.

Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	The same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions are assumed to be identical since authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PLRoB	Hard to determine. The methods described 2 litters per dose with 8 pups per litter. therefore, the total, not controlled for number of litters, is 16. In the relevant figure (Fig.6) in publication, the group sizes were not reported. In Table 2 it was reported that the methods used 'Two dams per group with their respective litters (eight pups per group)' but does not directly state that N=16 per group. They may or may not have been attrition.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The analytical work was not done. Purity reported as 99.8%. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Not reported. But the methods are acceptable. Same time points. No blinding – but not likely to bias receptor measurements.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Yes. But brain weights are not mentioned in the methods section and no details are given about the techniques used.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	No. This dosing regimen did not result in changes in body weight. No clinical signs of toxicity were observed Deltamethrin resulted in a small increase in body weights of about 15–20% on PND17 when comparing normothermic groups. This may have occurred due to the small number of litters (N=2).

Neurochemistry: Sodium channel

Magby JP, Richardson JR, 2017 – RefID: 1988

Study characteristics and population	Specific endpoint	Sodium channel: (Na alpha v.1.1) – Cortex; (Na alpha v.1.1) – Cortex vs Striatum; (Na alpha v.1.1) – Striatum; (Na alpha v.1.2) – Cortex; (Na alpha v.1.2) – Cortex vs Striatum; (Na alpha v.1.2) – Striatum; (Na alpha v.1.3) – Cortex; (Na alpha v.1.3) – Cortex vs Striatum; (Na alpha v.1.3) – Striatum; (Na alpha v.1.6) – Cortex; (Na alpha v.1.6) – Cortex vs Striatum; (Na alpha v.1.6) – Striatum; (Na beta 1) – Cortex; (Na beta 1) – Cortex vs Striatum; (Na beta 1) – Striatum; (Na beta 2) – Cortex; (Na beta 2) – Cortex vs Striatum; (Na beta 2) – Striatum; (Na beta 3) – Cortex; (Na beta 3) – Cortex vs Striatum; (Na beta 3) – Striatum; l (Na beta 4) – Cortex; (Na beta 4) – Cortex vs Striatum; (Na beta 4) – Striatum
	Specie:	Mouse
	Strain:	C57BL/6J
	Sex:	Male and Female
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	5 males
Exposure	Period of exposure:	From GD 6 to PDN 25
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin;
	Dose frequency:	Every 3 day
Measurement	Measurement time:	10 Post-natal month
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly reported, but the same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were assumed identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Results and methods agree on N/group.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The analytical work was not carried out. Purity was 99.5% from ChemService. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Same times, well established methods. Not blinded, but automatic method unlikely to impact bias
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No effects on maternal or pup body weight or clinical signs.

Neurochemistry: Transporter

Richardson JR, Taylor MM, Shalat SL, Guillot TS, 3rd Caudle WM, Hossain MM, Mathews TA, Jones SR, Cory-Slechta DA, Miller GW, 2015 – RefID: 361

Study characteristics and population	Specific endpoint	Transporter (Dopamine)
	Specie:	Mouse
	Strain:	C57BL/6J
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	–99
Exposure	Period of exposure:	From GD 0 to PDN 22

	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 0.3 mg/kg deltamethrin; 1 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	6 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Not directly reported, but likely based on methods used.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	Unable to ascertain. Insufficient information provided: 3 separate cohorts of animals, representing 9 litters per treatment group, with the litter used as the individual unit of analysis and no more than 1 mouse/sex/litter used for any behavioural test. There is no rationale or explanation for the very unbalanced group sizes. Also, there is no mention of whether there was balance across the '3 cohorts'. The figure in publication, reports 'n = 4–7 or 5–6' – so it is unclear what happened to the n = 9
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No purity reported, probably technical grade (source ChemService). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Three doses were tested.

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods, but no blinding reported. Methods were automated for motor activity assessments.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Some data are missing e.g. maternal toxicity and body weight.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	Not measured or reported if any toxicity resulted from the exposures. It is not likely at the doses used.

Vester AI, Chen M, Marsit CJ, Caudle WM, 2019 – RefID: 961

Study characteristics and population	Specific endpoint	Transporter (DAT):Cortical; Striatum; Transporter (Dopamine); Transporter (TH) – Cortical; Striatum; Transporter (VMAT2) – Cortical; Striatum
	Specie:	Mouse
	Strain:	C57BL/6NCrl
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	–99
Exposure	Period of exposure:	From Pre-Mating day 1 to PDN 21
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	8 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Stated randomisation of dams.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PHRoB	Only partially reported for lighting on/off, no information about T°, humidity, feed type and water source. Unclear whether peanut butter vehicle was given to controls. Same water used for CORT exposures in both groups.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine since there were no reports of the number of litters used . Number of samples reported varies from 5–9 per group depending on the figure.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, probably of technical grade (source Sigma). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose used.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Quantitative measurement with well established methods. Not blinded but the influence of blinding is low.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Pups body weight and survival were measured but not reported. It is unclear if dam body weights were recorded.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	No data on toxicity or body weight of pups or dams. Body weight and pup survival were measured but not reported. Dose used is at the high end of the no effect level for these endpoints when oral gavage exposure is used. Unclear about dosing via peanut butter. For statistical analysis males and females were separately tested. Authors said that they stratified by sex instead using a two-way ANOVA. Therefore, those samples violated the independent sample assumption as the litter was considered the smallest independent statistical unit. However, experts disagreed with this reasoning because standard statistical methods are available to test for sex within litter effects. ‘

Neuropathology: Brain weight

Asari MA, Abdullah MS, Ismail ZIM, 2010 – RefID: 680

Study characteristics and population	Specific endpoint	Brain weight: Absolute; Relative; Cerebellum weight: Absolute; Relative
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	6 males pups
Exposure	Period of exposure:	From PDN 2 to PDN 5
	Route of administration:	Intraperitoneal
	Vehicle:	Corn oil
	Doses:	0 mg/kg bw; 1 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	6 PDN; 14 PDN; 21 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported. Intra-litter dosing design was used so lack of randomisation could increase the risk of bias. In addition, no information on whether groups were balanced by body weight.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported but likely to be similar based on the experimental design. Same vehicle used.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Impossible to determine because the number of animals per group was not reported.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out. Deltamethrin was from Sigma at 98% purity. The solubility in corn is adequate at the dose tested. The administration was the same for all animals. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard techniques. Good methods descriptions. Small STDs. The age dependent changes in the control are the same as known biology of brain development. 4–6% lower body weights in treated group should not impact brain weight. Not certainty about the group sizes.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No. The 1mg/kg bw per day dose is well below the systemic toxicity levels.

Asari MA, Abdullah MS, Abdullah S, 2008 – RefID: 1525

Study characteristics and population	Specific endpoint	Cerebellum weight – Absolute; Relative
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male
	Housing condition:	Standard
	Funding source:	Public
	Group size:	10 male pups
Exposure	Period of exposure:	From PDN 2 to PDN 5
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg bw; 1 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.

Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	No information on feed, housing or husbandry. Only approval or local animal committee. Same vehicle.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Hard to determine. Number of starting dams per treatment is not reported. It is reported that N=10 per endpoint, but no information on number of litters used or if the N is referring to the number of litters.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out. Deltamethrin is 99% pure from Sigma Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Quantitative measurement of cerebellar weight was measured at the time point. The methods used were standard method and are not likely to impact bias.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. All data reported for all methods described in methods section.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	Body weights reported for pups with about 10% lower body weight gain on PND7. Since no significant effects were reported on cerebellar weight, these body weight changes are not likely to be a threat.. Statistical significance between group means was analysed by the unpaired two-tailed Student's t-test. There was no control for litter and this could bias for either false positive and false negative results.

Guo J, Xu J, Zhang J, An L, 2018 – RefID: 1803

Study characteristics and population	Specific endpoint	Brain weight – Absolute; Relative
	Specie:	Mouse
	Strain:	ICR
	Sex:	Male and Female

	Housing condition:	Not reported
	Funding source:	Public
	Group size:	6
Exposure	Period of exposure:	From GD 10.5 to GD 16.5
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg; 1.2 mg/kg
	Dose frequency:	nr
Measurement	Measurement time:	1 PDN; 16.5 GD
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	University guideline for animal care and study conduct was used. No information about housing conditions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Not able to determine. 6 dams/group was allocated but the number of fetuses/pups measured was not reported.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisations?	NR	Purity was not reported, source is Chinese company. The same vehicle was used. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	No evidence of binding – but a little impact on the brain weight measurements is assumed.

Selective outcome report	Q8: were all measured outcomes reported?	DHRoB	Not all measurements reported e.g. dam weight.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Fetal toxicity is present: body weight loss in pups about 20–25 % and embryo loss.

Patro N, Mishra SK, Chattopadhyay M, Patro IK, 1997 – RefID: 2096

Study characteristics and population	Specific endpoint	Brain weight – Absolute; Relative
	Specie:	Rat
	Strain:	Wistar
	Sex:	Male and Female
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	4
Exposure	Period of exposure:	From PDN 9 to PDN 13
	Route of administration:	Intraperitoneal
	Vehicle:	Propylene Glycol
	Doses:	0 mg/kg bw per day; 0.7 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	12 PDN; 15 PDN; 21 PDN; 30 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	NR	Same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were are assumed to be identical if authors did not report differences in housing or husbandry.

Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Not reported. There is no information in the paper on the number of animals per group.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	The analytical work was not conducted. Purity was not reported. Propylene glycol was used as vehicle and the intraperitoneal (i.p.) route as route of administration. Kinetics will differ from oral exposures. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Not reported. But the methods are acceptable and same time points were tested. No blinding – but this is not likely to bias the brain weight measurements.
Selective outcome report	Q8: were all measured outcomes reported?	DHRoB	Not all reported. (few micrographs).
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Yes. There was a decrease in pups body weight (between 38 and 41%) during preweaning period. Brain weight was 25% lower on PND30.

Pauluhn J, Schmuck G, 2003 – RefID: 2099

Study characteristics and population	Specific endpoint	Brain weight – Absolute; Relative
	Specie:	Mouse
	Strain:	NMRI
	Sex:	Male and Female
	Housing condition:	Standard (GLP)
	Funding source:	Mixed
	Group size:	8 pups per group
Exposure	Period of exposure:	From PDN 10 to PDN 16
	Route of administration:	Oral (via gavage)
	Vehicle:	peanut oil and egg lecithin
	Doses:	0 mg/kg; 0.7 mg/kg

	Dose frequency:	Day
Measurement	Measurement time:	17 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Yes. Animals were randomly assigned to groups -
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	The same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were are assumed to be identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Hard determine. The methods described total number of pups used and there is no methods section for brain weights.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The analytical work was not done. Purity reported as 99.8%. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Not directly reported. But the methods are acceptable. Same time points. No blinding – but not likely to bias receptor measurements. In addition, the number of litters per dose group (N=2) is too small.
Selective outcome report	Q8: were all measured outcomes reported?	PHRoB	Yes. Unplanned analysis. But brain weights are not mentioned in the methods section and no details are given about the techniques used.

Toxicity	Q9: were there other potential threats to internal validity?	PHRoB	No. This dosing regimen did not result in changes in body weight. No clinical signs of toxicity were observed. Deltamethrin resulted in a small increase in body weights of about 15–20% on PND17 when comparing normothermic groups. This may have occurred due to the small number of litters (N=2).

CONFIDENTIAL (2006) – RefID: 3201

Study characteristics and population	Specific endpoint	Brain weight – Absolute; Absolute (non-perfused); Relative; Relative (non-perfused)
	Specie:	Rat
	Strain:	Wistar
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Private
	Group size:	10/sex
Exposure	Period of exposure:	From GD 6 to PDN 21
	Route of administration:	Oral (via feed)
	Vehicle:	nr
	Doses:	0 mg/kg bw per day; 1.64 mg/kg bw per day; 16.1 mg/kg bw per day; 6.78 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN; 75 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. A protocol for randomisation was applied: 'Following at least six days of acclimation, P-generation females were weighed and those with a body weight more or less than 20% of the mean weight [18] were rejected. The remaining females were assigned to the control or an exposure group in sequence, as they were determined to be inseminated. This approach was used to ensure unbiased assignment of the animals to dose groups and that there would be approximately an equal number of litters from each dose group available for testing on a given day.'
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Supported by methods descriptions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. All exceptions were noted, and all data were presented in appendices.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Yes. Extensive analytical work was conducted on the diet. Brain concentrations were measured in offspring.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Yes. Not blinded but automated assessments.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. Extensive methods and results sections. All data included in appendices.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	Maternal body weight gain was marginally impacted, but not enough to bias results.

Neuropathology: Qualitative neuropathology examination

Asari MA, Abdullah MS, Ismail ZIM, 2010 – RefID: 680

Study characteristics and population	Specific endpoint	Qualitative neuropathology examination
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	6 males pups
Exposure	Period of exposure:	From PDN 2 to PDN 5
	Route of administration:	Intraperitoneal
	Vehicle:	Corn oil
	Doses:	1 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported. Intra-litter dosing design was used so lack of randomisation could increase the risk of bias. In addition, no information on whether groups were balanced by body weight.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodent's studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported but likely to be similar based on experimental design. Same vehicle used.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Impossible to determine because the number of animals per group was not reported.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out. Deltamethrin was from Sigma at 98% purity. The solubility in corn is adequate at the dose tested. The administration was the same for all animals. Only one dose was tested. In addition, intraperitoneal route was used which alter the kinetics compared to oral exposures.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Standard techniques. But not blinded subjective pathology.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No. The 1mg/kg bw per day dose is well below the systemic toxicity levels.

Guo J, Xu J, Zhang J, An L, 2018 – RefID: 1803

Study characteristics and population	Specific endpoint	Qualitative neuropathology examination
	Specie:	Mouse
	Strain:	ICR
	Sex:	Male and Female
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	
Exposure	Period of exposure:	From GD 10.5 to GD 16.5
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	1.2 mg/kg
	Dose frequency:	nr
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.

Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	University guideline for animal care and study conduct was used. No information about housing conditions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DHRoB	Not able to determine. Only 3 representative slides, subjectively chosen.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	Purity was not reported, source is Chinese company. The same vehicle was used. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DHRoB	No evidence of binding – qualitative assessment is prone to bias. .
Selective outcome report	Q8: were all measured outcomes reported?	DHRoB	Not all measurements reported e.g. dam weight.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Fetal toxicity is present: body weight loss in pups about 20–25 % and embryo loss.

Kumar K, Patro N, Patro I, 2013 – RefID: 1930

Study characteristics and population	Specific endpoint	Qualitative neuropathology examination
	Specie:	Rat
	Strain:	Wistar
	Sex:	Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	
Exposure	Period of exposure:	From GD 7 to GD 10

	Route of administration:	Intraperitoneal
	Vehicle:	DMSO
	Doses:	0.75 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported but same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were assumed identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DHRoB	No. Only representative photomicrographs.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out but purity reported as 98% from Sigma. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DHRoB	Well established methods and same time points. However, no blinding, subjective assessment and limited photomicrographs are likely to impact bias.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes

Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	No clinical signs reported. Decreases in body weight during lactation ranging from 35–41%.

Patro N, Mishra SK, Chattopadhyay M, Patro IK, 1997 – RefID: 2096

Study characteristics and population	Specific endpoint	Qualitative neuropathology examination
	Specie:	Rat
	Strain:	Wistar
	Sex:	Male and Female
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	
Exposure	Period of exposure:	From PDN 9 to PDN 13
	Route of administration:	Intraperitoneal
	Vehicle:	Propylene Glycol
	Doses:	0.7 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	NR	Same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were are assumed to be identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DHRoB	Not reported. There is no information in the paper on the number of animals per group, only representative data were presented.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	The analytical work was not conducted. Purity was not reported. Propylene glycol was used as vehicle and the intraperitoneal (i.p.) route as route of administration. Kinetics will differ from oral exposures. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Not reported. Methods are acceptable and same time points. However, no blinding and subjective assessment of micrographs are likely to impact the bias.
Selective outcome report	Q8: were all measured outcomes reported?	DHRoB	Not all reported.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Yes. There was a decrease in pups body weight (between 38 and 41%) during preweaning period. Brain weight was 25% lower during the post-natal period.

Patro N, Patro IK, 2005 – RefID: 2097

Study characteristics and population	Specific endpoint	Qualitative neuropathology examination
	Specie:	Rat
	Strain:	Wistar
	Sex:	Male and Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	
Exposure	Period of exposure:	From PDN 9 to PDN 13
	Route of administration:	Intraperitoneal
	Vehicle:	Propylene Glycol
	Doses:	0.7 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	NR	Same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were are assumed to be identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DHRoB	Not reported. There is no information in the paper on the number of animals per group. Only 4 photomicrographs from the entire study were presented.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	The analytical work was not conducted. Purity was not reported. Propylene glycol was used as vehicle and the intraperitoneal (i.p.) route as route of administration. Kinetics will differ from oral exposures. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DHRoB	Not reported. But the methods are acceptable and same time points were tested. No blinding, subjective histopathology and only 4 photomicrographs are presented. No other data were presented.
Selective outcome report	Q8: were all measured outcomes reported?	DHRoB	No. Only 4 pictures out of all the animals.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Yes. This dosing regime was previously reported by the same authors (R2096) to result in 38 to 41% lower body weight and 25% lower brain weights in pups during the post-natal period

Patro N, Shrivastava M, Tripathi S, Patro IK, 2009 – RefID: 2098

Study	Specific endpoint	Qualitative neuropathology examination

characteristics and population	Specie:	Rat
	Strain:	Wistar
	Sex:	Male and Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	
Exposure	Period of exposure:	From PDN 0 to PDN 7
	Route of administration:	Intraperitoneal
	Vehicle:	DMSO
	Doses:	0.7 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were are assumed to be identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DHRoB	Only representative slides were presented.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The analytical work was not conducted. Purity was not reported. DMSO was used as vehicle and the intraperitoneal (i.p.) route as route of administration. Kinetics will differ from oral exposures. Only one dose was tested.

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DHRoB	Not reported. But the methods are acceptable and same time points were tested. No blinding and subjective pathology.
Selective outcome report	Q8: were all measured outcomes reported?	NR	Not reported for pathology and not all time points shown.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Not reported. But, in a previous report from the same authors, this dose regimen resulted in 38 to 41% lower body weight and up to 25% decrease in brain weights in pups during preweaning period.

Neuropathology: Quantitative Histochemistry

Vester AI, Chen M, Marsit CJ, Caudle WM, 2019 – RefID: 961

Study characteristics and population	Specific endpoint	Quantitative Histochemistry: (Comt); (Dat1); (Nurr1); (Pitx3); (Th); (Vmat2)
	Specie:	Mouse
	Strain:	C57BL/6NCrl
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	2
Exposure	Period of exposure:	From Pre-Mating day 1 to PDN 21
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	8 Post-natal week
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Stated randomisation of dams.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PHRoB	Only partially reported for lighting on/off, no information about T°, humidity, feed type and water source. Unclear whether peanut butter vehicle was given to controls. Same water used for CORT exposures in both groups.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine since there were no reports of the number of litters used. Number of samples reported varies from 5–8 per group depending on the figure.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, probably of technical grade (source Sigma). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose was used.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Quantitative measurement with well established methods not prone to bias. It is not clear if assessors were blinded to the treatment.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Pups body weight and survival were measured but not reported. It is unclear if dam body weights were recorded. CORT was administered in water, but there is no mention of dams' water consumption.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	No data on toxicity or body weight of pups or dams. Body weight and pups survival were measured but not reported. Dose used is at the high end of the no effect level for these endpoints when oral gavage exposure is used. Unclear about dosing via peanut butter.

Asari MA, Abdullah MS, Abdullah S, 2008 – RefID: 1525

Study characteristics and population	Specific endpoint	Quantitative Histochemistry (no. of Purkinje cells)
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male
	Housing condition:	Standard
	Funding source:	Public

	Group size:	10 male pups
Exposure	Period of exposure:	From PDN 2 to PDN 5
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg bw; 1 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PHRoB	No information on housing or husbandry. Approval or local animal committee. Same vehicle.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Hard to determine. Number of starting dams per treatment is not reported. It is reported that N=10 per endpoint, but no information on number of litters used or if the N is referring to the number of litters.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out. Deltamethrin is 99% pure from Sigma Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established quantitative method that used random sampling but subjective assessments. Same time point used. It is assumed that this did not adversely impact the outcomes, especially since the findings were negative. No blinding.

Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. All data reported for all methods described in methods section.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	Body weights reported for pups with about 10% lower body weight gain on PND7. Since no significant effects were reported on cerebellar anatomy, these body weight changes are not likely to be a threat. Statistical significance between group means was analysed by the unpaired two-tailed Student's t-test. There was no control for litter and this could bias either false positive and false negative results.

Guo J, Xu J, Zhang J, An L, 2018 – RefID: 1803

Study characteristics and population	Specific endpoint	Quantitative Histochemistry: (BrdU Ki67); (BrdU NeuN); (BrdU); (Ki67); (Pax6); (PPH); (Tbr1); (Tbr2); (TUNEL stain)
	Specie:	Mouse
	Strain:	ICR
	Sex:	Male and Female
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	14
Exposure	Period of exposure:	From GD 10.5 to GD 16.5
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg; 1.2 mg/kg
	Dose frequency:	nr
Measurement	Measurement time:	15.5 GD; 16.5 GD; 18.5 GD
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	University guideline for animal care and study conduct was used. No information about housing conditions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Not able to determine. The number of measurements was not reported.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	Purity was not reported, source is Chinese company. The same vehicle was used. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	No evidence of binding – but a little impact on the quantitative methods is assumed.
Selective outcome report	Q8: were all measured outcomes reported?	DHRoB	Not all measurements reported e.g. dam weight.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Fetal toxicity is present: body weight loss in pups about 20–25 % and embryo loss.

Neuropathology: Quantitative morphometric evaluation

Asari MA, Abdullah MS, Ismail ZIM, 2010 – RefID: 680

Study characteristics and population	Specific endpoint	Quantitative morphometric evaluation: EGL; IGL; ML
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	6 males pups
Exposure	Period of exposure:	From PDN 2 to PDN 5
	Route of administration:	Intraperitoneal
	Vehicle:	Corn oil

	Doses:	0 mg/kg bw; 1 mg/kg bw
	Dose frequency:	Day
Measurement	Measurement time:	6 PDN; 14 PDN; 21 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported. Intra-litter dosing design was used so lack of randomisation could increase the risk of bias. In addition, no information on whether groups were balanced by body weight.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not reported but likely to be similar based on experimental design. Same vehicle used.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Impossible to determine. It was reported 6 animals per groups but there table has no information about the number.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out. Deltamethrin was from Sigma at 98% purity. The solubility in corn is adequate at the dose tested. The administration was the same for all animals. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Standard techniques. Good methods descriptions. Small STDs. The age dependent changes in the control are the same as known biology of brain development. Not certainty about the group sizes.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	No. The 1mg/kg/day dose is well below systemic toxicity levels.

Guo J, Xu J, Zhang J, An L, 2018 – RefID: 1803

Study characteristics and population	Specific endpoint	Quantitative morphometric evaluation: CP; IZ; VZ/SVZ
	Specie:	Mouse
	Strain:	ICR
	Sex:	Male and Female
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	15
Exposure	Period of exposure:	From GD 10.5 to GD 16.5
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg; 1.2 mg/kg
	Dose frequency:	nr
Measurement	Measurement time:	18.5 GD
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	University guideline for animal care and study conduct was used. No information about housing conditions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	N =3 but started out with N=6.

Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	Purity was not reported, source is Chinese company. The same vehicle was used. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	No evidence of binding – but quantification is automatic.
Selective outcome report	Q8: were all measured outcomes reported?	DHRoB	Not all measurements reported e.g. dam weight, dam toxicity. In addition, is not possible to deduce if the results of all treated mice are reported in the paper.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Dams toxicity is present: body weight loss in pups about 20–25 % and embryo loss.

Kumar K, Patro N, Patro I, 2013 – RefID: 1930

Study characteristics and population	Specific endpoint	Quantitative morphometric evaluation: EGL; IGL; Length of dendritic arbour; ML; PCL; Reelin expression; Spine density/10 μm of dendrite; Width of dendritic arbour
	Specie:	Rat
	Strain:	Wistar
	Sex:	Female
	Housing condition:	Standard
	Funding source:	Public
	Group size:	3
Exposure	Period of exposure:	From GD 7 to GD 10
	Route of administration:	Intraperitoneal
	Vehicle:	DMSO
	Doses:	0 mg/kg bw per day; 0.75 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	0 PDN; 3 PDN; 7 PDN; 12 PDN; 15 PDN; 21 PDN; 30 PDN; 60 PDN; 90 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.

Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PLRoB	Not directly reported but same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were assumed identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	PHRoB	No. Methods and results do not agree (N=6 in methods; N=3 in Table 2) .
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No analytical work was carried out but purity reported as 98% from Sigma. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Well established methods and same time points. No blinding and subjective assessment but measures were automatic quantified.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes.
Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	No clinical signs reported. Decreases in body weight during lactation ranging from 35–41%.

Patro N, Mishra SK, Chattopadhyay M, Patro IK, 1997 – RefID: 2096

Study characteristics and population	Specific endpoint	Quantitative morphometric evaluation : CBL (total cortex layer); EGL; IGL; ML; PCL
	Specie:	Rat
	Strain:	Wistar
	Sex:	Male and Female
	Housing condition:	Not reported
	Funding source:	Public
	Group size:	–99

Exposure	Period of exposure:	From PDN 9 to PDN 13
	Route of administration:	Intraperitoneal
	Vehicle:	Propylene Glycol
	Doses:	0 mg/kg bw per day; 0.7 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	12 PDN; 15 PDN; 21 PDN; 30 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	NR	Not reported.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	NR	Same vehicle was used in control and experimental animals. Non-treatment-related experimental conditions were are assumed to be identical if authors did not report differences in housing or husbandry.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Not reported. There is no information in the paper on the number of animals per group.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	NR	The analytical work was not conducted. Purity was not reported. Propylene glycol was used as vehicle and the intraperitoneal (i.p.) route as route of administration. Kinetics will differ from oral exposures. Only one dose was tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PHRoB	Not reported. Methods are acceptable and same time points. However, no blinding and subjective assessment with ocular device.
Selective outcome report	Q8: were all measured outcomes reported?	DHRoB	Not all data reported.

Toxicity	Q9: were there other potential threats to internal validity?	DHRoB	Yes. There was a decrease in pups body weight (between 38 and 41%) during preweaning period. Brain weight was 25% lower during the post-natal period.

CONFIDENTIAL (2006) – RefID: 3201

Study characteristics and population	Specific endpoint	Quantitative morphometric evaluation: Cerebrum length (gross measurement); Caudate Putamen; Cerebellum; Cerebellum (gross measurements); Frontal Cortex; Hippocampal Gyrus; Parietal Cortex
	Specie:	Rat
	Strain:	Wistar
	Sex:	Female; Male
	Housing condition:	Standard (GLP)
	Funding source:	Private
	Group size:	10/sex
Exposure	Period of exposure:	From GD 6 to PDN 21
	Route of administration:	Oral (via feed)
	Vehicle:	nr
	Doses:	0 mg/kg bw per day; 1.64 mg/kg bw per day; 16.1 mg/kg bw per day; 6.78 mg/kg bw per day
	Dose frequency:	Day
Measurement	Measurement time:	21 PDN; 75 PDN
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. A protocol for randomisation was applied: 'Following at least six days of acclimation, P-generation females were weighed and those with a body weight more or less than 20% of the mean weight [18] were rejected. The remaining females were assigned to the control or an exposure group in sequence, as they were determined to be inseminated. This approach was used to ensure unbiased assignment of the animals to dose groups and that there would be approximately an equal number of litters from each dose group available for testing on a given day.'

Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. Supported by methods descriptions.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. All exceptions were noted, and all data were presented in appendices.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	DLRoB	Yes. Extensive analytical work was conduction on the diet. Brain concentrations were measured in offspring.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Yes. Not blinded but automated assessments
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. Extensive methods and results sections. All data included in appendices.
Toxicity	Q9: were there other potential threats to internal validity?	DLRoB	Maternal body weight gain was marginally impacted, but not enough to bias results.

Clinical chemistry: Hormones

Vester AI, Chen M, Marsit CJ, Caudle WM, 2019 – RefID: 961

Study characteristics and population	Specific endpoint	Hormones
	Specie:	Mouse
	Strain:	C57BL/6NCrl
	Sex:	Female
	Housing condition:	Standard (GLP)
	Funding source:	Public
	Group size:	–99

Exposure	Period of exposure:	From Pre-Mating day 1 to PDN 21
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil mixed with peanut butter
	Doses:	0 mg/kg deltamethrin; 3 mg/kg deltamethrin
	Dose frequency:	Every 3 day
Measurement	Measurement time:	–99 nr
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	PLRoB	Stated randomisation of dams.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for animal studies.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	PHRoB	Only partially reported for lighting on/off, no information about T°, humidity, feed type and water source. Unclear whether peanut butter vehicle was given to controls. Same water used for CORT exposures in both groups.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	NR	Unable to determine since there were no reports of the number of litters used. Number of samples reported varies from 5–9 per group depending on the figure.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	No purity reported, probably of technical grade (source Sigma). The analytical work was not carried out, the dose was administered in peanut butter and it was not clear the impact on kinetic. Only one dose was used.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Quantitative measurement with well established methods not prone to bias. Assessors were not blinded to the treatment, but the influence is low. In addition, standard curves, plate differences (if any) and limit of quantification were not reported.

Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Pups body weight and survival were measured but not reported. It is unclear if dam body weights were recorded.
Toxicity	Q9: were there other potential threats to internal validity?	PLRoB	No data on toxicity or body weight of pups or dams. Body weight and pup's survival were measured but not reported. Dose used is at the high end of the no effect level for these endpoints when oral gavage exposure is used. Unclear about dosing via peanut butter. For statistical analysis males and females were separately tested. Authors said that they stratified by sex instead using a two-way ANOVA. Therefore, those samples violated the independent sample assumption as the litter was considered the smallest independent statistical unit. However, experts disagreed with this reasoning because standard statistical methods are available to test for sex within litter effects.

Neurophysiology: LTP

Pitzer EM, Sugimoto C, Gudelsky GA, Huff Adams CL, Williams MT, Vorhees CV, 2019 – RefID: 2121

Study characteristics and population	Specific endpoint	LTP (Long term potentiation)
	Specie:	Rat
	Strain:	Sprague-Dawley
	Sex:	Male and Female
	Housing condition:	Non-standard
	Funding source:	Public
	Group size:	CO: 7 M; 1.0 mg/kg DLM: 8 M
Exposure	Period of exposure:	From PDN 3 to PDN 20
	Route of administration:	Oral (via gavage)
	Vehicle:	Corn oil
	Doses:	0 mg/kg deltamethrin; 0.25 mg/kg deltamethrin
	Dose frequency:	Day
Measurement	Measurement time:	25 PDN
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement

Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Yes. Pups were randomly assigned to 4 treatment groups.
Allocation concealment	Q2: was allocation [of animals] to study groups adequately concealed?	PLRoB	Not relevant for rodents' studies.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes. The study was well conducted and described.
Blind research personnel	Q4: was the research personnel* blinded to the study group during the study?	NR	Not reported.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	A lot in information on numbers of animals. Checking numbers of animals in graphs they seem to be consistent with methods section.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PHRoB	The test substance was supplied as technical grade from the manufacturer (Bayer Crop Science) with a purity of 99.9%. Concentrations were well below the known solubility in corn oil. Moreover, duration and number of concentrations are adequate. Only one dose and a control were tested.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	The study was well conducted and run by a well-regarded laboratory. Blinding was not reported, but most tests were automated and not likely to bias results.
Selective outcome report	Q8: were all measured outcomes reported?	DLRoB	Yes. All data reported in text or figures as group means.
Toxicity	Q9: were there other potential threats to internal validity?	PHRoB	There was no maternal exposure, so no risk of maternal toxicity. However, there were large body weight effects in the two high dose groups, during the preweaning period (up to about 28 and 33% in the mid and high doses at the end of weaning). The effect on body weight persisted through adult ages, but the magnitude was decreased (~10 and 12% by 112 days of age)

Critical appraisal table-human

Attention-deficit hyperactivity disorder (ADHD)

Dalsager L, Fage-Larsen B, Bilenberg N, Jensen TK, Nielsen F, Kyhl HB, Grandjean P, Andersen HR, 2019 – RefID: 876

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	948
	Exclusion criteria:	nr
	N enrolled subject:	948
	Sex of subject:	Male and Female
	Age of subject:	1.7–4.1 years
	Funding source:	Mixed
Exposure	Metabolites:	3-PBA
	Period od exposure:	During gestation
Measurement	Measurement time:	2–4 Year
	Endpoint:	ADHD

Statistical analysis	Statistical analysis:	/; Logistic regression: association between metabolite concentrations and ADHD assessed with ADHD score DICHOTOMISED, assessed against the baseline (ref) exposure as a continuous ln2-transformed variable. Z-test to calculate a p trend across the tertile exposure groups. The estimates are presented as odds ratios. Covariates included in the adjusted analyses selected a priori based on a directed acyclic graph (DAG) . Factors that may influence either maternal exposure to insecticides in pregnancy or ADHD symptom score in the children were included in the DAG.; Logistic regression: association between metabolite concentrations and ADHD assessed with ADHD score DICHOTOMISED, assessed against the baseline (ref) exposure expressed in TERTILES. Z-test to calculate a p trend across the tertile exposure groups. The estimates are presented as odds ratios. Covariates included in the adjusted analyses selected a priori based on a directed acyclic graph (DAG) . Factors that may influence either maternal exposure to insecticides in pregnancy or ADHD symptom score in the children were included in the DAG.; Logistic regression: association between metabolite concentrations and ADHD assessed with ADHD score DICHOTOMISED, assessed against the baseline (ref) exposure as a continuous ln2-transformed variable. The estimates are presented as odds ratios. Covariates included in the adjusted analyses selected a priori based on a directed acyclic graph (DAG) . Factors that may influence either maternal exposure to insecticides in pregnancy or ADHD symptom score in the children were included in the DAG.; Negative binomial regression: association between metabolite concentrations and ADHD assessed as ORDINAL DATA and assessed against the baseline (ref) exposure expressed as a continuous ln2-transformed variable. The estimates are presented as ratios reflecting the expected relative change in the ADHD score for a doubling in the urinary metabolite concentration for the ln2-transformed variable. Covariates included in the adjusted analyses selected a priori based on a directed acyclic graph (DAG). Factors that may influence either maternal exposure to insecticides in pregnancy or ADHD symptom score in the children were included in the DAG; Negative binomial regression: association between metabolite concentrations and ADHD assessed as ORDINAL DATA and assessed against the baseline (ref) exposure expressed as a continuous ln2-transformed variable. The estimates are presented as ratios reflecting the expected relative change in the ADHD score for a doubling in the urinary metabolite concentration for the ln2-transformed variable. Covariates included in the adjusted analyses selected a priori based on a directed acyclic graph (DAG) . Factors that may influence either maternal exposure to insecticides in pregnancy or ADHD symptom score in the children were included in the

		DAG; Negative binomial regression: association between metabolite concentrations and ADHD assessed as ORDINAL DATA and assessed against the baseline (ref) exposure expressed in TERTILES. Z-test to calculate a p trend across the tertile exposure groups. The estimates are presented as ratios reflecting the expected relative change in the ADHD score compared to the low tertile. Covariates included in the adjusted analyses selected a priori based on a directed acyclic graph (DAG). Factors that may influence either maternal exposure to insecticides in pregnancy or ADHD symptom score in the children were included in the DAG; Negative binomial regression: association between metabolite concentrations and ADHD assessed as ORDINAL DATA and assessed against the baseline (ref) exposure expressed in TERTILES. Z-test to calculate a p trend across the tertile exposure groups. The estimates are presented as ratios reflecting the expected relative change in the ADHD score compared to the low tertile. Covariates included in the adjusted analyses selected a priori based on a directed acyclic graph (DAG). Factors that may influence either maternal exposure to insecticides in pregnancy or ADHD symptom score in the children were included in the DAG.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	The study participants were derived from the Odense Child Cohort, which is an ongoing prospective birth cohort study on children's health in Odense, Denmark. So, pregnant women were recruited from the same eligible population, with the same method and inclusion and exclusion criteria.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	Information on maternal age and educational level was obtained from the first questionnaire administered during pregnancy. Perceived stress during pregnancy was reported in a second questionnaire. Furthermore, information on parity, smoking, child sex, date of birth, birth weight and prematurity was collected from the hospital birth records. For the statistical analysis, covariates included in the adjusted analyses were selected a priori based on a DAG. Factors that may influence either maternal exposure to insecticides in pregnancy or ADHD symptom score in the children were included in the DAG. However, not all co-exposures were measured (OP metabolites were measured but not lead).

Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PLRoB	An invitation to fill online the CBCL:1½-5 questionnaire was mailed to 2551 parents (15 did not wish to be contacted). However, a total of 1942 (76.6%) answered the questionnaire.
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	DHRoB	It not possible to estimate associations between cis-DBCA (specific PYR metabolites) and ADHD scores due to the small number of subjects with concentrations above the LODs (3%)
Outcome assessment	Q5: Can we be confident in the outcome assessment?	PLRoB	Mothers completed online the Child Behaviour Checklist for ages 1½-5 years (CBCL:1½-5). The CBCL is a parent-report questionnaire on which the child is rated on various behavioural and emotional problems. CBCL:1½-5 is a well established and frequently used parent checklist with solid psychometric properties, including strong stability over time. However, CBCL is not a specific diagnostic tool for ADHD and it was not administered by experienced neuropsychologists.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	The ADHD score was analysed as ordinal data and also as a dichotomised variable (scoring â‰¥ 90th percentile on the CBCL:1½-5) using appropriated regression analysis.
Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	DLRoB	-preprocessing: for metabolite concentration: substitution of <LOD with LOD/RADQ(2), for low freq dichotomisation, otherwise ln2() transformation; for score dichotomisation; -descr stat: Kruskal–Wallis test and chi-squared test: statistical tests are appropriate for the considered variables -statmodel: negative binomial regression was used to assess the association between ln2(concentration) and ADHD scores; logistic regression model was used to assess association between ln2(concentration) and dichotomised scores. -variable selection: DAG -modifying variable: stratification by sex -sensitivity analysis: excluding preterm children; adjusting for breast feeding

Behavioural

Viel JF, Rouget F, Warembourg C, Monfort C, Limon G, Cordier S, Chevrier C, 2017 – RefID: 129

Study	Study type	Prospective cohort

characteristics and population	N enrolled mothers:	287
	Exclusion criteria:	EXCLUDED: - <35 weeks of amenorrhea
	N enrolled subject:	287
	Sex of subject:	Male and Female
	Age of subject:	6 years
	Funding source:	Mixed
Exposure	Metabolites:	3-PBA; cis-DBCA
	Period od exposure:	During gestation
Measurement	Measurement time:	6 Year
	Endpoint:	Behaviour – Externalising score; Behaviour – Internalising score; Behaviour – Prosocial behaviour
Statistical analysis	Statistical analysis:	Associations between dichotomised behavioural subscales (abnormal/borderline vs normal) as outcomes and prenatal urinary pyrethroid metabolite concentrations were examined using multiple logistic regression models with the following selection and analysis strategy. Metabolite concentrations were dichotomised if the proportion of non-detected values was greater than 50% (3-PBA) or divided into 3 groups if the proportion of non-detected was in the range 30–50% (cis-DBCA) To preserve the size of the analytic sample, missing values for covariates were replaced by the modal value from participants with non-missing values. Imputation was required for 6 mothers (6 missing data values, i.e. 0.2%) and 14 children (40 missing data values, i.e. 1.5%). Child's sex and maternal education and DE and DM phosphate metabolites in maternal urine samples and the corresponding childhood concentration of metabolite were included in models a priori. The remaining variables that predicted both the behavioural scores and the pyrethroid metabolite levels with p -value < 0.2 were selected as model covariates. Reverse-scale Cox regression model was performed to handle non-detected values. In this method, the measured metabolite is treated as the modelled outcome. The method makes full use of quantifiable metabolite measurements and appropriately treats non-detected values as censored. The corresponding hazard ratio parameter is interpretable as an odds ratio (OR), that is the odds of the health outcome at concentration t divided by the odds of the health outcome for the aggregate of concentrations below t, assuming that this OR is the same across all concentrations. For hazard ratio parameters only their corresponding p-values are reported.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	Mother-child birth cohort (PELAGIE, France) with detailed description of including/excluding criteria, exposure and outcome measurement. The rationale behind the random selection of a subcohort is explained and reported in a previous study of the same group
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PHRoB	Although authors claim that acid-leachable lead in the living room was measured as a co-exposure, statistical models shown in Table 3 did not adjust for this variable. MW: Lead was only measured in dust samples at the home visit at age 6, but it seems no adjustments for this were made in any of the calculations. Information was collected on sociodemographic characteristics, lifestyle factors and mothers' child health, behaviour and environmental co-exposures (organophosphate metabolites in maternal urine and lead in home dust). Also, maternal intelligent scoring was performed along with interviews for home environment assessments
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	DLRoB	The neuropsychological tool (SDQ) was not completed by 3 children. For the remaining 284 children, 13 missing data values for the neurobehavioral scores were replaced, for a given subscale, by the mode estimated from the children with an exact match on the three other subscales (thus, multiple imputation was not used).
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	PHRoB	Exposure was characterised using biomonitoring techniques. Urinary levels of pyrethroid metabolites (3-PBA, 4-F-3-PBA, cis-DCCA and trans-DCCA, and cis-DBCA) were analysed which included specific and non-specific and specific metabolites for deltamethrin. However, only a single urine sample was analysed, corresponding to the first trimester of pregnancy

Outcome assessment	Q5: Can we be confident in the outcome assessment?	PLRoB	SDQ is a validated screening instrument for epidemiologic research in children 4–16 y. Parents, mostly mothers, completed a list of 25 questions to describe their child's behaviour in the previous 6 months. Blinding doses not apply as this is a birth cohort design. Experienced neuropsychologist did not administer the questionnaires as only parents knew their children behaviour in the past 6 months.
Selective outcome report	Q6: Were all measured outcomes reported?	DLRoB	Three subscales were considered from the SDQ: prosocial behaviour, internalising disorders and externalising disorders. These were reported in the results and statically assessed separately.
Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	PLRoB	Multiple logistic regression analysis was used. Models were adjusted for maternal and children factors as well as for environmental factors and co-exposures. However, exposure data were dichotomised. The 3 endpoints for SDQ questionnaire were regressed against those factors and the different pyrethroid metabolites measured.

Eskenazi B, An S, Rauch SA, Coker ES, Maphula A, Obida M, Crause M, Kogut KR, Bornman R, Chevrier J, 2018 – RefID: 1117

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	705
	Exclusion criteria:	EXCLUDED: <18 years; not speaking TshiVenda as the main language at home; not living within 20 km (12.4mi) of the hospital and not planned to remain in the area; diagnosis with malaria during pregnancy
	N enrolled subject:	705
	Sex of subject:	Male and Female
	Age of subject:	2 years
	Funding source:	Public
Exposure	Metabolites:	/; 3-PBA; cis-DBCA
	Period od exposure:	na; Prior to delivery; Prior to delivery OR Postdelivery before leaving the hospital
Measurement	Measurement time:	1 Year
	Endpoint:	Social-Emotional

Statistical analysis	Statistical analysis:	Adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). p-Values of 0.05 considered statistically significant; Bayesian kernel machine regression (BKMR) model to consider individual insecticide exposure–responses (and potential nonlinearity) with co-occurring insecticides, and evaluate each exposure's relative importance with respect to the outcome via Bayesian variable selection, the exposure–response of the overall mixture, interaction between any two insecticide measures and each chemical exposure–response while holding the other insecticide measure at various percentiles of exposure. Posterior inclusion probabilities (PIPs) used to highlight the relative ranking of variable importance for each pesticide class as well as each pesticide within a particular class of insecticides. PIPs result from the Bayesian Markov chain Monte Carlo (MCMC) iterations (25, 000). A PIP for an exposure (or group PIP) is calculated as the posterior mean of the indicator variable across the MCMC iterations, in which the indicator variable equals 1 if the variable (or group) is selected into the model at a given iteration or 0 otherwise. Group PIP = indicates the posterior probability that an exposure grouping (e.g. pyrethroids) was included into the 'true' model from the multiple iterations (25, 000) of the MCMC sampler. Conditional = indicates the posterior probability that a particular chemical exposure (e.g. cis-DCCA) within an exposure grouping (e.g. pyrethroids) was included into the 'true' model from the multiple iterations (25, 000) of the MCMC sampler, conditional on the exposure grouping being included. R (version 3.3.1; R Development Core Team); Sex-stratified adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). Interactions considered statistically significant at p<0:1. p-Values of 0.05 considered statistically significant.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	PLRoB	Longitudinal birth cohort: Venda Health Examination of Mothers, Babies and the Environment (VHEMBE). Pregnant women were recruited from the same eligible population and had to fulfil specific selection criteria, exposure and outcome were quantified making thus comparison possible (unbiased study). No explanation is given why some women did not complete the questionnaire.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	Information was gathered on sociodemographic characteristics, reproductive and medical history, personal habits (smoking and use of alcohol or drugs), occupational and residential history, household composition and use of pesticides around the home, at work, in their fields, on their livestock or for malaria control. The study tested for effect modification by child sex using stratification and cross-product terms. A number of sensitivity analyses were made. Appropriate adjustments were made for modifying variables and confounders in the final multivariate statistical models. Covariates for the multivariable models were identified using a directed acyclic graph (DAG). Models were rerun considering child blood lead concentrations, child haemoglobin levels and maternal HIV status as covariates. However, co-exposure for OP metabolites was not assessed
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	DLRoB	A total of 689 children were assessed for outcomes at 1-y visit and 681 at 2-y. The Cognitive, Language (Receptive and Expressive), and Motor (Fine and Gross) subtests were administered at both visits, and the Social-Emotional subtest only at the 1-y assessment. The attrition is less than 5%.
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	PHRoB	A spot urine sample was collected for 432 women before delivery and for 263 women postdelivery (but before leaving the hospital). Pyrethroid metabolites (cis-DBCA and the non-specific 3-PBA metabolite) were measured using GC-MS

Outcome assessment	Q5: Can we be confident in the outcome assessment?	DLRoB	Two bilingual English-TshiVenda psychologists, trained by a TshiVenda developmental neuropsychologist, administered the Bayley Scales of Infant Development, third edition (BSID-III). A portion of the assessments were videotaped for quality control. Each week, one video was randomly selected for review by all three psychologists to assure consistency in administration and scoring. It is considered a well established method.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	No protocol available, but all of the study's measured outcomes were measured according to the research plan and adequately reported, although at 2 y assessment, the Social-Emotional subtest was not administered and no clear explanation was given. However, this is explicitly acknowledged in the methodology.

Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	DLRoB	-preprocessing: log10 of concentration -missing values: -descr stat: yes -statmodel 1: longitudinal models using generalised estimating equations (GEEs)to assess relationship across 1 and 2 visit (GAM to test linearity) -variable selection: DAG -modifying variable: stratification for sex, cross-product term sex* -sensitivity analysis: restriction to participants collecting urine sample pre-delivery; restriction to children having lead and haemoglobin level measured -stat model 2: Bayesian kernel machine regression for bi pollutant interaction -variable selection: hierarchical var selection. Additional note for Bayesian kernel machine regression model: this model is an innovative approach for addressing mixture components having complex non-linear and non-additive relationship with health outcome. The hierarchical variable selection, incorporate information on the structure of the mixtures into the model allowing for grouping highly correlated components (p, p'DDT and p, p'-DDE in one group and pyrethroid in another group). Model results include only posterior inclusion probabilities (for group and for components in group) describing the probability of inclusion of the mixture component(s). In theory the model estimates the exposure–response function too. (probably the full characterisation of the form of the exposure–response function required too many data). Limit of the approach: it could be very sensitive to the specification of the mixture prior.

Furlong MA, Barr DB, Wolff MS, Engel SM, 2017 – RefID: 1118

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	162
	Exclusion criteria:	EXCLUDED:- non-primiparous; – medical complications

	N enrolled subject:	163
	Sex of subject:	Male and Female
	Age of subject:	9 years
	Funding source:	Public
Exposure	Metabolites:	3-PBA
	Period od exposure:	During gestation
Measurement	Measurement time:	4–5, 6, 7–9 Year
	Endpoint:	Behaviour- Internalising Composite; Behaviour – Adaptive Skills Composite; Behaviour – Atypicality; Behaviour – Behavioural Regulation Index; Behaviour – Behavioural Symptoms Index; Behaviour – Externalising Composite; Behaviour – Global Executive Composite; Behaviour – Withdrawal
Statistical analysis	Statistical analysis:	Linear mixed model, random effects for subjects. Assumption: all missing covariate data missing at random. Directed acyclic graphs (DAGs) to identify possible colliders and mediators. They adjusted for the minimally sufficient set and variables that were hypothesised to be highly predictive of the outcome, after noting adjustment for creatinine, sum of DMPs and visit in the DAG. In sensitivity analyses exclusion of organophosphate pesticides (OPs) from the models, assessed a binary variable for detection of malathion (an OP) as a confounder, and excluded individuals with Behavioural Assessment System for Children (BASC) F Index scores >4, which indicate unreliable answering patterns. Assessment of interactions between 3-PBA and race, and between 3-PBA and sex for each scale and set an alpha of 0.10 to denote presence of a notable interaction. All analyses were performed in R v3.3.1.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement

Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	Longitudinal (prospective) birth cohort: Mount Sinai Children's Environmental Health Center. Primiparous women were enrolled between 1998 and 2002, and had to fulfil specific selection criteria. Exposure and outcome were quantified making thus comparison possible (unbiased study). The study assessed whether characteristics at enrolment were different by follow-up status. (for inclusion on the appraisal rational) also: Large Longitudinal birth cohort is considered well established study design with DL RoB for selection of participants and enough details in how people were selected.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	During the third trimester, participants completed questionnaires about home, demographics and behavioural characteristics during pregnancy. Directed acyclic graphs (DAGs) were constructed to identify possible colliders and mediators (maternal education, maternal marital status at follow-up, race/ethnicity, quality of the home environment, maternal IQ, sum DMP pesticide use, smoking and alcohol during pregnancy, creatinine and preterm birth). Co-exposure to OP metabolites was assessed but not to lead

Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PHRoB	404 primiparous women in late pregnancy were recruited from 1998 to 2001. From them, 162 mother/child pairs with complete exposure and behavioural outcomes data were studied and their data reported in the results. From 361 pregnant women at enrolment, 168 returned for follow-up at 4–5-, 6-, or 7–9-year visit and 162 had complete exposure and behavioural outcomes. Hence, this study had substantial loss-to-follow-up. Follow-up loses are common in cohort studies, and selection bias needs to be checked for. In this study, women with higher levels of 3-PBA (and the other two pyrethroid metabolites studied) were less likely to return for follow-up, what might have biased results. Although the appraisal rated Q3 as PLRoB, we should reconsider this rating and perhaps to downgrade to e.g. PHRoB. It is also of concern that only 32 participants with complete covariate data attended all three follow-ups, but there are no additional data on this.
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	DHRoB	Prenatal pyrethroid metabolites (3-PBA) were analysed by the Centers for Disease Control and Prevention using methods described elsewhere. High performance liquid chromatography-tandem mass spectrometry. Biomarkers were analysed from a single spot urine sample in the third trimester. Non-specific PYR metabolites were measured.
Outcome assessment	Q5: Can we be confident in the outcome assessment?	PLRoB	The Behavioural Assessment System for Children (BASC) and the Behaviour Rating Inventory of Executive Function (BRIEF) were used. Test-retest reliabilities and internal consistencies are good (Cronbach's alphas were calculated) The BASC and the BRIEF were both completed at the 4-, 6- and 7-year visits. The questioner is design for parents to assess the behaviour of the children. It is considered well established method. No expertise is needed for assessing the question.

Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	All of the study's measured outcomes were measured according to the research plan and adequately reported in Table 3. Enrolment (n = 361) and follow-up (n = 162
Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	PLRoB	-preprocessing: concentrations were dichotomised, outcomes were scaled -missing values: The study assumed that all missing covariate data were missing at random. -descr stat: to test if enrolled are equal to follow-up, Chi-squared for categorical var, t-test for continuous var (Normality not checked) -statmodel: Associations between the pyrethroid metabolites and each of the composites and subscales were estimated with linear mixed models, with random effects for subject. -variable selection: DAG, Statistical models were adjusted for the minimally sufficient set and variables that were hypothesised to be highly predictive of the outcome. -modifying variable: cross-product 3PBA*race, 3PBA*sex -sensitivity analysis: excluding OPs from model, excluding observation with BASC score >4 (not reliable) Not details are providing for the missing data.

Cognitive impairment

Xue Z, Li X, Su Q, Xu L, Zhang P, Kong Z, Xu J, Teng J, 2013 – RefID: 433

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	497
	Exclusion criteria:	EXCLUDED: - unqualified samples of urine - stillbirths
	N enrolled subject:	497
	Sex of subject:	Male and Female
	Age of subject:	1 year
	Funding source:	Unclear
Exposure	Metabolites:	Total permethrin; Total Permethrin (sum of cis-Cl2CA, trans-Cl2CA, 3-PBA)

	Period od exposure:	Prior to delivery
Measurement	Measurement time:	1 Year
	Endpoint:	Development quotient; mental development index
Statistical analysis	Statistical analysis:	/; Variance analysis. All the statistical tests were two-sided. P < 0.05 was taken to indicate a difference of statistical significance. Epidata 3.1 and SSPS
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	This is a birth cohort where all women were recruited with the same method and using the same inclusion and exclusion criteria. Exposure was assessed by urine biomonitoring for all pregnant women.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	DHRoB	Information was collected using a self-made questionnaire: basic information on pregnancy, occupational history, spouse, family, living environment (including pesticide exposure), lifestyle, pregnancy history, history of diseases, medical history and vitamin supplement intake. Information on growth and development, feeding, diseases together with basic information on the family, environment and lifestyle was also gathered from mother using another questionnaire. The above variables are effect modifying variables and potential confounders of MDI and DQ. However, these confounders were not properly analysed.
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	DLRoB	The basic features of the infants that were not followed-up, such as gender, developmental status at birth and basic demography of parents and families, were compared with those who were followed-up, and no significant differences were found. Thus, selection bias can be ruled out. (Follow-up rate 88%). Table 4 shows that the outcome was assessed in all children, without attrition.
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	DHRoB	Only a single spot urine sample was collected prenatally from mothers. Then, samples were subjected to liquid-liquid extraction and further gas chromatography mass spectrometry analysis. However, only non-specific metabolites measured

Outcome assessment	Q5: Can we be confident in the outcome assessment?	DLRoB	The Development Screen Test (DST) scale was used to assess the intellectual development of infants. The assessment included movement, social adaptation, and intelligence, and there were 120 test items in total. The MDI and DQ were obtained after taking the scores of these three parts into consideration. DST was designed by Dr Bharath Raj (1983) and is considered a reliable and valid test to screen the mental development of children in the Indian context. The DST measures mental development of children from birth to 15y of age (https://shodhganga.inflibnet.ac.in/bitstream/10603/110045/11/11_chapter-4.pdf). The survey was conducted by qualified investigators and lasted 30 min. The collection and recording of information of newborns were completed by staff in the hospital.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	The Development Screen Test (DST) scale was applied to all infants to assess their intellectual development. The assessment provided information on 2 endpoints: mental development index (MI) and development quotient (DQ). Information on these was available for all followed-up infants. However, no indication of the protocol is provided
Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	PHRoB	-preprocessing: concentration <LOD converted in LOD/2 -missing values: NULL -statistical model: Kruskal–Wallis test and one-way ANOVA test to assess DQ changes in the different exposure groups -variable selection: NULL -modifying variable: NULL -sensitivity analysis: NULL Confounding issues assessed in question 2.

Fluegge KR, Nishioka M, Wilkins JR, 3rd 2016 – RefID: 480

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	140
	Exclusion criteria:	nr
	N enrolled subject:	140

	Sex of subject:	Male and Female
	Age of subject:	3 months
	Funding source:	Public
Exposure	Metabolites:	3-PBA
	Period od exposure:	During gestation
Measurement	Measurement time:	3 Month
	Endpoint:	Mental development index
Statistical analysis	Statistical analysis:	REGRESSION ANALYSIS: generalised linear model using maximum likelihood estimation results to assess the association between prenatal metabolites and MENTAL endpoint. IMPUTATION: Linear interpolation to estimate second and/or third trimester weights once prepregnancy weight and pregnancy weight gain were determined. Imputation using a multivariate normal model (MVN) and imputation by chained equations (ICE) using cross imputation (all variables to be included in a model were used to predict all other variables in the model, known as a fully conditional specification (FCS)), for each imputed weight variable. Multiple imputation (MI) procedures used as simulation techniques to replace each missing data point with a set of m>1 plausible value (based on known relevant predictors). These imputations were created through a Bayesian process [...]
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	The study design consists of a birth cohort with 140 mother-infant pairs. Maternal urine was collected in 2nd and 3rd trimesters of gestation and infant urine was at 2 months of age. BSID-II testing was completed at 3 months of age to all children. Standard demographic information, pregnancy weight, potentially confounding family-based factors (e.g. race, socioeconomic status, maternal education), and relevant clinical information pertaining to the pregnancy and birth event were collected. Therefore, baseline characteristics of mothers-infant pair were similar and comparable.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	DHRoB	They did not control for the confounders, (Table 5). No co-exposures were considered in the models despite OP metabolites were analysed

Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PLRoB	The 140 mother-infant pairs from this birth cohort were followed-up for two years. However, Bayley's mental and motor score at 3 months was reported for 119 infants (Table 2), but the proportion lost to follow-up would not appreciably bias results.
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	DHRoB	Exposure was consistently assessed through biomonitoring of general and specific pyrethroid metabolites at two trimesters of pregnancy using well established methods. However, the method validation is not provided and no reference is given to a previous study addressing this validation. Besides, although specific PYR metabolites were analysed, they were not associated with neurodevelopmental outcomes very likely because all maternal samples were below the LOD for DBCA
Outcome assessment	Q5: Can we be confident in the outcome assessment?	PLRoB	Infant neurodevelopment was evaluated using the Bayley Scales of Infant Development (BSID-II) at three months of age. Although this is a well-known valid and reliable neurobehavioral scale, no information is provided on whether it was administered by trained neuropsychologists.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	The Bayley Scale for Infant Development (BSID) assessed 2 neurological domains: motor and mental development (MDI and MDI, respectively). Quantitative data for both of them are reported in the results. However, protocol not provided.
Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	DLRoB	-preprocessing: NULL (only value >LOD were included) -missing values: imputation using linear interpolation to estimate second and/or third trimester mother weights; using multivariate normal model; using chained equation (correlation of the two imputation methods was tested and all regression models were repeated including binary variable indicating whether mother's weights were imputed) -statmodel: generalised linear model -variable selection: forward selection of maternal and pregnancy-related variables, while metabolites were included only if median>LOD -sensitivity analysis: excluding imputed weight values

Eskenazi B, An S, Rauch SA, Coker ES, Maphula A, Obida M, Crause M, Kogut KR, Bornman R, Chevrier J, 2018 – RefID: 1117

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	705
	Exclusion criteria:	EXCLUDED: <18 years; not speaking TshiVenda as the main language at home; not living within 20 km (12.4mi) of the hospital and not planned to remain in the area; diagnosis with malaria during pregnancy
	N enrolled subject:	705
	Sex of subject:	Male and Female
	Age of subject:	2 years
	Funding source:	Public
Exposure	Metabolites:	/; 3-PBA; cis-DBCA
	Period od exposure:	na; Prior to delivery; Prior to delivery OR Postdelivery before leaving the hospital
Measurement	Measurement time:	1 Year; 2 Year
	Endpoint:	Cognitive

Statistical analysis	Statistical analysis:	Adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). p-Values of 0.05 considered statistically significant; Bayesian kernel machine regression (BKMR) model to consider individual insecticide exposure–responses (and potential nonlinearity) with co-occurring insecticides, and evaluate each exposure's relative importance with respect to the outcome via Bayesian variable selection, the exposure–response of the overall mixture, interaction between any two insecticide measures and each chemical's exposure–response while holding the other insecticide measure at various percentiles of exposure. Posterior inclusion probabilities (PIPs) used to highlight the relative ranking of variable importance for each pesticide class as well as each pesticide within a particular class of insecticides. PIPs result from the Bayesian Markov chain Monte Carlo (MCMC) iterations (25, 000). A PIP for an exposure (or group PIP) is calculated as the posterior mean of the indicator variable across the MCMC iterations, in which the indicator variable equals 1 if the variable (or group) is selected into the model at a given iteration or 0 otherwise. Group PIP = indicates the posterior probability that an exposure grouping (e.g. pyrethroids) was included into the 'true' model from the multiple iterations (25, 000) of the MCMC sampler. Conditional = indicates the posterior probability that a particular chemical exposure (e.g. cis-DCCA) within an exposure grouping (e.g. pyrethroids) was included into the 'true' model from the multiple iterations (25, 000) of the MCMC sampler, conditional on the exposure grouping being included. R (version 3.3.1; R Development Core Team); Sex-stratified adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). Interactions considered statistically significant at p<0:1. p-Values of 0.05 considered statistically significant; Sex-stratified adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations

		(GEEs). STATA version 13 (Stata Corp). Interactions considered statistically significant at p<0:1. p-Values of 0.05 considered statistically significant.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	PLRoB	Longitudinal birth cohort: Venda Health Examination of Mothers, Babies and the Environment (VHEMBE). Pregnant women were recruited from the same eligible population and had to fulfil specific selection criteria, exposure and outcome were quantified making thus comparison possible (unbiased study). No explanation is given why some women did not complete the questionnaire.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	Information was gathered on sociodemographic characteristics, reproductive and medical history, personal habits (smoking and use of alcohol or drugs), occupational and residential history, household composition and use of pesticides around the home, at work, in their fields, on their livestock or for malaria control. The study tested for effect modification by child sex using stratification and cross-product terms. A number of sensitivity analyses were made .Appropriate adjustments were made for modifying variables and confounders in the final multivariate statistical models. Covariates for the multivariable models were identified using a directed acyclic graph (DAG). Models were rerun considering child blood lead concentrations, child haemoglobin levels and maternal HIV status as covariates. However, co-exposure for OP metabolites was not assessed
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	DLRoB	A total of 689 children were assessed for outcomes at 1-y visit and 681 at 2-y. The Cognitive, Language (Receptive and Expressive), and Motor (Fine and Gross) subtests were administered at both visits, and the Social-Emotional subtest only at the 1-y assessment The attrition is less than 5%.

Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	PHRoB	A spot urine sample was collected for 432 women before delivery and for 263 women postdelivery (but before leaving the hospital). Pyrethroid metabolites (cis- DBCA and the non-specific 3-PBA metabolite) were measured using GC-MS
Outcome assessment	Q5: Can we be confident in the outcome assessment?	DLRoB	Two bilingual English-TshiVenda psychologists, trained by a TshiVenda developmental neuropsychologist, administered the Bayley Scales of Infant Development, third edition (BSID-III). A portion of the assessments were videotaped for quality control. Each week, one video was randomly selected for review by all three psychologists to assure consistency in administration and scoring. It is considered a well established method.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	No protocol available, but all of the study's measured outcomes were measured according to the research plan and adequately reported, although at 2 y assessment, the Social-Emotional subtest was not administered and no clear explanation was give. However, this is explicitly acknowledged in the methodology.

Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	DLRoB	-preprocessing: log10 of concentration -missing values: -descr stat: yes -statmodel 1: longitudinal models using generalised estimating equations (GEEs)to assess relationship across 1 and 2 visit (GAM to test linearity) -variable selection: DAG -modifying variable: stratification for sex, cross-product term sex* -sensitivity analysis: restriction to participant collecting urine sample pre-delivery; restriction to children having lead and haemoglobin level measured -stat model 2: Bayesian kernel machine regression for bi pollutant interaction -variable selection: hierarchical var selection Additional note for Bayesian kernel machine regression model: this model is an innovative approach for addressing mixture components having complex non-linear and non-additive relationship with health outcome. The hierarchical variable selection, incorporate information on the structure of the mixtures into the model allowing for grouping highly correlated components (p, p'DDT and p, p'-DDE in one group and pyrethroid in another group). Model results include only posterior inclusion probabilities (for group and for components in group) describing the probability of inclusion of the mixture component(s). In theory the model estimate the exposure–response function too. (probably the full characterisation of the form of the exposure–response function required too many data). Limit of the approach: it could be very sensitive to the specification of the mixture prior.

Furlong MA, Barr DB, Wolff MS, Engel SM, 2017 – RefID: 1118

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	162
	Exclusion criteria:	EXCLUDED:- non-primiparous; – medical complications

	N enrolled subject:	163
	Sex of subject:	Male and Female
	Age of subject:	9 years
	Funding source:	Public
Exposure	Metabolites:	3-PBA
	Period od exposure:	During gestation
Measurement	Measurement time:	4–5, 6, 7–9 Year
	Endpoint:	Behaviour – Metacognition Index
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Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	Longitudinal (prospective) birth cohort: Mount Sinai Children's Environmental Health Center. Primiparous women were enrolled between 1998 and 2002, and had to fulfil specific selection criteria. Exposure and outcome were quantified making thus comparison possible (unbiased study). The study assessed whether characteristics at enrolment were different by follow-up status. (for inclusion on the appraisal rational) also: Large Longitudinal birth cohort is considered well established study design with DL RoB for selection of participants and enough details in how people was selected.

Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	During the third trimester, participants completed questionnaires about home, demographics and behavioural characteristics during pregnancy. Directed acyclic graphs (DAGs) were constructed to identify possible colliders and mediators (maternal education, maternal marital status at follow-up, race/ethnicity, quality of the home environment, maternal IQ, sum DMP pesticide use, smoking and alcohol during pregnancy, creatinine and preterm birth). Co-exposure to OP metabolites was assessed but not to lead
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PHRoB	404 primiparous women in late pregnancy were recruited from 1998 to 2001. From them, 162 mother/child pairs with complete exposure and behavioural outcomes data were studied and their data reported in the results. From 361 pregnant women at enrolment, 168 returned for follow-up at 4–5, 6, 7–9 year visit and 162 had complete exposure and behavioural outcomes. Hence, this study had substantial loss-to-follow-up. Follow-up loses are common in cohort studies, and selection bias needs to be checked for. In this study, women with higher levels of 3-PBA (and the other two pyrethroid metabolites studied) were less likely to return for follow-up, what might have biased results. Although the appraisal rated Q3 as PLRoB, we should reconsider this rating and perhaps to downgrade to e.g. PHRoB. It is also of concern that only 32 participants with complete covariate data attended all three follow-ups, but there are no additional data on this.
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Outcome assessment	Q5: Can we be confident in the outcome assessment?	PLRoB	The Behavioural Assessment System for Children (BASC) and the Behaviour Rating Inventory of Executive Function (BRIEF) were used. Test-retest reliabilities and internal consistencies are good (Cronbach's alphas were calculated) The BASC and the BRIEF were both completed at the 4-, 6- and 7-year visits. The questioner is design for parents to assess the behaviour of the children. It is considered well established method. No expertise is needed for assessing the questions.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	All of the study's measured outcomes were measured according to the research plan and adequately reported in Table 3. Enrolment (n = 361) and follow-up (n = 162
Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	PLRoB	-preprocessing: concentrations were dichotomised, outcomes were scaled -missing values: The study assumed that all missing covariate data were missing at random. -descr stat: to test if enrolled are equal to follow-up, Chi-squared for categorical var, t-test for continuous var (Normality not checked) -statmodel: Associations between the pyrethroid metabolites and each of the composites and subscales were estimated with linear mixed models, with random effects for subject. -variable selection: DAG, Statistical models were adjusted for the minimally sufficient set and variables that were hypothesised to be highly predictive of the outcome. -modifying variable: cross-product 3PBA*race, 3PBA*sex -sensitivity analysis: excluding OPs from model, excluding observation with BASC score >4 (not reliable) Not details are providing for the missing data.

Viel JF, Warembourg C, Le Maner-Idrissi G, Lacroix A, Limon G, Rouget F, Monfort C, Durand G, Cordier S, Chevrier C, 2015 – RefID: 1152

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	287
	Exclusion criteria:	EXCLUDED: - Pregnancy <35 weeks of amenorrhea
	N enrolled subject:	287
	Sex of subject:	Male and Female
	Age of subject:	6 years
	Funding source:	Mixed
Exposure	Metabolites:	3-PBA; cis-DBCA
	Period od exposure:	During gestation
Measurement	Measurement time:	6 Year
	Endpoint:	Verbal Comprehension Index; Working Memory Index
Statistical analysis	Statistical analysis:	Associations between cognitive scores (WISC-VCI or WISC-WMI as dependent variable) and maternal prenatal urinary pyrethroid metabolite concentrations were examined using linear regression models. Prenatal metabolite levels were categorised into two groups for 3-BPA (< limit of detection [LOD], â‰¥LOD) and into three groups for cis-DBCA (<LOD, and for those with a detectable level, subdivided below and above the median).To preserve the size of the analytic sample, missing values for covariates were replaced by the modal value from participants with non-missing values. Imputation was required for 6 mothers (for a total of 6 values) and 14 children (for a total of 40 values). In addition, missing values for the neuropsychological scores were replaced by the values predicted from the subdomain scores when available (WISC-VCI=4, WISC-WMI=12, WAIS-III=2). As a result, the number of children with available neurocognitive scores was 287 for WISC-VCI and 283 for WISC-WMI. Non-parametric Spearman correlation coefficients were calculated between metabolite levels; Tests for monotonic trend between metabolite levels and cognitive scores were performed using a reverse-scale Cox regression model proposed to handle non-detected values. In this method, the measured metabolite is treated as the modelled outcome, switching the roles of exposure and health effect. The method begins by reversing the concentration scale and then applying Cox regression analysis with non-detected values as right- censored data. Only p-value were reported. p-values < 0.05 were considered statistically significant
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	The PELAGIE cohort (France) is a birth cohort consisting of 3421 pregnant women. They were recruited from the same eligible population (Brittany, France), with the same method of ascertainment using the same inclusion and exclusion criteria), and recruited within the same time frame (between 2002 and 2006), A subsample of 428 mothers were randomly selected, contacted and found eligible. From them, 287 (67%) mothers agreed to participate with their children in the neuropsychological follow-up
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	DLRoB	Efforts were made to account for confounding by identifying as collecting data on certain variables. Mothers completed a self-administered questionnaire to provide information on sociodemographic characteristics, lifestyle factors, their child's health, and their child's environmental exposures. Mothers were also administered the Wechsler Adult Intelligence Scale – 3th revision (WAIS-III). Midwives and paediatricians at the maternity units provided the study staff with medical information about the pregnancy, delivery, birth weight and neonatal health. Maternal factors that were considered were the following: age (continuous), place of residence (rural, urban), parity (0, >1), prepregnancy body mass index (<25, >25 kg/m²), education (<12, >12 years), WAIS-III VIQ (continuous), tobacco smoking at the beginning of pregnancy (no, yes), usual fish consumption before pregnancy (<2, >2 times a week), length of pregnancy (continuous) and non-exclusive breastfeeding (none, <16, >16 weeks). Are considered enough.

Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PHRoB	A random subcohort of 591 mothers was selected for pesticide determination in urine samples from the mothers who delivered live-born singleton infants, to obtain a final sample of size similar to those used in previous OP insecticide exposure studies. Among the 571 eligible families, 446 were successfully contacted by phone and 18 were further excluded because of previous child neuropsychological or behavioural tests (to avoid bias due to the learning effect). A total of 287 (67%) mothers agreed to participate with their child in the neuropsychological follow-up. Home visits were organised by two psychologists who were blinded to exposure status and supervised by four paediatric neuropsychologists in meetings held every two months. However, Table 3 shows data only for 155 mothers (exposure) and neuropsychological assessment of their children. No explanation is given for the remaining 133 mother/children pairs.
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	PHRoB	Exposure was consistently assessed using well established methods that directly measure exposure (i.e. biomonitoring or prenatal urinary samples). Non-specific (3-PBA) and specific deltamethrin metabolites (cis-DBCA) were assessed using ultra-performance liquid chromatography and triple quadrupole mass spectrometry (UPLC/MS-MS) for 3-PBA and gas chromatography and triple quadrupole spectrometry for cis-DBCA. However, only one single prenatal urine sample collected during early pregnancy (6–19 gestational weeks) was analysed. Specific metabolites were measured in maternal urine

Outcome assessment	Q5: Can we be confident in the outcome assessment?	DLRoB	The Wechsler Intelligence Scale for Children, 4th edition (WISC IV) was used to assess cognitive abilities. Scores were calculated for two domains: the Verbal Comprehension Index and the Working Memory Index. Home visits were organised by two psychologists who were blinded to exposure status and supervised by four paediatric neuropsychologists in meetings held every two months. WISC is a well established method and there is a need of neuropsychologist.
Selective outcome report	Q6: Were all measured outcomes reported?	DLRoB	DLRoB: WISC protocol is not explicitly reported but a reference is given. Association between specific PYR metabolite and Verbal comprehension index and Working memory index is given in Table 3 (there is no selective reporting)
Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	PLRoB	-preprocessing: concentration were converted using dichotomisation, or 3 classes or tertiles. -missing values: for analytic samples: replaced by modal values; for neuro scores: predicted from subdomain scores; -descr stat: Spearman correlation test for metabolite levels -statmodel 1: linear multiple regression model (reverse-scale Cox regression model to test for monotonic trend between metabolite levels and cognitive scores) -variable selection: a priori and elimination if p<0.2 and forced -modifying variable: NULL -sensitivity analysis: NULL -stat model 2: A sound and flexible statistical technique was used to handle biomarker values falling below LODs (reverse-scale Cox regression model to account for censored values) No best practice for the missing values. They predicted the value in the neuropsychological report not enough details are provided.

Watkins DJ, Fortenberry GZ, Sanchez BN, Barr DB, Panuwet P, Schnaas L, Osorio-Valencia E, Solano-Gonzalez M, Ettinger AS, Hernandez-Avila M, Hu H, Tellez-Rojo MM, Meeker JD, 2016 – RefID: 1432

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	187
	Exclusion criteria:	EXCLUDED: - Plans to leave the area within the next 5 years; - Daily consumption of alcoholic beverages during pregnancy; – Addiction to illegal drugs; - Habitual use of prescription drugs; - Diagnosis of high risk pregnancy; - Pre-eclampsia; - Gestational diabetes; - Renal or heart disease; - History of infertility; - Diabetes; - Psychosis; - Suffering from seizures requiring medical treatment
	N enrolled subject:	172
	Sex of subject:	Male and Female
	Age of subject:	3 years
	Funding source:	Public
Exposure	Metabolites:	3-PBA
	Period od exposure:	During gestation
Measurement	Measurement time:	2 Year; 3 Year
	Endpoint:	Mental development index

Statistical analysis	Statistical analysis:	Urinary 3-PBA concentrations below LOD for many urine samples, hence exposure grouped into 2 categories for descriptive analyses (detected vs not detected) and into 3 categories for regression analyses. The lowest category of the three-level variable comprised all samples <LOD (n = 103); the medium and high categories were formed by dividing the samples with detectable 3-PBA into 2 equally sized groups (n = 42 each). Chi-squared and t-tests to determine differences in categorical and continuous demographic characteristics between mothers who had detectable levels of urinary3-PBA and those that did not. Linear regression to investigate associations between the categorical urinary 3-PBA measure (low, medium and high) and developmental assessment scores measured at 24 and 36 months. Linear trend estimates for the categorical 3-PBA measure calculated by entering the3-level ordinal exposure variable into regression models as a continuous variable [CHECK THIS] Statistical Analysis Software (SAS) (version 9.4; SAS Institute Inc., Cary, NC, USA)
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	Birth cohort of Mexican women enrolled between 1997 and 2001 (Mexico City). Women were enrolled during their first trimester of pregnancy from the National Institute of Perinatology, Hospital General and participated in follow-up visits until their children were 5 years of age. Women were thus recruited from the same eligible population, recruited with the same method of ascertainment using the same inclusion and exclusion criteria.

Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	Maternal education (years), IQ score, socioeconomic status (SES) score, blood lead, and child sex were included in multivariable models as potential confounders, and urinary specific gravity was included as a covariate to adjust for urine dilution. Maternal IQ was calculated based on scores on the Information, Comprehension, Similarities and Block Designs scales of the Spanish Wechsler Adult Intelligence Scale. Besides, strict exclusion criteria for initial enrolment included: daily consumption of alcoholic beverages during pregnancy; addiction to illegal drugs; habitual use of prescription drugs; diagnosis of high risk pregnancy, pre-eclampsia, gestational diabetes or renal or heart disease; a history of infertility, diabetes, or psychosis; or suffering from seizures requiring medical treatment. Although lead was measured and adjusted for, co-exposure to OP metabolites was not measured and adjusted for.
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PLRoB	Outcome can be considered almost complete as from 187 mother-children pairs, 181 and 180 completed the mental development index at 24 and 36 months of age (respectively) and 180 and 169 the psychomotor development index at 24 and 36 months of age, respectively. The little missing outcome data were not related to outcome. Only 6 cases were lost and evidence is provided.

Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	DHRoB	Maternal urine samples (single spot samples from 3rd trimester) were transported on dry ice to Emory University for analysis of 3-PBA (non-specific metabolite) using previously described methods (Olsson et al., 2004). Samples were subjected to enzyme hydrolysis and then extracted using SPE cartridges and eluates were concentrated and analysed using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). It should be noted that a random subset of women (n = 21) selected from the 187 mother-child pairs, we measured 3-PBA in urine samples collected during each trimester to examine variability in repeat samples over the course of pregnancy. Only non-specific metabolites were measured in a single assessment in the 3rd trimester
Outcome assessment	Q5: Can we be confident in the outcome assessment?	PLRoB	A well established method was used. The Bayley Scales for Infant Development – Spanish version (BSID-IIS) to assess developmental functioning of infants and children at 24 and 36 months of age using the mental development index (MDI) and Psychomotor Development Index (PDI). Research personnel who administered the assessments were trained and supervised by an expert member of the research team. Standardisation and quality control checks were conducted by reviews of videotaped evaluations. Not provided information whether the research personnel were neuropsychologists or not.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	The study outcomes (MDI and PSI scores) outlined in the methods were reported for the two timelines studied. From 187 mother-children pairs, 181 and 180 completed the mental development index (MDI) at 24 and 36 months of age (respectively) and 180 and 169 the psychomotor development index (PDI) at 24 and 36 months of age, respectively. No protocol available. But all in M and M is reported in results section.

Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	PLRoB	-preprocessing: for descriptive statistics concentration were dichotomised while for regression analysis con were converted in 3 classes -descr stat: chi-squared test for categorical variable and t-test for continuous variable (normality not checked) to test difference in group having metabolite levels below/above LOD -statmodel: linear regression model to assess association between metabolite levels and mental index or psychomotor index in two visit. Association was assessed using class as continuous values and considering 4 separate models (approach considered less appropriate than a longitudinal model accounting for visit 1 and 2) -variable selection: NR -modifying variable: stratification by sex -sensitivity analysis: NULL

Communication

Eskenazi B, An S, Rauch SA, Coker ES, Maphula A, Obida M, Crause M, Kogut KR, Bornman R, Chevrier J, 2018 – RefID: 1117

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	705
	Exclusion criteria:	EXCLUDED: <18 years; not speaking TshiVenda as the main language at home; not living within 20 km (12.4mi) of the hospital and not planned to remain in the area; diagnosis with malaria during pregnancy
	N enrolled subject:	705
	Sex of subject:	Male and Female
	Age of subject:	2 years
	Funding source:	Public
Exposure	Metabolites:	/; 3-PBA; cis-DBCA
	Period od exposure:	na; Prior to delivery; Prior to delivery OR Postdelivery before leaving the hospital
Measurement	Measurement time:	1 Year; 2 Year
	Endpoint:	Expressive Communication; Language Composite; Receptive Communication

Statistical analysis	Statistical analysis:	Adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). p-Values of 0.05 considered statistically significant; Bayesian kernel machine regression (BKMR) model to consider individual insecticide exposure–responses (and potential nonlinearity) with co-occurring insecticides, and evaluate each exposure's relative importance with respect to the outcome via Bayesian variable selection, the exposure–response of the overall mixture, interaction between any two insecticide measures and each chemical's exposure–response while holding the other insecticide measure at various percentiles of exposure. Posterior inclusion probabilities (PIPs) used to highlight the relative ranking of variable importance for each pesticide class as well as each pesticide within a particular class of insecticides. PIPs result from the Bayesian Markov chain Monte Carlo (MCMC) iterations (25, 000). A PIP for an exposure (or group PIP) is calculated as the posterior mean of the indicator variable across the MCMC iterations, in which the indicator variable equals 1 if the variable (or group) is selected into the model at a given iteration or 0 otherwise. Group PIP = indicates the posterior probability that an exposure grouping (e.g. pyrethroids) was included into the 'true' model from the multiple iterations (25, 000) of the MCMC sampler. Conditional = indicates the posterior probability that a particular chemical exposure (e.g. cis-DCCA) within an exposure grouping (e.g. pyrethroids) was included into the 'true' model from the multiple iterations (25, 000) of the MCMC sampler, conditional on the exposure grouping being included. R (version 3.3.1; R Development Core Team); Sex-stratified adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). Interactions considered statistically significant at p<0:1. p-Values of 0.05 considered statistically significant; Sex-stratified adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations

		(GEEs). STATA version 13 (Stata Corp). Interactions considered statistically significant at p<0:1. p-Values of 0.05 considered statistically significant.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	PLRoB	Longitudinal birth cohort: Venda Health Examination of Mothers, Babies and the Environment (VHEMBE). Pregnant women were recruited from the same eligible population and had to fulfil specific selection criteria, exposure and outcome were quantified making thus comparison possible (unbiased study). No explanation is given why some women did not complete the questionnaire.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	Information was gathered on sociodemographic characteristics, reproductive and medical history, personal habits (smoking and use of alcohol or drugs), occupational and residential history, household composition and use of pesticides around the home, at work, in their fields, on their livestock or for malaria control. The study tested for effect modification by child sex using stratification and cross-product terms. A number of sensitivity analyses were made. Appropriate adjustments were made for modifying variables and confounders in the final multivariate statistical models. Covariates for the multivariable models were identified using a directed acyclic graph (DAG). Models were rerun considering child blood lead concentrations, child haemoglobin levels and maternal HIV status as covariates. However, co-exposure for OP metabolites was not assessed
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	DLRoB	A total of 689 children were assessed for outcomes at 1-y visit and 681 at 2-y. The Cognitive, Language (Receptive and Expressive), and Motor (Fine and Gross) subtests were administered at both visits, and the Social-Emotional subtest only at the 1-y assessment The attrition is less than 5%.

Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	PHRoB	A spot urine sample was collected for 432 women before delivery and for 263 women postdelivery (but before leaving the hospital). Pyrethroid metabolites (cis- DBCA and the non-specific 3-PBA metabolite) were measured using GC-MS
Outcome assessment	Q5: Can we be confident in the outcome assessment?	DLRoB	Two bilingual English-TshiVenda psychologists, trained by a TshiVenda developmental neuropsychologist, administered the Bayley Scales of Infant Development, third edition (BSID-III). A portion of the assessments were videotaped for quality control. Each week, one video was randomly selected for review by all three psychologists to assure consistency in administration and scoring. It is considered a well established method.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	No protocol available, but all of the study's measured outcomes were measured according to the research plan and adequately reported, although at 2 y assessment, the Social-Emotional subtest was not administered and no clear explanation was given. However, this is explicitly acknowledged in the methodology.

Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	DLRoB	-preprocessing: log10 of concentration -missing values: -descr stat: yes -statmodel 1: longitudinal models using generalised estimating equations (GEEs)to assess relationship across 1 and 2 visit (GAM to test linearity) -variable selection: DAG -modifying variable: stratification for sex, cross-product term sex* -sensitivity analysis: restriction to participant collecting urine sample pre-delivery; restriction to children having lead and haemoglobin levels measured -stat model 2: Bayesian kernel machine regression for bi pollutant interaction -variable selection: hierarchical var selection Additional note for Bayesian kernel machine regression model: this model is an innovative approach for addressing mixture components having complex non-linear and non-additive relationship with health outcome. The hierarchical variable selection, incorporate information on the structure of the mixtures into the model allowing for grouping highly correlated components (p, p'DDT and p, p'-DDE in one group and pyrethroid in another group). Model results include only posterior inclusion probabilities (for group and for components in group) describing the probability of inclusion of the mixture component(s). In theory the model estimates the exposure–response function too. (probably the full characterisation of the form of the exposure–response function required too many data). Limit of the approach: it could be very sensitive to the specification of the mixture prior.

Impaired psychomotor development

Fluegge KR, Nishioka M, Wilkins JR, 3rd 2016 – RefID: 480

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	140

	Exclusion criteria:	nr
	N enrolled subject:	140
	Sex of subject:	Male and Female
	Age of subject:	3 months
	Funding source:	Public
Exposure	Metabolites:	3-PBA
	Period od exposure:	During gestation
Measurement	Measurement time:	3 Month
	Endpoint:	Motor development score
Statistical analysis	Statistical analysis:	REGRESSION ANALYSIS: generalised linear model using maximum likelihood estimation results to assess the association between prenatal metabolites and MOTOR endpoint. IMPUTATION: Linear interpolation to estimate second and/or third trimester weights once prepregnancy weight and pregnancy weight gain were determined. Imputation using a multivariate normal model (MVN) and imputation by chained equations (ICE) using cross imputation (all variables to be included in a model were used to predict all other variables in the model, known as a fully conditional specification (FCS)), for each imputed weight variable. Multiple imputation (MI) procedures used as simulation techniques to replace each missing data point with a set of m>1 plausible value (based on known relevant predictors). These imputations were created through a Bayesian process [...]
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	The study design consists of a birth cohort with 140 mother-infant pairs. Maternal urine was collected in 2nd and 3rd trimesters of gestation and infant urine was at 2 months of age. BSID-II testing was completed at 3 months of age to all children. Standard demographic information, pregnancy weight, potentially confounding family-based factors (e.g. race, socioeconomic status, maternal education), and relevant clinical information pertaining to the pregnancy and birth event were collected. Therefore, baseline characteristics of mothers-infant pair were similar and comparable.

Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	DHRoB	They did not control for the confounders, (Table 5). No co-exposures were considered in the models despite OP metabolites were analysed
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PLRoB	The 140 mother-infant pairs from this birth cohort were followed-up for two years. However, Bayley's mental and motor score at 3 months was reported for 119 infants (Table 2), but the proportion lost to follow-up would not appreciably bias results.
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	DHRoB	Exposure was consistently assessed through biomonitoring of general and specific pyrethroid metabolites at two trimesters of pregnancy using well established methods. However, the method validation is not provided and no reference is given to a previous study addressing this validation. Besides, although specific PYR metabolites were analysed, they were not associated with neurodevelopmental outcomes very likely because all maternal samples were below the LOD for DBCA
Outcome assessment	Q5: Can we be confident in the outcome assessment?	PLRoB	Infant neurodevelopment was evaluated using the Bayley Scales of Infant Development (BSID-II) at three months of age. Although this is a well-known valid and reliable neurobehavioral scale, no information is provided on whether it was administered by trained neuropsychologists.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	The Bayley Scale for Infant Development (BSID) assessed 2 neurological domains: motor and mental development (MDI and MDI, respectively). Quantitative data for both of them are reported in the results. However, protocol not available

Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	DLRoB	-preprocessing: NULL (only value >LOD were included) -missing values: imputation using linear interpolation to estimate second and/or third trimester mother weights; using multivariate normal model; using chained equation (correlation of the two imputation methods was tested and all regression models were repeated including binary variable indicating whether mother's weights were imputed) -statmodel: generalised linear model -variable selection: forward selection of maternal and pregnancy-related variables, while metabolites were included only if median>LOD -sensitivity analysis: excluding imputed weight values

Eskenazi B, An S, Rauch SA, Coker ES, Maphula A, Obida M, Crause M, Kogut KR, Bornman R, Chevrier J, 2018 – RefID: 1117

Study characteristics and population	Study type	Prospective cohort
	N enrolled mothers:	705
	Exclusion criteria:	EXCLUDED: <18 years; not speaking TshiVenda as the main language at home; not living within 20 km (12.4mi) of the hospital and not planned to remain in the area; diagnosis with malaria during pregnancy
	N enrolled subject:	705
	Sex of subject:	Male and Female
	Age of subject:	2 years
	Funding source:	Public
Exposure	Metabolites:	/; 3-PBA; cis-DBCA
	Period od exposure:	na; Prior to delivery; Prior to delivery OR Postdelivery before leaving the hospital
Measurement	Measurement time:	1 Year; 2 Year
	Endpoint:	Fine motor; Gross motor; Motor Composite

Statistical analysis	Statistical analysis:	Adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). p-values of 0.05 considered statistically significant; Bayesian kernel machine regression (BKMR) model to consider individual insecticide exposure–responses (and potential nonlinearity) with co-occurring insecticides, and evaluate each exposure's relative importance with respect to the outcome via Bayesian variable selection, the exposure–response of the overall mixture, interaction between any two insecticide measures and each chemical's exposure–response while holding the other insecticide measure at various percentiles of exposure. Posterior inclusion probabilities (PIPs) used to highlight the relative ranking of variable importance for each pesticide class as well as each pesticide within a particular class of insecticides. PIPs result from the Bayesian Markov chain Monte Carlo (MCMC) iterations (25, 000). A PIP for an exposure (or group PIP) is calculated as the posterior mean of the indicator variable across the MCMC iterations, in which the indicator variable equals 1 if the variable (or group) is selected into the model at a given iteration or 0 otherwise. Group PIP = indicates the posterior probability that an exposure grouping (e.g. pyrethroids) was included into the 'true' model from the multiple iterations (25, 000) of the MCMC sampler. Conditional = indicates the posterior probability that a particular chemical exposure (e.g. cis-DCCA) within an exposure grouping (e.g. pyrethroids) was included into the 'true' model from the multiple iterations (25, 000) of the MCMC sampler, conditional on the exposure grouping being included. R (version 3.3.1; R Development Core Team); Sex-stratified adjusted linear regression – multivariable model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). Interactions considered statistically significant at p<0:1. p-Values of 0.05 considered statistically significant; Sex-stratified adjusted linear regression – multivariable

		model. Covariates identified using a directed acyclic graph (DAG). Generalised additive models (GAMs) with a 3-degree of freedom cubic spline and residual plots confirmed the appropriateness of the linearity assumption for the models. To assess relationships across the 1- and 2-y visits, longitudinal models using generalised estimating equations (GEEs). STATA version 13 (Stata Corp). Interactions considered statistically significant at p<0:1. p-Values of 0.05 considered statistically significant.
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	PLRoB	Longitudinal birth cohort: Venda Health Examination of Mothers, Babies and the Environment (VHEMBE). Pregnant women were recruited from the same eligible population and had to fulfil specific selection criteria, exposure and outcome were quantified making thus comparison possible (unbiased study). No explanation is given why some women did not complete the questionnaire.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	Information was gathered on sociodemographic characteristics, reproductive and medical history, personal habits (smoking and use of alcohol or drugs), occupational and residential history, household composition and use of pesticides around the home, at work, in their fields, on their livestock or for malaria control. The study tested for effect modification by child sex using stratification and cross-product terms. A number of sensitivity analyses were made. Appropriate adjustments were made for modifying variables and confounders in the final multivariate statistical models. Covariates for the multivariable models were identified using a directed acyclic graph (DAG). Models were rerun considering child blood lead concentrations, child haemoglobin levels and maternal HIV status as covariates. However, co-exposure for OP metabolites was not assessed

Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	DLRoB	A total of 689 children were assessed for outcomes at 1-y visit and 681 at 2-y. The Cognitive, Language (Receptive and Expressive), and Motor (Fine and Gross) subtests were administered at both visits, and the Social-Emotional subtest only at the 1-y assessment The attrition is less than 5%.
Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	PHRoB	A spot urine sample was collected for 432 women before delivery and for 263 women postdelivery (but before leaving the hospital). Pyrethroid metabolites (cis-DBCA and the non-specific 3-PBA metabolite) were measured using GC-MS
Outcome assessment	Q5: Can we be confident in the outcome assessment?	DLRoB	Two bilingual English-TshiVenda psychologists, trained by a TshiVenda developmental neuropsychologist, administered the Bayley Scales of Infant Development, third edition (BSID-III). A portion of the assessments were videotaped for quality control. Each week, one video was randomly selected for review by all three psychologists to assure consistency in administration and scoring. It is considered a well established method.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	No protocol available, but all of the study's measured outcomes were measured according to the research plan and adequately reported, although at 2 y assessment, the Social-Emotional subtest was not administered and no clear explanation was given. However, this is explicitly acknowledged in the methodology.

Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	DLRoB	-preprocessing: log10 of concentration -missing values: -descr stat: yes -statmodel 1: longitudinal models using generalised estimating equations (GEEs)to assess relationship across 1 and 2 visit (GAM to test linearity) -variable selection: DAG -modifying variable: stratification for sex, cross-product term sex* -sensitivity analysis: restriction to participants collecting urine sample pre-delivery; restriction to children having lead and haemoglobin levels measured -stat model 2: Bayesian kernel machine regression for bi pollutant interaction -variable selection: hierarchical var selection Additional note for Bayesian kernel machine regression model: this model is an innovative approach for addressing mixture components having complex non-linear and non-additive relationship with health outcome. The hierarchical variable selection, incorporate information on the structure of the mixtures into the model allowing for grouping highly correlated components (p, p'DDT and p, p'-DDE in one group and pyrethroid in another group). Model results include only posterior inclusion probabilities (for group and for components in group) describing the probability of inclusion of the mixture component(s). In theory the model estimates the exposure–response function too. (probably the full characterisation of the form of the exposure–response function required too many data). Limit of the approach: it could be very sensitive to the specification of the mixture prior.

Watkins DJ, Fortenberry GZ, Sanchez BN, Barr DB, Panuwet P, Schnaas L, Osorio-Valencia E, Solano-Gonzalez M, Ettinger AS, Hernandez-Avila M, Hu H, Tellez-Rojo MM, Meeker JD, 2016 – RefID: 1432

Study	Study type	Prospective cohort

characteristics and population	N enrolled mothers:	187
	Exclusion criteria:	EXCLUDED: - Plans to leave the area within the next 5 years; - Daily consumption of alcoholic beverages during pregnancy; – Addiction to illegal drugs; - Habitual use of prescription drugs; - Diagnosis of high risk pregnancy; - Pre-eclampsia; - Gestational diabetes; - Renal or heart disease; - History of infertility; - Diabetes; - Psychosis; - Suffering from seizures requiring medical treatment
	N enrolled subject:	172
	Sex of subject:	Male and Female
	Age of subject:	3 years
	Funding source:	Public
Exposure	Metabolites:	3-PBA
	Period od exposure:	During gestation
Measurement	Measurement time:	3 Year; 2 Year
	Endpoint:	Psychomotor development index
Statistical analysis	Statistical analysis:	Urinary 3-PBA concentrations below LOD for many urine samples, hence exposure grouped into 2 categories for descriptive analyses (detected vs not detected) and into 3 categories for regression analyses. The lowest category of the three-level variable comprised all samples <LOD (n = 103); the medium and high categories were formed by dividing the samples with detectable 3-PBA into 2 equally sized groups (n = 42 each). Chi-squared and t-tests to determine differences in categorical and continuous demographic characteristics between mothers who had detectable levels of urinary3-PBA and those that did not. Linear regression to investigate associations between the categorical urinary 3-PBA measure (low, medium and high) and developmental assessment scores measured at 24 and 36 months. Linear trend estimates for the categorical 3-PBA measure calculated by entering the3-level ordinal exposure variable into regression models as a continuous variable [CHECK THIS] Statistical Analysis Software (SAS) (version 9.4; SAS Institute Inc., Cary, NC, USA)
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	Birth cohort of Mexican women enrolled between 1997 and 2001 (Mexico City). Women were enrolled during their first trimester of pregnancy from the National Institute of Perinatology, Hospital General and participated in follow-up visits until their children were 5 years of age. Women were thus recruited from the same eligible population, recruited with the same method of ascertainment using the same inclusion and exclusion criteria.
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	Maternal education (years), IQ score, socioeconomic status (SES) score, blood lead, and child sex were included in multivariable models as potential confounders, and urinary specific gravity was included as a covariate to adjust for urine dilution. Maternal IQ was calculated based on scores on the Information, Comprehension, Similarities and Block Designs scales of the Spanish Wechsler Adult Intelligence Scale. Besides, strict exclusion criteria for initial enrolment included: daily consumption of alcoholic beverages during pregnancy; addiction to illegal drugs; habitual use of prescription drugs; diagnosis of high risk pregnancy, pre-eclampsia, gestational diabetes or renal or heart disease; a history of infertility, diabetes, or psychosis; or suffering from seizures requiring medical treatment. Although lead was measured and adjusted for, co-exposure to OP metabolites was not measured and adjusted for
Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PLRoB	Outcome can be considered almost complete as from 187 mother-children pairs, 181 and 180 completed the mental development index at 24 and 36 months of age (respectively) and 180 and 169 the psychomotor development index at 24 and 36 months of age, respectively. The little missing outcome data were not related to outcome. Only 6 cases were lost and no evidence is provided.

Exposure characterisation	Q4: Can we be confident in the exposure characterisation?	HRoB	Maternal urine samples (single spot samples from 3rd trimester) were transported on dry ice to Emory University for analysis of 3-PBA (non-specific metabolite) using previously described methods (Olsson et al., 2004). Samples were subjected to enzyme hydrolysis and then extracted using SPE cartridges and eluates were concentrated and analysed using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). It should be noted that a random subset of women (n = 21) selected from the 187 mother-child pairs, we measured 3-PBA in urine samples collected during each trimester to examine variability in repeat samples over the course of pregnancy. Only non-specific metabolites were measured in a single assessment in the 3rd trimester
Outcome assessment	Q5: Can we be confident in the outcome assessment?	PLRoB	A well established method was used. The Bayley Scales for Infant Development Spanish version (BSID-IIS) to assess developmental functioning of infants and children at 24 and 36 months of age using the mental development index (MDI) and Psychomotor Development Index (PDI). Research personnel who administered the assessments were trained and supervised by an expert member of the research team. Standardisation and quality control checks were conducted by reviews of videotaped evaluations. Information not provided on whether the research personnel were neuropsychologists or not.
Selective outcome report	Q6: Were all measured outcomes reported?	PLRoB	The study outcomes (MDI and PSI scores) outlined in the methods were reported for the two timelines studied. From 187 mother-children pairs, 181 and 180 completed the mental development index (MDI) at 24 and 36 months of age (respectively) and 180 and 169 the psychomotor development index (PDI) at 24 and 36 months of age, respectively. No protocol available. But all in M and M is reported in results section.

Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	PLRoB	-preprocessing: for descriptive statistics concentration were dichotomised while for regression analysis con were converted in 3 classes -descr stat: chi-squared test for categorical variable and t-test for continuous variable (normality not checked) to test difference in group having metabolite levels below/above LOD -statmodel: linear regression model to assess association between metabolite levels and mental index or psychomotor index in two visits. Association was assessed using class as continuous values and considering 4 separate models (approach considered less appropriate than a longitudinal model accounting for visit 1 and 2) -variable selection: NR -modifying variable: stratification by sex -sensitivity analysis: NULL

ASD and non-typical development (non-TD)

Barkoski JM, Philippat C, Tancredi D, Schmidt RJ, Ozonoff S, Barr DB, Elms W, Bennett D, Hertz-Picciotto I, 2020- RefID: 3034541

Study characteristics and population	Study type	Birth cohort
	N enrolled mothers:	201 (121 boys and 80 girls)
	N enrolled subject:	201 (2007–2014) who had data for pesticide metabolite concentrations from urine samples collected during pregnancy and child neurodevelopmental assessments
	Sex of subject:	Male and Female
	Age of subject:	3 years
	Funding source:	Public
Exposure	Metabolites:	3-PBA
	Period of exposure:	During gestation
Measurement	Measurement time:	3 Year
	Endpoint:	ASD, typically developing (TD), or non-TD (and not meeting criteria for ASD)

Statistical analysis	Statistical analysis:	Multinomial logistic regression was used to obtain relative risk ratios (RRR) linking 3-PBA concentrations averaged across each trimester and over pregnancy with child's outcome, either ASD or non-TD vs TD. Models were adjusted for specific gravity, maternal prepregnancy BMI, prenatal vitamin use, birth year, home-ownership, and TCPy (3,5,6-trichloro-2-pyridinol) pregnancy concentrations
Risk of bias appraisal		Tier: 3

Bias domain	Question	Score	Judgement
Selection	Q1: Did the selection of study participants result in appropriate comparison groups?	DLRoB	The MARBLES (Markers of Autism Risk in Babies-Learning Early Signs) cohort was used. Because familial recurrence risk of ASD is high, MARBLES enrolled pregnant women with a family history of ASD. Children were clinically assessed at 3 years of age and classified into 3 outcome categories: ASD, typically developing (TD), or non-TD (and not meeting criteria for ASD). Repeated maternal second and third trimester urine samples were analysed for pyrethroid metabolite 3-phenoxybenzoic acid (3-PBA).
Confounding	Q2: Did the study design or analysis account for important confounding and modifying variables?	PLRoB	The study selected potential confounders based on the associations reported in the literature of covariates with pyrethroid exposure and with child neurodevelopment. The study used a directed acyclic graph (DAG) to represent assumed causal relationships between the exposure, outcome and covariates. Models were adjusted for specific gravity, maternal prepregnancy BMI, prenatal vitamin use, birth year, home-ownership, and TCPy (3,5,6-trichloro-2-pyridinol) pregnancy concentrations.

Attrition or exclusion	Q3: Were outcome data complete without attrition or exclusion from analysis?	PLRoB	The study included 201 mother-children pairs, but 7 had missing information on covariates. From the 201 mother/child pairs, the multivariate analysis was performed with 133 for the 2nd trimester and 183 for the 3rd trimester. The sample size of 201 corresponded to those pairs that had data for maternal urine pesticide metabolite concentrations and child neurodevelopmental assessment. The total number of mother-children pairs from which the study sample was drawn is not reported.
sure characterisation	Q4: Can we be confident in the exposure characterisation?	PHRoB	Several maternal urine samples were collected during each trimester of pregnancy. Participants were instructed to collect three first morning void (FMV) urine samples (one week apart) and one 24-hour urine sample. Only non-specific metabolites were measured in urine which to some extent can be found preformed in the environment.
Outcome assessment	Q5: Can we be confident in the outcome assessment?	DLRoB	A well established method was used, participants were assessed at the MIND Institute by expert examiners at four ages, including 36 months, when a comprehensive diagnostic assessment is conducted. The Mullen Scales of Early Learning (MSEL), a norm-referenced developmental measure, was administered at each of these time points. Four subscales (visual reception, fine motor skills, receptive language, and expressive language) are combined into an overall composite score. During the child's 3-year visit, expert clinicians evaluated children on the Autism Diagnostic Observation Scale (ADOS). Three categories of children's outcomes at 3 years were defined using an algorithmic approach previously developed by the Baby Siblings Research Consortium which takes into account scores on clinical assessments including the ADOS and MSEL.

Selective outcome report	Q6: Were all measured outcomes reported?	DLRoB	The 3 study outcomes outlined in the methods (ASD, non-TD and TD) were reported in the results Table 1. Also, Table 3 presents results of the multinomial logistic regression analysis for ASD and non-TC, using TD children as the reference group.
Statistical method	Q7: Were there other potential threats to internal validity? Were the statistical methods appropriate?	DLRoB	-preprocessing: due to a skewed distribution, pyrethroid metabolite (3-PBA) concentrations were natural log-transformed, and those cases with levels <LOD were assigned a value of LOD/√2. Outcomes were dichotomised. Although trans-DCCA was also determined, it was not included in the analysis as only 35% of samples were >LOQ (correlation between trans-DCCA and 3-PBA was 0.88). -missing values: no information on whether all missing covariate data were missing at random. -descr stat: not reported whether all mother-child pairs enrolled were equal to follow-up. Associations between specific gravity-corrected 3-PBA (nat. log-transformed, continuous variable) and categorical demographic variables assessed using Kruskal–Wallis test and associations with continuous demographic variables using linear regression. -statmodel: Associations between the pyrethroid metabolite 3-PBA and ASD and non-TD were estimated using (crude and adjusted) multinomial logistic regression analysis with TD as reference group. -variable selection: DAG used to represent assumed causal relationships between the exposure, outcome and covariates, Statistical models were adjusted for those covariates changed the exposure beta coefficient of the model by 10% or more, and for natural log-transformed TCPy.

Appendix B3.3 Outcome of the ROB IVB (In vitro battery)

DNT Appraisal In vitro battery table

Proliferation in primary human NPC

Area/BrdU incorporation–proliferation

Study characteristics and test system	Specific endpoint	Area/BrdU incorporation–proliferation
	Test system:	Primary cells
	Species:	Human
	Origin of the test system:	Lonza Verviers SPRL (Verviers, Belgium).
	Stage of development of the primary cells:	Embryonic
	Medium:	Proliferation medium (B27), consisting of DMEM (Life Technologies, Darmstadt, Germany) and Hams F12 (Life Technologies) (3:1) supplemented with 2% B27 (Life Technologies), 1% penicillin and streptomycin (Pan Biotech, Aidenbach, Germany), 20 ng/ml epidermal growth factor (EGF, Life Technologies) and 20 ng/ml recombinant human fibroblast growth factor (FGF, R&D systems, Wiesbaden, Germany).
	Funding source:	Public
	Number of biological replicate:	5 replicate wells per condition
Exposure	Exposure duration:	72 hours
	Concentration:	7 concentrations 1/3 dilution up to 20 μM
	Treatment frequency:	Single

Measurement	Measurement time:	7 hours
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Exposure is conducted automated.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, conducted at the same time with the same conditions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Acceptability parameters were predefined and criteria for exclusion of single wells, concentration, conditions and endpoints were pre-established and reported in the acceptability criteria of the report.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or medium. Source was ToxCast analytical grade. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	Using a luminescence-based BrdU Assay (from Roche) and a luminometer (Baumann et al., 2014, 2015; Nimtz et al., 2019). Data are analysed through a classification model reported in Section 8.3.9 of the IVB report.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity and cell viability are tested at the same time and reported for each condition of the experiment.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	5 biological replicates per condition

Migration assay (NPC2 test)

NPC2a migration distance radial glia (72 h)/NPC2a migration distance radial glia (120 h)/NPC2b migration distance neurons (120 h)/NPC2c migration distance oligodendrocytes (120 h)

Study characteristics and test system	Specific endpoint	NPC2a migration distance radial glia (72 h)/NPC2a migration distance radial glia (120 h)/NPC2b migration distance neurons (120 h)/NPC2c migration distance oligodendrocytes (120 h)
	Test system:	Primary cells
	Species:	Human
	Origin of the test system:	Lonza Verviers SPRL (Verviers, Belgium).
	Stage of development of the primary cells:	Embryonic
	Medium:	The differentiation medium consists of DMEM (Life Technologies) and Hams F12 (Life Technologies) at a ratio of 3 to 1 supplemented with 1% N2 (Life Technologies) and 1% penicillin and streptomycin (Pan Biotech). NPCs migrate for 5 days and so differentiate into radial glia cells, neurons, oligodendrocytes and astrocytes (Baumann et al., 2015; Nimtz et al., 2019; Bal-Price et al., 2018). For letting spheres migrate and differentiate, they are incubated at 37°C and 5% CO2 at a pH between 7.2 and 7.6. Cells are fed on day 3 by replacing half the medium with fresh medium.
	Funding source:	Public
	Number of biological replicate:	5 replicate wells per condition
Exposure	Exposure duration:	72 hours/120 hours
	Concentration:	7 concentrations 1/3 dilution up to 20 μM
	Treatment frequency:	Single (replacement of half of the medium is carried out on day 3)
Measurement	Measurement time:	72 hours/120 hours
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Exposure is conducted automatised.

Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, conducted at the same time with the same conditions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Acceptability parameters were predefined and criteria for exclusion of single wells, concentration, conditions and endpoints were pre-established and reported in the acceptability criteria of the report.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or medium. Source was ToxCast analytical grade. Exposure, culture, times etc. are all considered adequate.

Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	PLRoB	NPC2a manually measuring the radial migration from the sphere core on brightfield images (72 h). NPC2a assessed by automatically identifying the migration area of each sphere of Hoechst stained nuclei on fluorescence images (120 h). Migration distance neurons (120 h) (NPC2b) is the mean distance of all neurons from the edge of the sphere core to the position of each neuron (see 'neuronal differentiation') and is given as ratio of the 'migration distance radial glia (120 h)'. Migration distance oligodendrocytes (120 h) (NPC2c) is the mean distance of all oligodendrocytes from the edge of the sphere core to the position of each oligodendrocyte (see 'oligodendrocyte differentiation') and is given as ratio of the 'migration distance radial glia (120 h)'. Cell number (120 h) is measured as the number of Hoechst-positive objects in the migration area detected on the fluorescence images from each sphere after 120 h. Nuclei detection is carried out using the HCS Studio: Cellomics Scan software Data are analysed through a classification model reported in Section 8.3.9 of the IVB report.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity and cell viability are tested at the same time and reported for each condition of the experiment.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	5 biological replicates per condition

Neuronal differentiation (NPC3 Test)

NPC3 neuronal differentiation

Study characteristics and test system	Specific endpoint	NPC3 neuronal differentiation
	Test system:	Primary cells
	Species:	Human
	Origin of the test system:	Lonza Verviers SPRL (Verviers, Belgium).
	Stage of development of the primary cells:	Embryonic
	Medium:	The differentiation medium consists of DMEM (Life Technologies) and Hams F12 (Life Technologies) at a ratio of 3 to 1 supplemented with 1% N2 (Life Technologies) and 1% penicillin and streptomycin (Pan Biotech). NPCs migrate for 5 days and so differentiate into radial glia cells, neurons, oligodendrocytes and astrocytes (Baumann et al., 2015; Nimtz et al., 2019; Bal-Price et al., 2018). For letting spheres migrate and differentiate, they are incubated at 37°C and 5% CO2 at a pH between 7.2 and 7.6. Cells are fed on day 3 by replacing half the medium with fresh medium.
	Funding source:	Public
	Number of biological replicate:	5 replicate wells per condition
Exposure	Exposure duration:	120 hours
	Concentration:	7 concentrations 1/3 dilution up to 20 μM
	Treatment frequency:	Single (replacement of half of the medium is carried out on day 3)
Measurement	Measurement time:	120 hours
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Exposure is conducted automatised.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, conducted at the same time with the same conditions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Acceptability parameters were predefined and criteria for exclusion of single wells, concentration, conditions and endpoints were pre-established and reported in the acceptability criteria of the report.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or medium. Source was ToxCast analytical grade. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Number of all TUBB3-positive cells in per cent of the amount of Hoechst-positive nuclei in the migration area after 120 h of differentiation. Automatised. The annotation of neurons is carried out by a CNN running on Keras implemented in Python 3.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity and cell viability are tested at the same time and reported for each condition of the experiment.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	5 biological replicates per condition

Neurite length and neurite area (NPC4 Test)

Neurite length (120 h) (NPC4) and neurite area (120 h) (NPC4)

Study characteristics and test system	Specific endpoint	Neurite length (120 h) (NPC4)/Neurite area (120 h) (NPC4)
	Test system:	Primary cells
	Species:	Human
	Origin of the test system:	Lonza Verviers SPRL (Verviers, Belgium).
	Stage of development of the primary cells:	Embryonic
	Medium:	The differentiation medium consists of DMEM (Life Technologies) and Hams F12 (Life Technologies) at a ratio of 3 to 1 supplemented with 1% N2 (Life Technologies) and 1% penicillin and streptomycin (Pan Biotech). NPCs migrate for 5 days and so differentiate into radial glia cells, neurons, oligodendrocytes and astrocytes (Baumann et al., 2015; Nimtz et al., 2019; Bal-Price et al., 2018). For letting spheres migrate and differentiate, they are incubated at 37°C and 5% CO2 at a pH between 7.2 and 7.6. Cells are fed on day 3 by replacing half the medium with fresh medium.
	Funding source:	Public
	Number of biological replicate:	5 replicate wells per condition
Exposure	Exposure duration:	120 hours
	Concentration:	7 concentrations 1/3 dilution up to 20 μM
	Treatment frequency:	Single (replacement of half of the medium is carried out on day 3)
Measurement	Measurement time:	120 hours
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Exposure is conducted automatised.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, conducted at the same time with the same conditions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Acceptability parameters were predefined and criteria for exclusion of single wells, concentration, conditions and endpoints were pre-established and reported in the acceptability criteria of the report.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or medium. Source was ToxCast analytical grade. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	N Length, is assessed as neurite length in µm based on the skeletonisation of each annotated neuron (see 'neuronal differentiation') that reaches a predefined intensity threshold. N Area is assessed as the area in amount of pixel for each skeletonised neuron (see neurite length)
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity and cell viability are tested at the same time and reported for each condition of the experiment.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	5 biological replicates per condition

Oligodendrocyte differentiation (120 h) (NPC5)

Study characteristics and test system	Specific endpoint	Oligodendrocyte differentiation (120 h) (NPC5)
	Test system:	Primary cells
	Species:	Human
	Origin of the test system:	Lonza Verviers SPRL (Verviers, Belgium).
	Stage of development of the primary cells:	Embryonic
	Medium:	The differentiation medium consists of DMEM (Life Technologies) and Hams F12 (Life Technologies) at a ratio of 3 to 1 supplemented with 1% N2 (Life Technologies) and 1% penicillin and streptomycin (Pan Biotech). NPCs migrate for 5 days and so differentiate into radial glia cells, neurons, oligodendrocytes and astrocytes (Baumann et al., 2015; Nimtz et al., 2019; Bal-Price et al., 2018). For letting spheres migrate and differentiate, they are incubated at 37°C and 5% CO2 at a pH between 7.2 and 7.6. Cells are fed on day 3 by replacing half the medium with fresh medium.
	Funding source:	Public
	Number of biological replicate:	5 replicate wells per condition
Exposure	Exposure duration:	120 hours
	Concentration:	7 concentrations 1/3 dilution up to 20 μM
	Treatment frequency:	Single (replacement of half of the medium is carried out on day 3)
Measurement	Measurement time:	120 hours
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Exposure is conducted automatised.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.

Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, conducted at the same time with the same conditions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.
Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Acceptability parameters were predefined and criteria for exclusion of single wells, concentration, conditions and endpoints were pre-established and reported in the acceptability criteria of the report.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or medium. Source was ToxCast analytical grade. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	It is determined as the number of all O4-positive cells in per cent of the amount of all Hoechst-positive nuclei in the migration area after 120 h of differentiation. The annotation of oligodendrocytes is carried out by a CNN running on Keras implemented in Python 3.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity and cell viability are tested at the same time and reported for each condition of the experiment.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	5 biological replicates per condition

The cMINC neural crest cell migration assay (UKN2 Test)

Study characteristics and test system	Specific endpoint	Human induced pluripotent stem cell (hiPSC)
	Test system:	Pluripotential stem cells
	Species:	Human
	Origin of the test system:	Line IMR90_clone #4 (WiCell, Wisconsin)
	Stage of development of the primary cells:	Stem cells
	Medium:	The medium is fully described in Section 8.26 in the report.
	Funding source:	Public
	Number of biological replicate:	At least 4 technical replicates and 3 biological replicates.
Exposure	Exposure duration:	24 hours
	Concentration:	At least 5 serial dilutions ½ or 1/3
	Treatment frequency:	Single (replacement of half of the medium is carried out on day 3)
Measurement	Measurement time:	24 hours
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Exposure is conducted automatised.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, conducted at the same time with the same conditions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Acceptability parameters were predefined and criteria for exclusion of single wells, concentration, conditions and endpoints were pre-established and reported in the acceptability criteria of the report.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or medium. Source was ToxCast analytical grade. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	For image acquisition, an automated microplate reading microscope (Array-Scan VTI, Cellomics, Thermo Fisher Scientific) equipped with a Hamamatsu ORCA-ER camera (resolution 1024 × 1024) is used. Images were recorded in 2 channels at excitation/emission wavelengths of 365 ± 50/535 ± 45 to detect H-33342 in channel 1 and 474 ± 40/535 ± 45 to detect calcein in channel 2. In both channels, a fixed exposure time and an intensity histogram-derived threshold is used for object identification (Stiegler et al., 2011).
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity are tested at the same time and reported for each condition of the experiment.
Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	3 biological replicates per condition

NeuriTox neurite outgrowth of CNS neurons test (UKN4 test)

Study characteristics and test system	Specific endpoint	Immortalised primary (LUHMES) cells derived from an 8-week-old mesencephalon were used.
	Test system:	Primary cells
	Species:	Human
	Origin of the test system:	LUHMES
	Stage of development of the primary cells:	Fetal
	Medium:	The medium is fully described in Section 8.2.7. Lund human mesencephalic (LUHMES) cells are handled in culture as described before (Lotharius et al., 2005; Scholz et al., 2011).
	Funding source:	Public
	Number of biological replicate:	At least 3 technical replicates and 3 biological replicates.
Exposure	Exposure duration:	24 hours
	Concentration:	At least 5 serial dilutions ½ or 1/3
	Treatment frequency:	Single (replacement of half of the medium is carried out on day 3)
Measurement	Measurement time:	24 hours
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Exposure is conducted automatised.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, conducted at the same time with the same conditions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Acceptability parameters were predefined and criteria for exclusion of single wells, concentration, conditions and endpoints were pre-established and reported in the acceptability criteria of the report.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or medium. Source was ToxCast analytical grade. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Cells are stained with H-33342 and calcein-AM and imaged via a high-content imaging microscope (Cellomics ArrayScanVTI; Thermo Fisher Scientific) to assess viability and neurite area (NA) i.e. neurite outgrowth of the cells as described before in detail (Krug et al., 2013; Stiegler et al., 2011). An imaging algorithm is used to distinguish between live and dead cells. All stained nuclei (H-33342 positive) are used to calculate the number of cells in total in channel 1. All H-33342 and calcein double-positive cells are then defined as viable objects in channel 2. **Viability in each well was estimated by dividing the amount of viable cells to total cell number. To assess the NA, the algorithm calculates a likely area of the cell soma and subtracts this area from all calcein-positive pixels imaged, resulting in the NA (** Figure C. 23C). As the seeded precursor cells show no such area, this calculated NA represents the neurite outgrowth of the cells within 24 h. In a final step for data display, the single well data were always normalised to the mean of untreated controls.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity are tested at the same time and reported for each condition of the experiment.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	3 biological replicates per condition

PeriTox neurite outgrowth of neural crest cell test (UKN5 test)

Study characteristics and test system	Specific endpoint	hiPSC line SBAD2. SBAD2 cells were derived and characterised at the University of Newcastle from Lonza fibroblasts CC-2511,
	Test system:	hiPSC
	Species:	Human
	Origin of the test system:	Lot 293971 with the tissue acquisition number 24245 (Baud et al., 2017).
	Stage of development of the primary cells:	Stem cells
	Medium:	The medium is fully described in Section 8.2.8
	Funding source:	Public
	Number of biological replicate:	At least 3 technical replicates and 3 biological replicates.
Exposure	Exposure duration:	24 hours
	Concentration:	At least 5 serial dilutions ½ or 1/3
	Treatment frequency:	Single (replacement of half of the medium is carried out on day 3)
Measurement	Measurement time:	24 hours
Risk of bias appraisal		Tier: 1

Bias domain	Question	Score	Judgement
Randomisation	Q1: was administered dose or exposure level adequately randomised?	DLRoB	Exposure is conducted automatised.
Allocation concealment	Q2: was allocation [of e.g. cells] to study groups adequately concealed?	PLRoB	Lack of adequate blinding during the study would not appreciably bias results for cell maintenance and cell dosing personnel.
Exposure condition across groups	Q3: were experimental conditions identical across study groups?	DLRoB	Yes, conducted at the same time with the same conditions.
Blind research personnel	Q4: was the research personnel blinded to the study group during the study?	PLRoB	Not reported, but unlikely to impact the automated procedures or the growth of cultures.

Attrition or exclusion	Q5: were outcome data complete without attrition or exclusion from analysis?	DLRoB	Yes. Acceptability parameters were predefined and criteria for exclusion of single wells, concentration, conditions and endpoints were pre-established and reported in the acceptability criteria of the report.
Exposure characterisation	Q6: can we be confident in the EXPOSURE characterisation?	PLRoB	No analytical work was carried out on source, stock or medium. Source was ToxCast analytical grade. Exposure, culture, times etc. are all considered adequate.
Outcome assessment	Q7: can we be confident in the OUTCOME assessment?	DLRoB	Cells are stained with H-33342 and calcein-AM and imaged via a high-content imaging microscope (Cellomics ArrayScanVTI; Thermo Fisher Scientific) to assess viability and neurite area (NA) i.e. neurite outgrowth of the cells as described before in detail (Krug et al., 2013; Stiegler et al., 2011). An imaging algorithm is used to distinguish between live and dead cells. All stained nuclei (H-33342 positive) are used to calculate the number of cells in total in channel 1. All H-33342 and calcein double-positive cells are then defined as viable objects in channel 2. **Viability in each well was estimated by dividing the amount of viable cells to total cell number. To assess the NA, the algorithm calculates a likely area of the cell soma and subtracts this area from all calcein-positive pixels imaged, resulting in the NA (** Figure C. 23C). As the seeded precursor cells show no such area, this calculated NA represents the neurite outgrowth of the cells within 24 h. In a final step for data display, the single well data were always normalised to the mean of untreated controls.
Selective outcome report	Q8: were all measured outcomes reported?	PLRoB	Yes.
Cytotoxicity	Q9a: were there other potential threats to internal validity? CYTOTOXICITY	DLRoB	Cytotoxicity are tested at the same time and reported for each condition of the experiment.

Replicates and Repetitions	W9b: were there other potential threats to internal validity? REPETITIONS/REPLICATES	DLRoB	3 biological replicates per condition

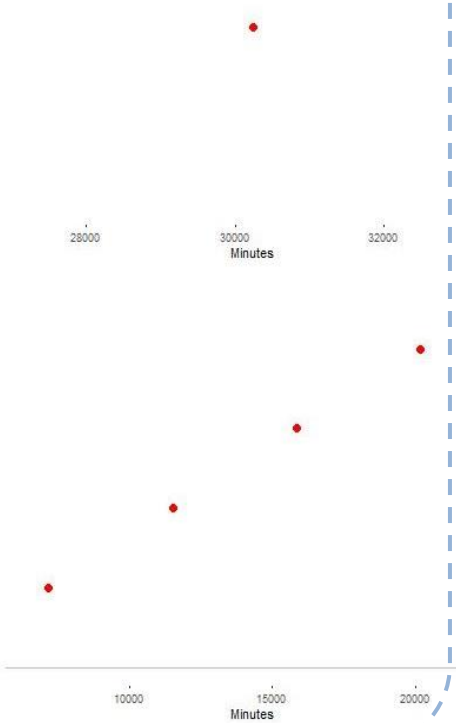
Appendix B4.1 Graph report In vivo and in vitro deltamethrin

How to read the graph

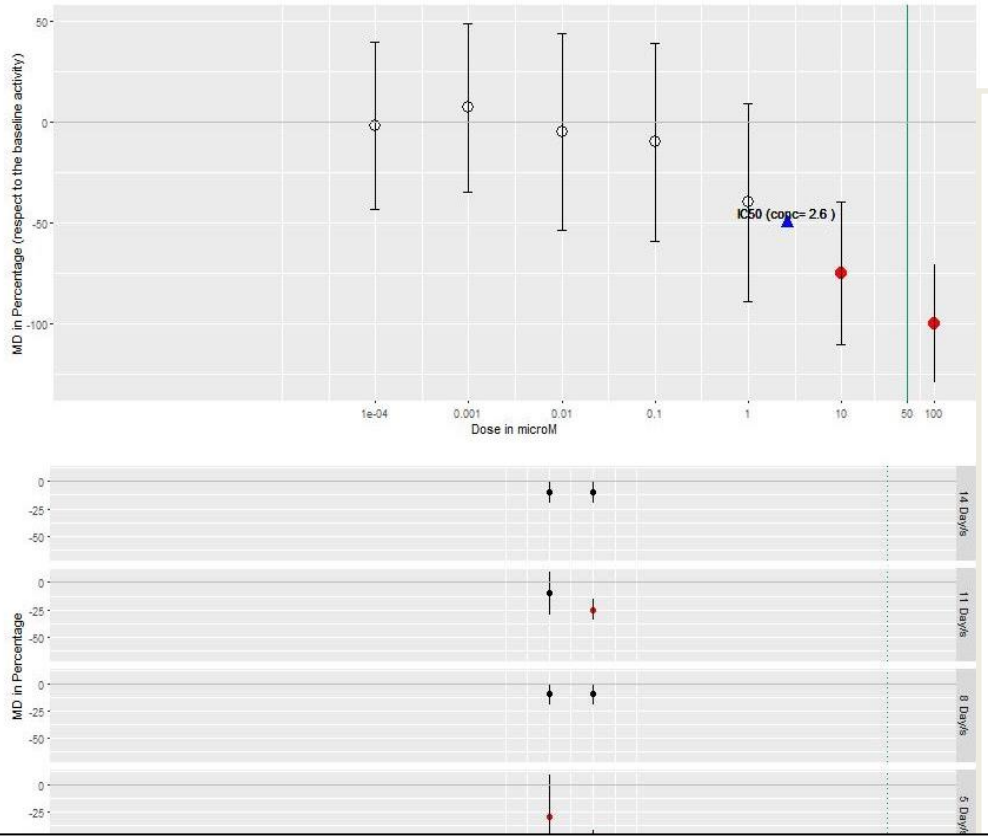
MBR (mean burst rate)
Refid 926
rat | Primary cells
Cortical | Fetal (=gestation)
Concentration Frequency: Single
Exposure duration: 20 Minutes
Time of measure: 30240 Minutes
Tier: 1
Q9a: PLRoB
NBioReplicates: 9

MBR (mean burst rate)
Refid 2452
mouse | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 7 Day
Time of measure:
Tier: 1
Q9a: PLRoB
NBioReplicates: 3

Study characteristics



Time /multiple times when the endpoint was measured.



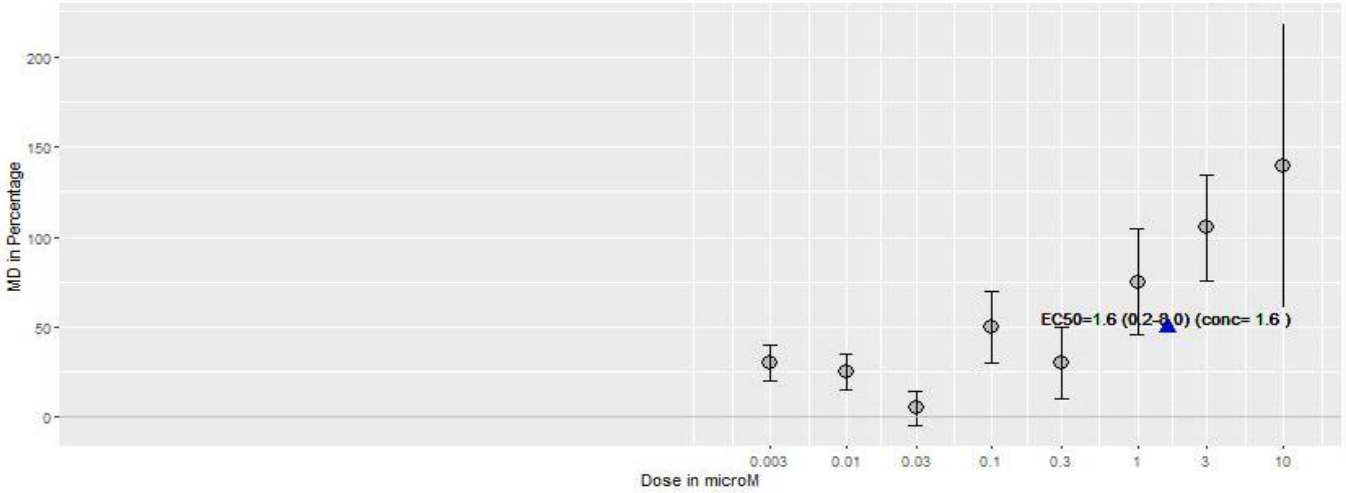
- difference statistically significative
- difference NOT statistically significative statistically
- significance not reported
- IC50
- Cytotoxicity, agreed
- Cytotoxicity, extrapolated

The graph provides information about the effect, expressed as difference between treated and control, in relation to the dose. On x axis the dose administered to treated group is reported. On y axis the corresponding mean effect (mean of endpoint measurements in treated group minus mean of endpoint measurements in control group) is illustrated as a circle. Circe filling colour depends on the statistical significance of the difference: red when the difference between treated and control group is statistically significative, white when the difference is not statistically significative, grey when the significance is not reported. Whenever possible, the confidence interval of the mean difference is displayed. Grey horizontal line at y=0 describes when there is no difference between control and treated. In addition, blue triangle indicates the value for IC50 and vertical green line provides information on the cytotoxicity as reported in the paper (solid green line) or as extrapolated by experts (dashed green line).

In vitro

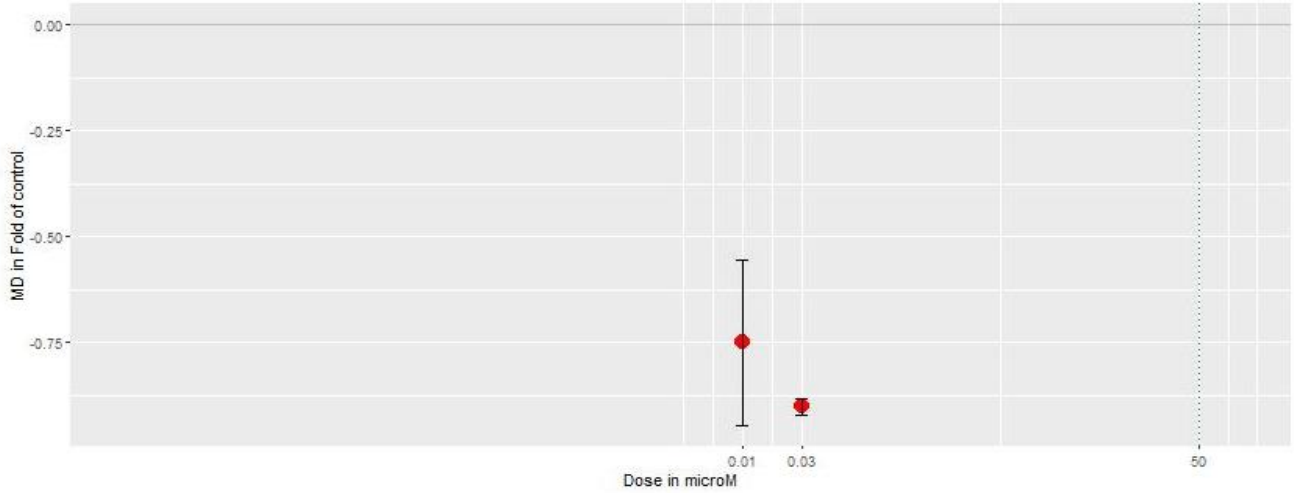
In vitro – MIE

1. MIE in vitro. Binding to RyRs. Binding and functional activation of RyRs. Ryanodine – [3H] Ry binding to RyRs in cortex



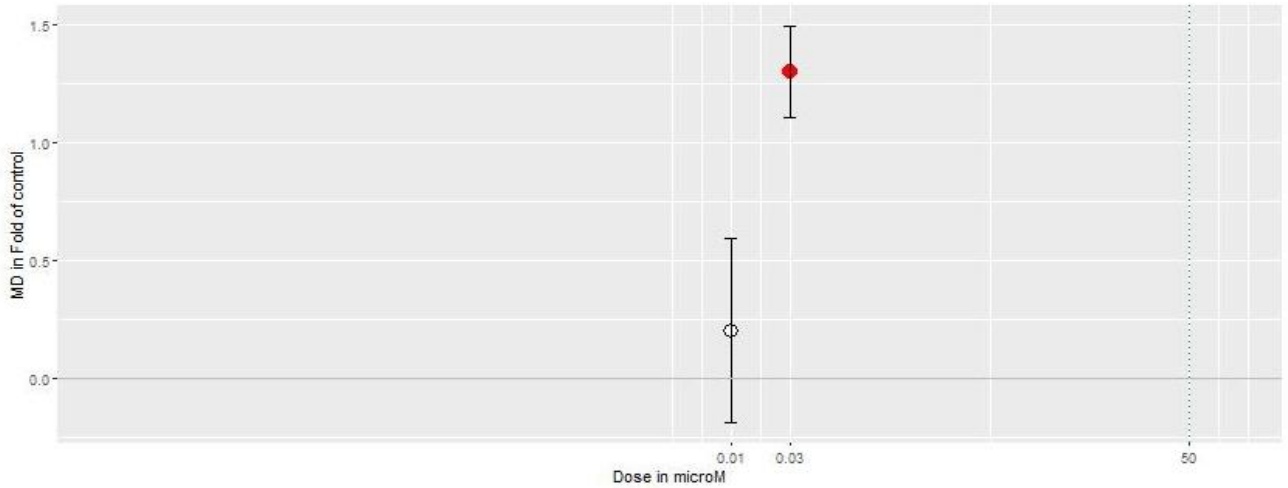
Ryanodine - [3H]Ry binding to RyRs in cortex
Refid 2452
mouse | Primary cells
Cortical | Post-natal
Concentration Frequency: Single
Exposure duration: 3 Hours
Time of measure: -9 nr
Tier: 1
Q9a: PLRoB
NBioReplicates: 3

2. MIE in vitro. Binding to RyRs. Binding and functional activation of RyRs. Ryanodine – Mean close time (Tc).



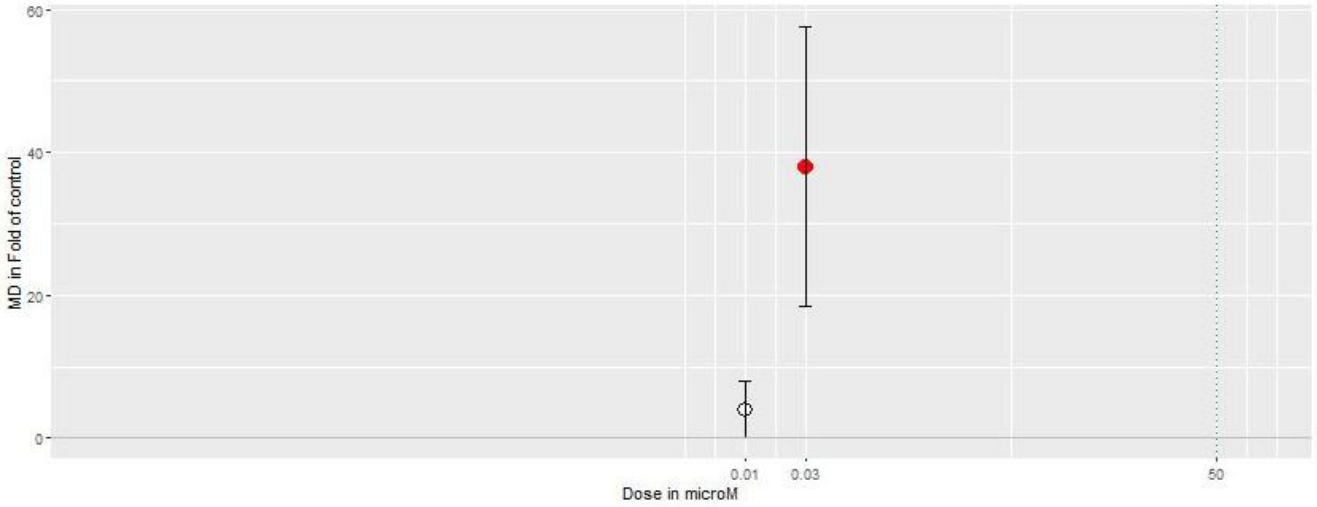
Ryanodine - Mean close time (Tc)
Refid 2452
mouse | Primary cells
Cortical | Post-natal
Concentration Frequency: Single
Exposure duration: 15 Minutes
Time of measure: -9 nr
Tier: 1
Q9a: PLRoB
NBioReplicates: 3

3. MIE in vitro. Binding to RyRs. Binding and functional activation of RyRs. Ryanodine – Mean open time (T0)



Ryanodine - Mean open time (T0)
Refid 2452
mouse | Primary cells
Cortical | Post-natal
Concentration Frequency: Single
Exposure duration: 15 Minutes
Time of measure: -9 nr
Tier: 1
Q9a: PLRoB
NBioReplicates: 3

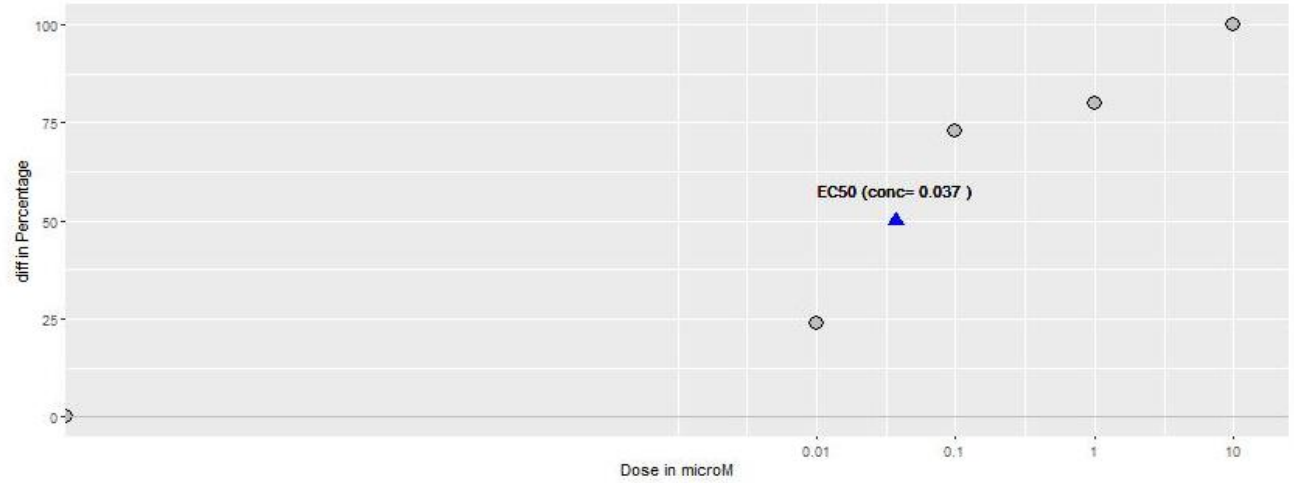
4. MIE in vitro. Binding to RyRs. Binding and functional activation of RyRs. Ryanodine – Open Probability (P0)



Ryanodine - Open Probability (P0)
Refid 2452
mouse | Primary cells
Cortical | Post-natal
Concentration Frequency: Single
Exposure duration: 15 Minutes
Time of measure: -9 nr
Tier: 1
Q9a: PLRoB
NBioReplicates: 3

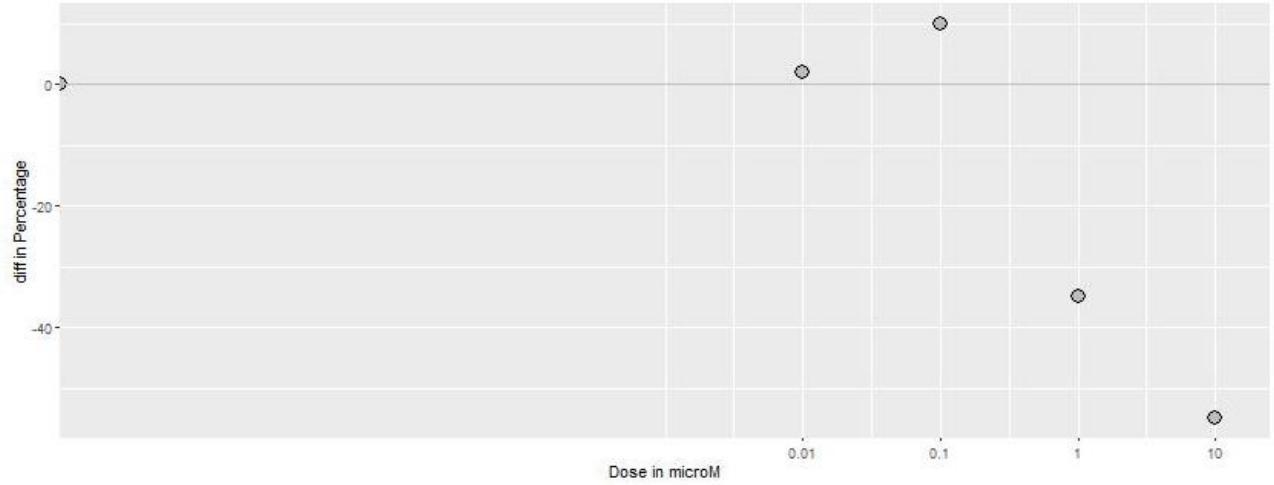
In vitro – KE1–KE2–KE3

1. KE in vitro. KE1–KE2–KE3. Patch Clamp. Interevent Interval



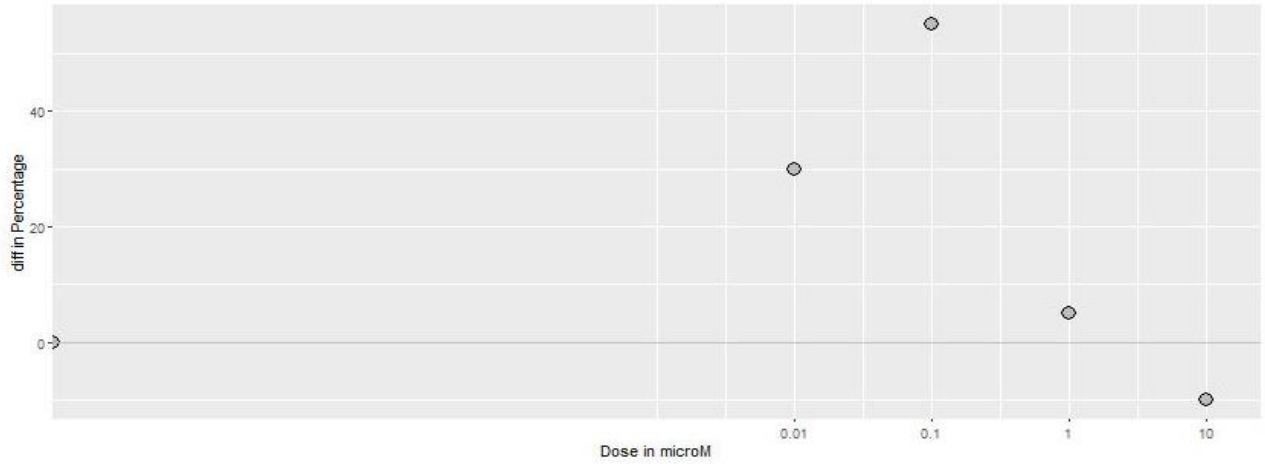
Membrane excitability (sEPSC) - Interevent interval
Refid 3052
rat | Primary cells
Hippocampal pyramidal neurons | Post-natal
Concentration Frequency: Single
Exposure duration: 10 Minutes
Time of measure: 11520 Minutes
Tier: 1
Q9a: PLRoB
NBioReplicates: 4

2. KE in vitro. KE1–KE2–KE3. Patch Clamp. Events/burst



Membrane excitability (Events/Burst)
Refid 3052
rat | Primary cells
Hippocampal pyramidal neurons | Post-natal
Concentration Frequency: Single
Exposure duration: 10 Minutes
Time of measure: 11520 Minutes
Tier: 1
Q9a: PLRoB
NBioReplicates: 4

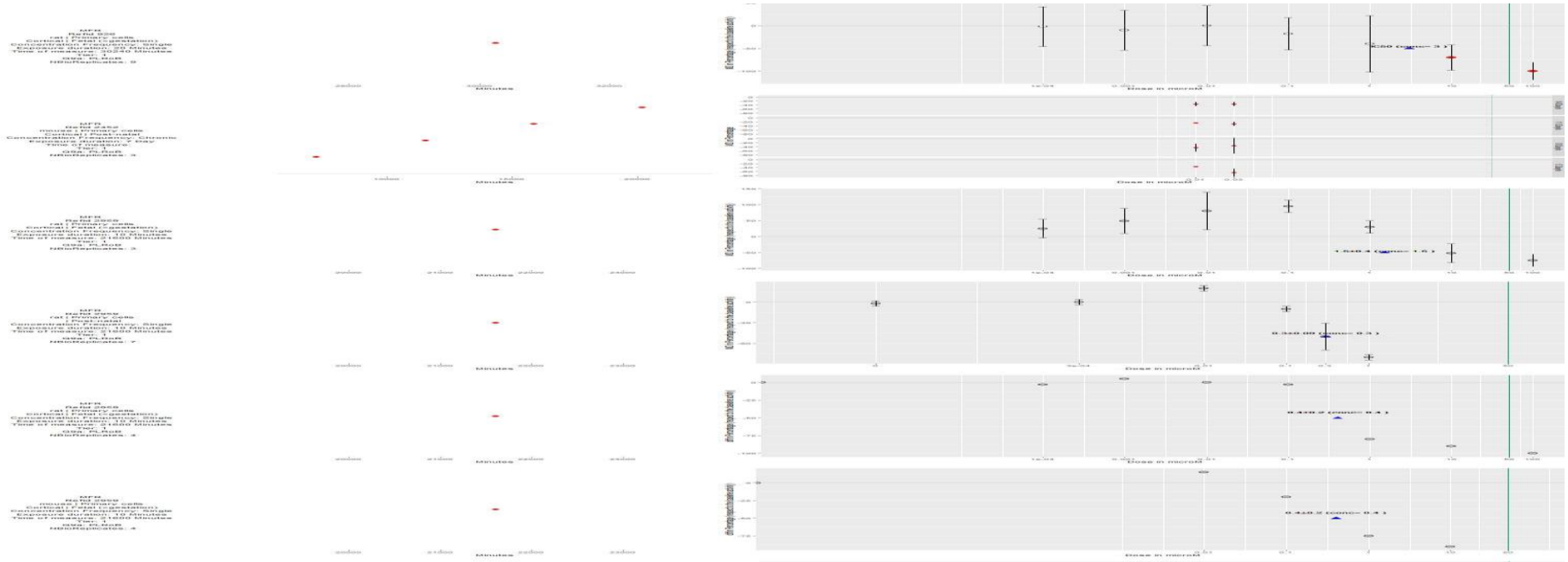
3. KE in vitro. KE1–KE2–KE3. Patch Clamp. Burst Duration



Membrane excitability (Burst duration)
Refid 3052
rat | Primary cells
Hippocampal pyramidal neurons | Post-natal
Concentration Frequency: Single
Exposure duration: 10 Minutes
Time of measure: 11520 Minutes
Tier: 1
Q9a: PLRoB
NBioReplicates: 4

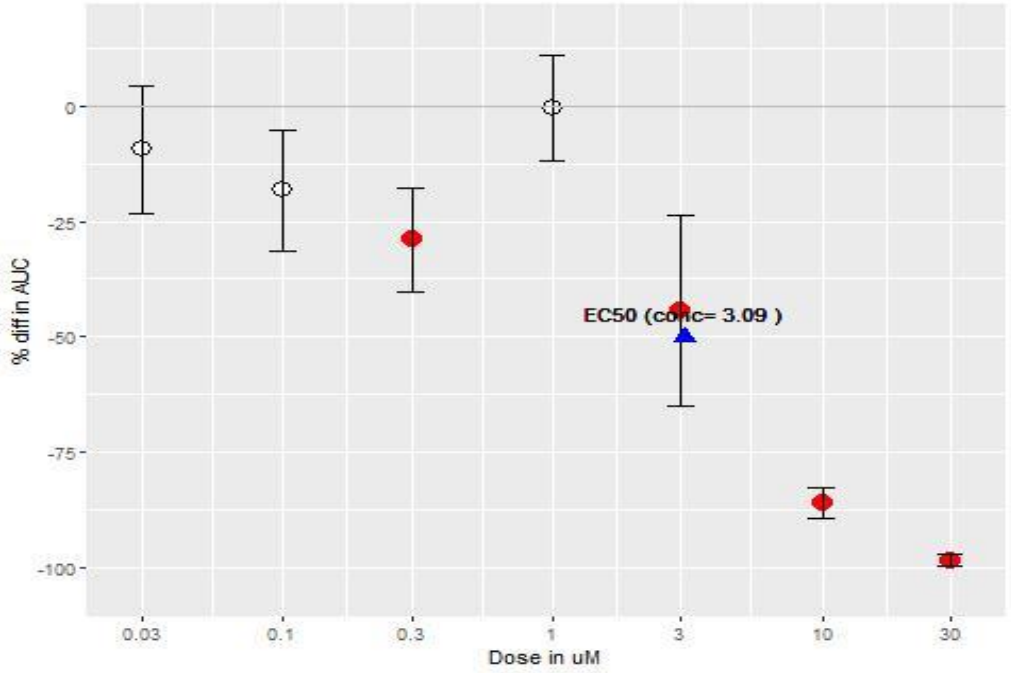
In vitro – KE4 (MEA)

4. 1/4. 2/4. 3. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – General activity. MFR (Mean Firing Rate) – from literature

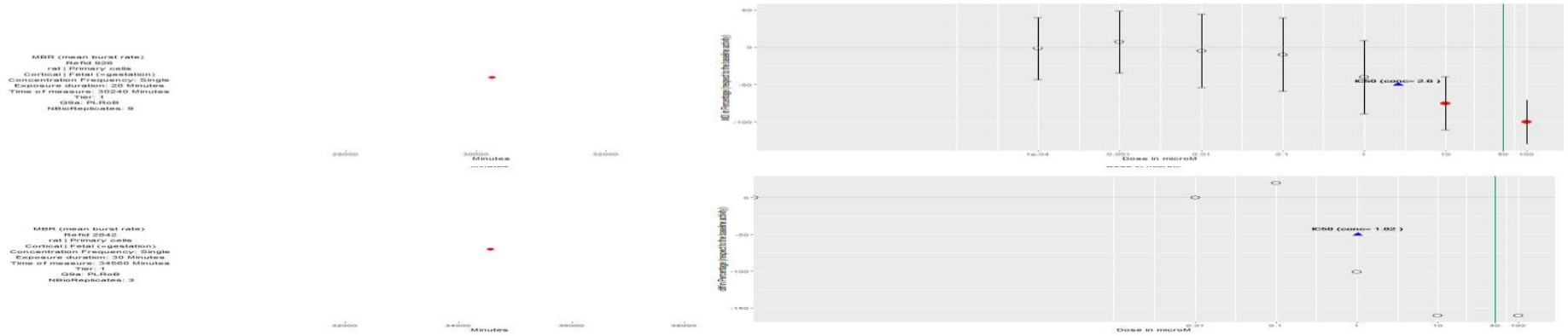


4. 4. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – General activity. MFR (Mean Firing Rate) – from US EPA

meanfiringrate_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3

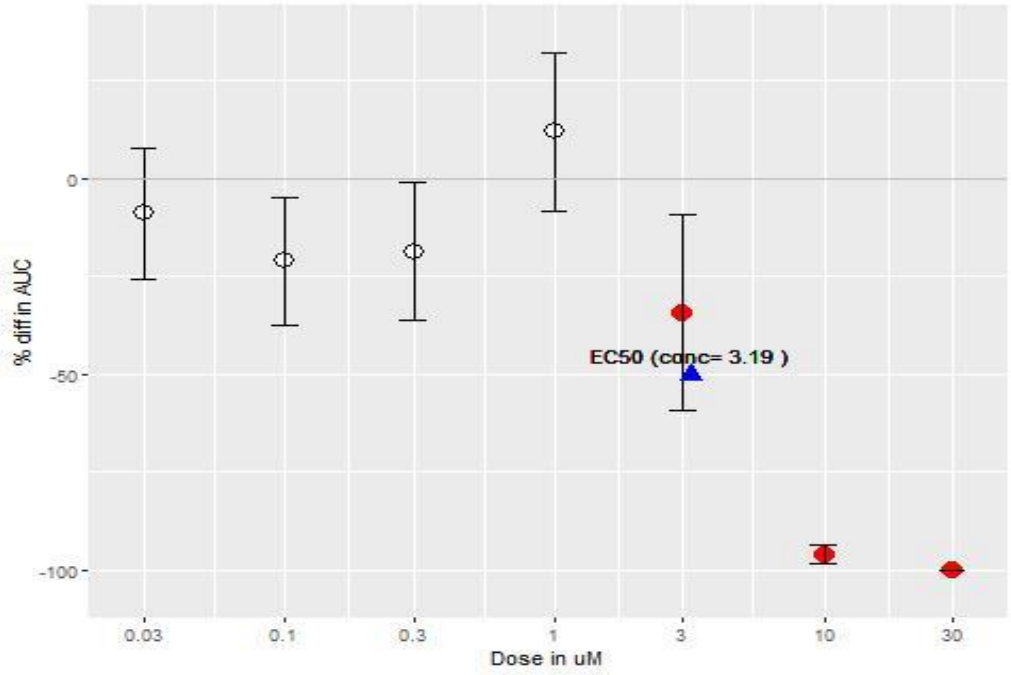


5. 1/5. 2 KE in vitro. KE4 – Altered Neuronal Network Function. MEA – General activity. MBR (Mean Burst Rate) – from literature

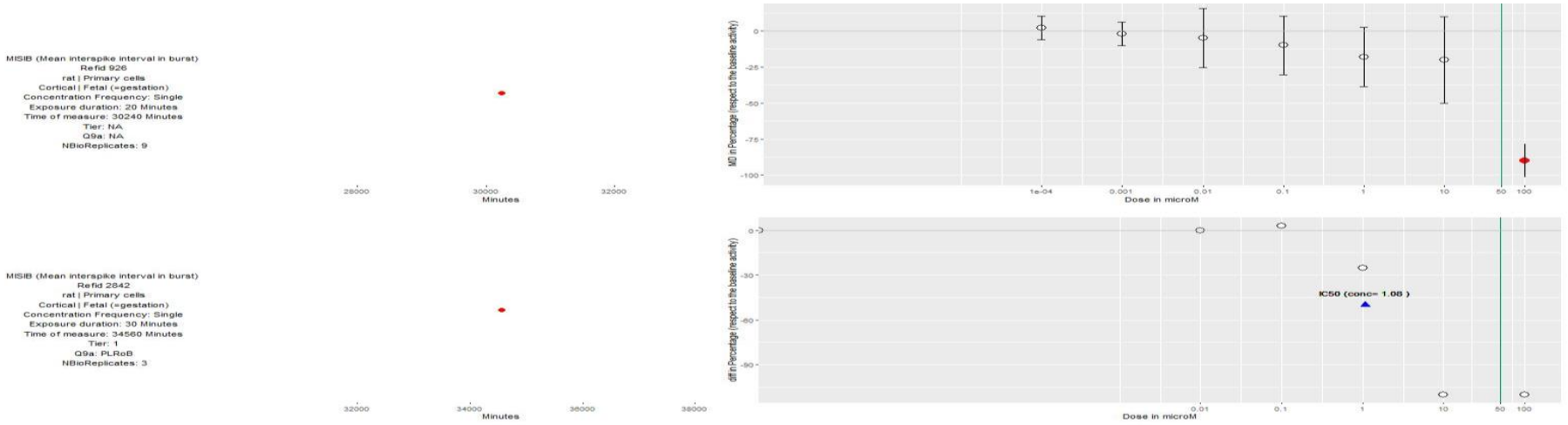


5. 3 KE in vitro. KE4 – Altered Neuronal Network Function. MEA – General activity. MBR (Mean Burst Rate) – from US EPA

burst.per.min_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3

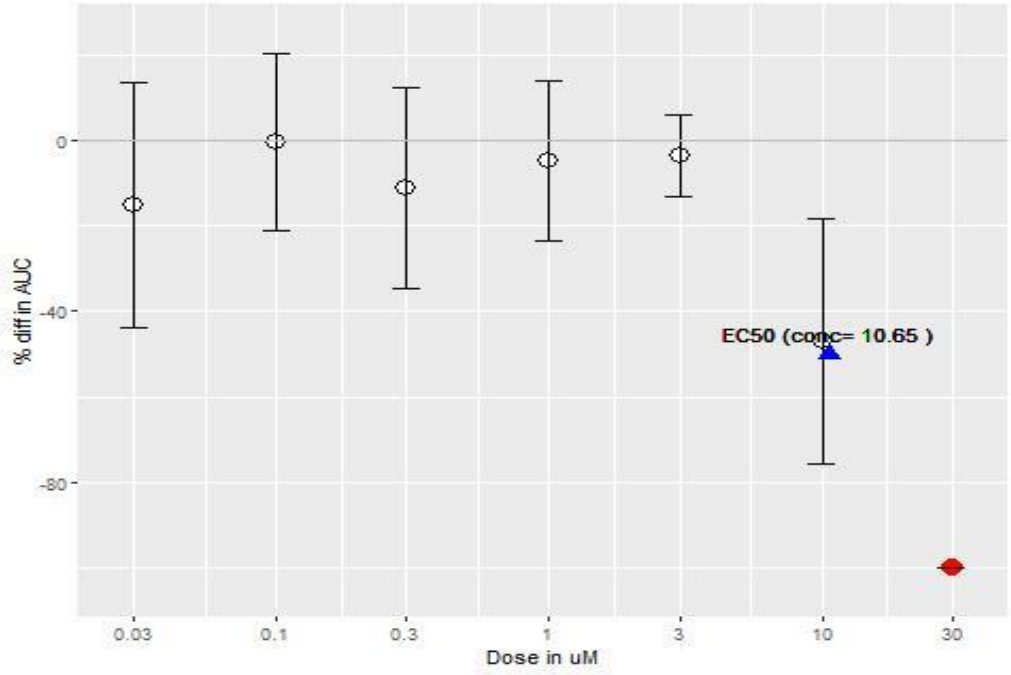


6. 1/6. 2. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. Mean Interspike Interval (MISB) – from literature

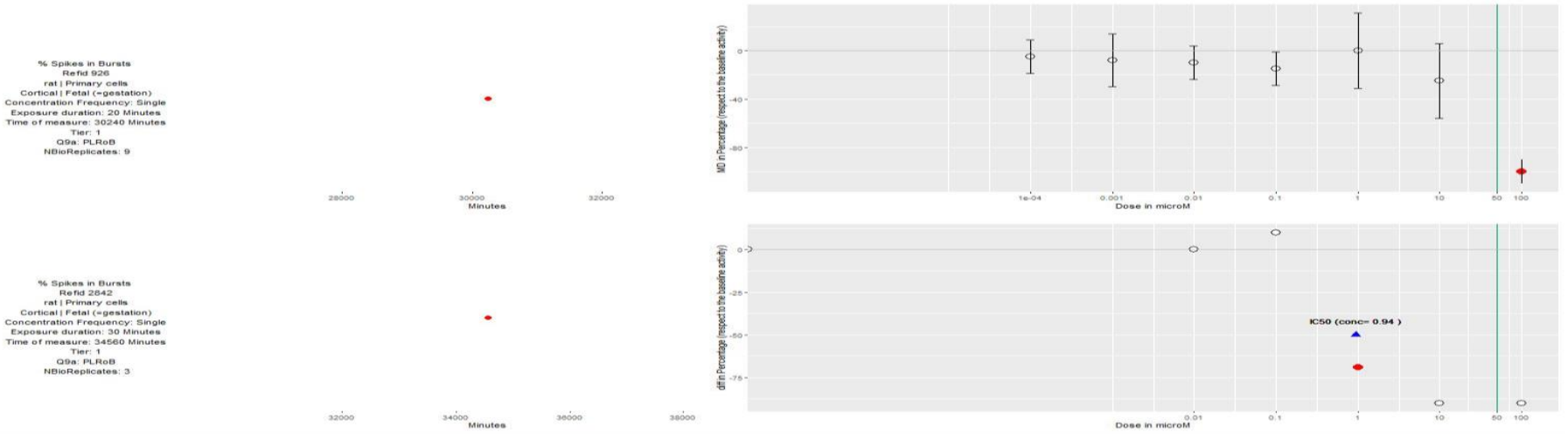


6. 3. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. Mean Interspike Interval (MISB) – from US EPA

mean.isis_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3

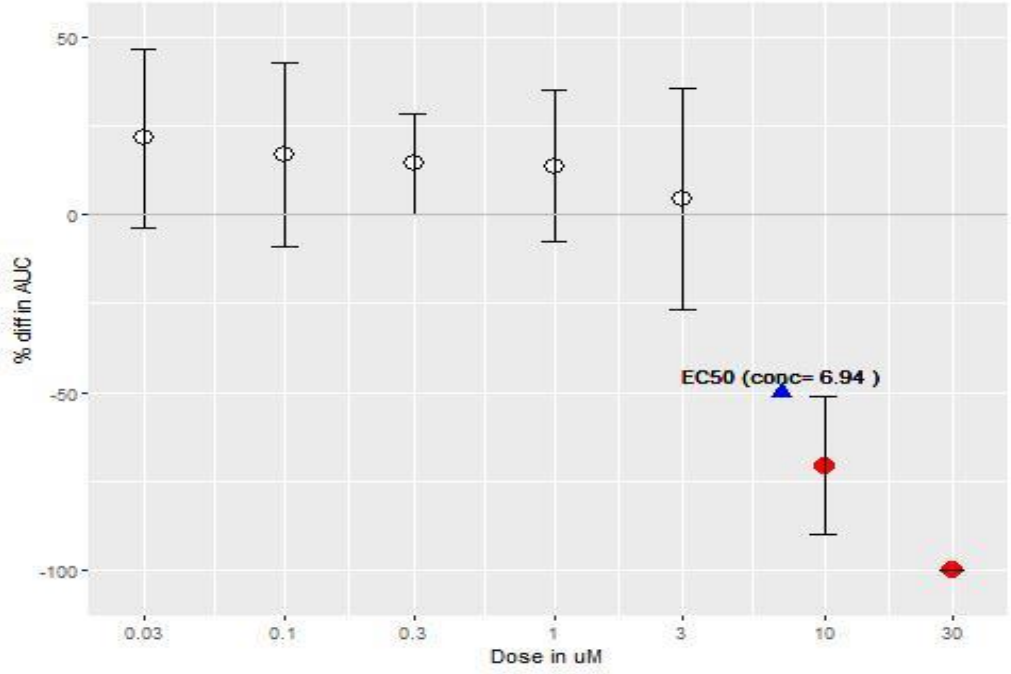


7. 1/7. 2 KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. % spikes in burst – from literature

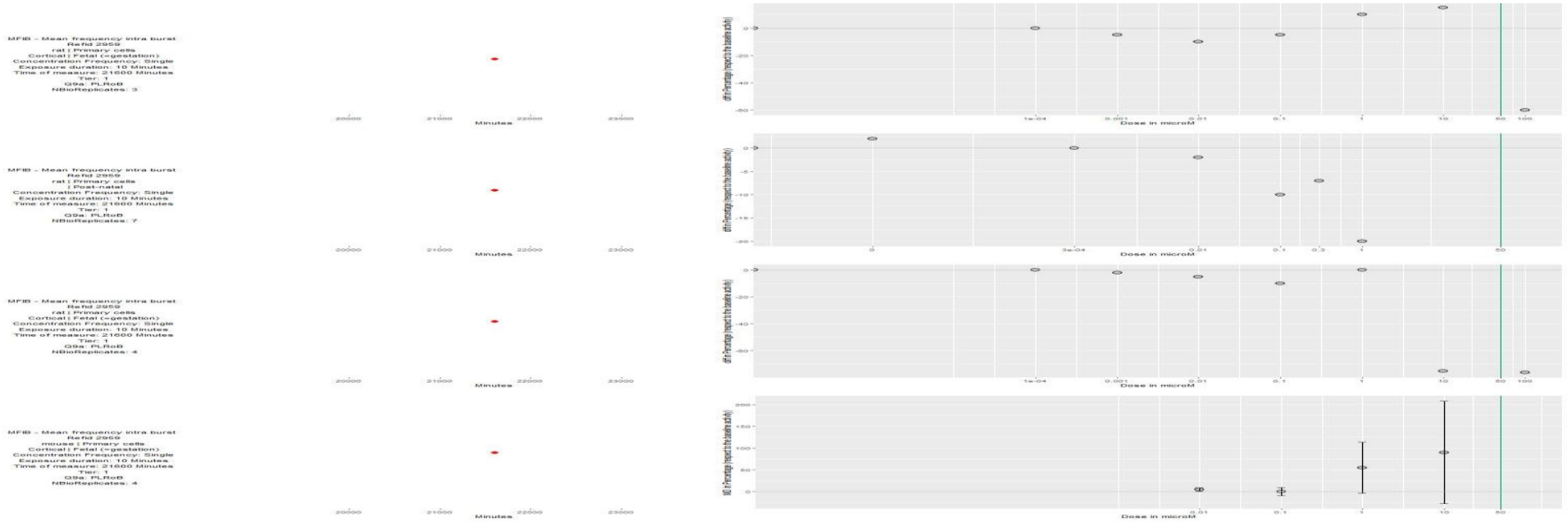


7. 3. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. % spikes in burst – from US EPA

per.spikes.in.burst_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3



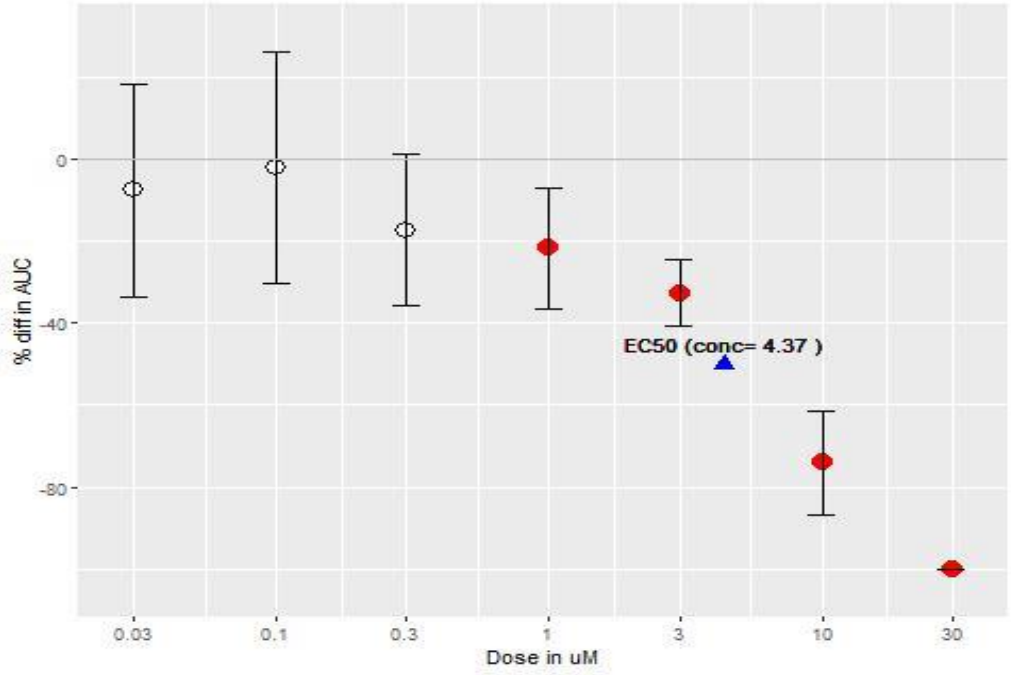
21. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. Mean Firing Rate in Burst (spikes per second) – from literature



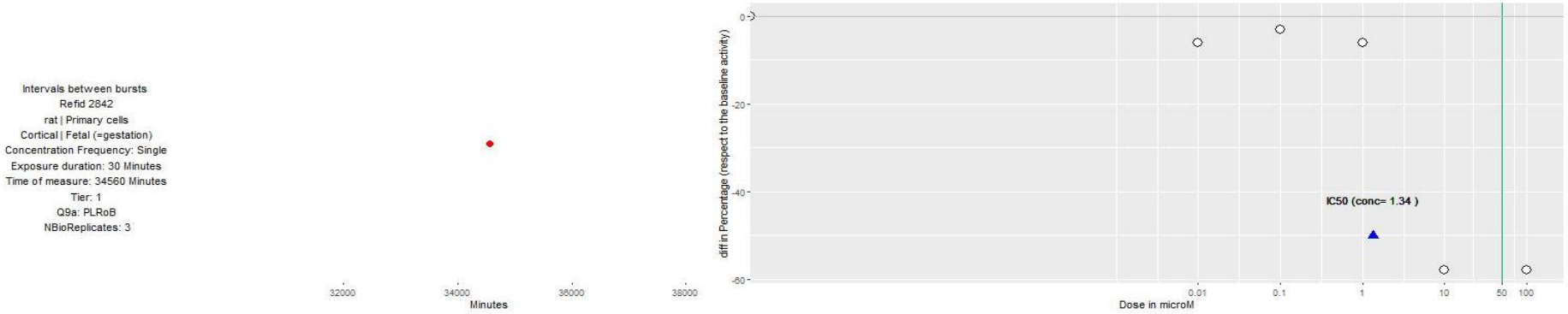
8. 1/8. 2/8. 3. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. Burst Duration – from literature

8. 4. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. Burst Duration – from US EPA

mean.dur_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3

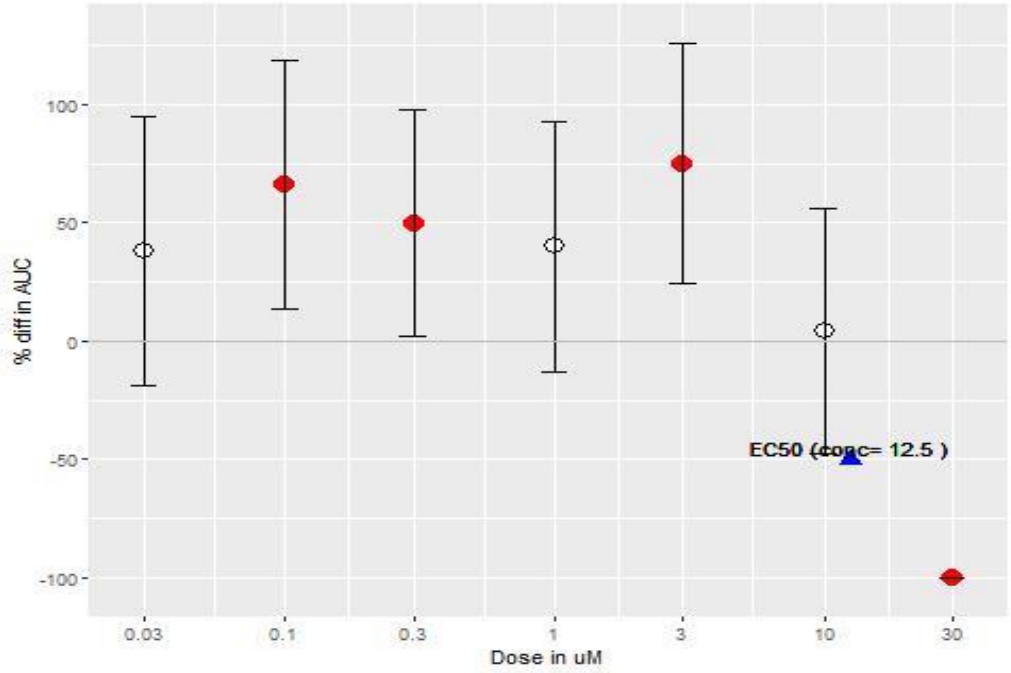


9. 1. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. Intervals between Burst– from literature



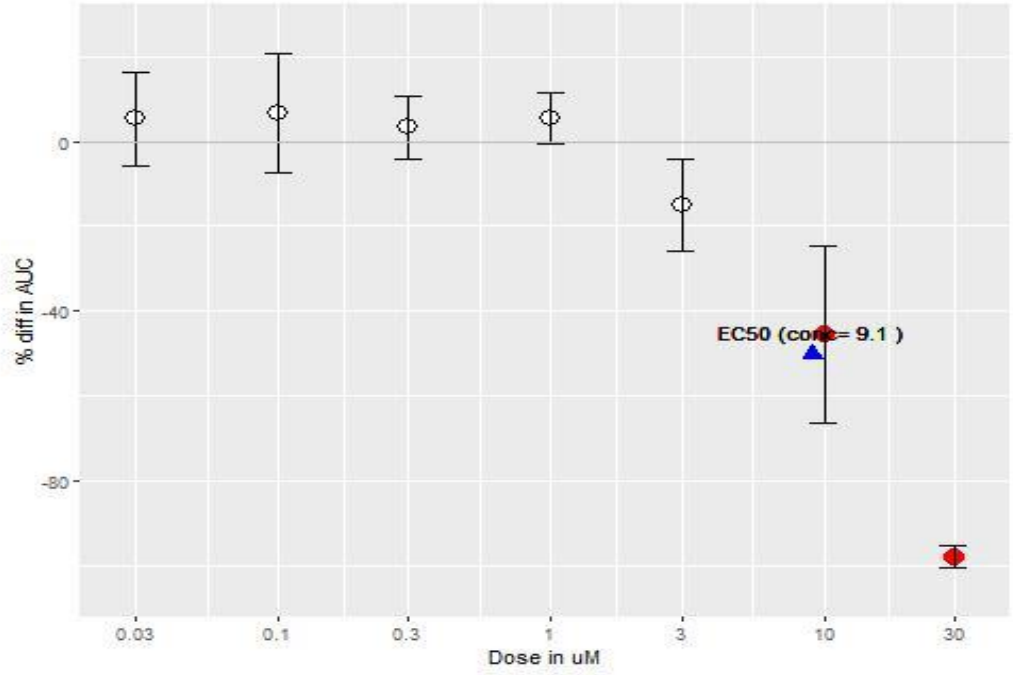
9. 2. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Bursting Activity. Intervals between Burst – from US EPA

mean.IBIs_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3



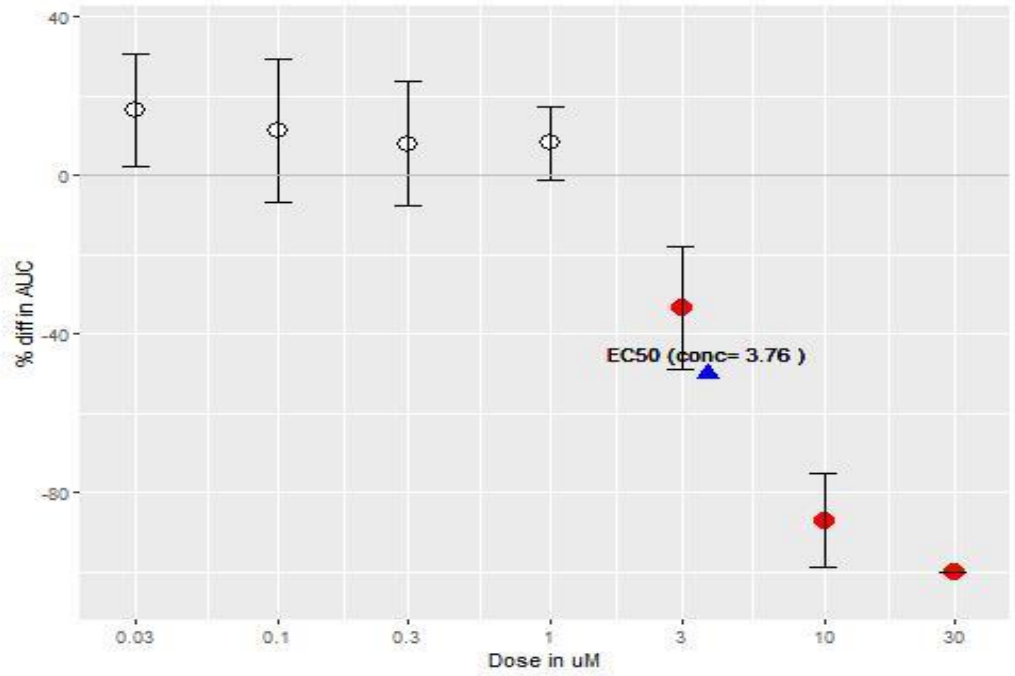
10. 1. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – General Activity. Number of active electrodes – from US EPA

nAE_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3



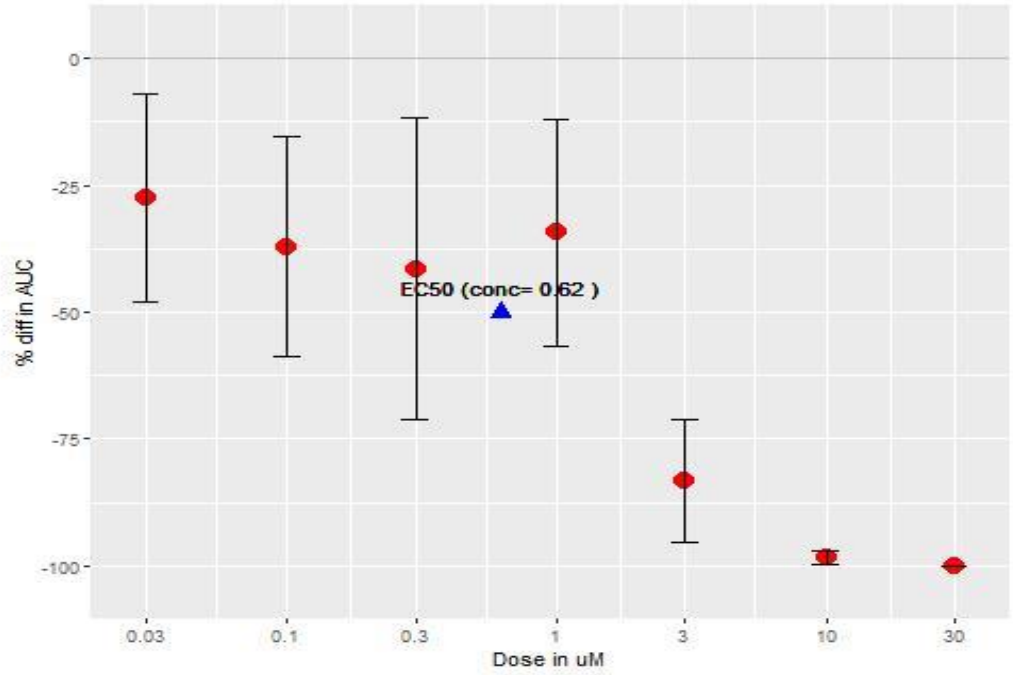
11. 1. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – General Activity. Number of actively Bursting electrodes (#ABE) – from US EPA

nABE_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3



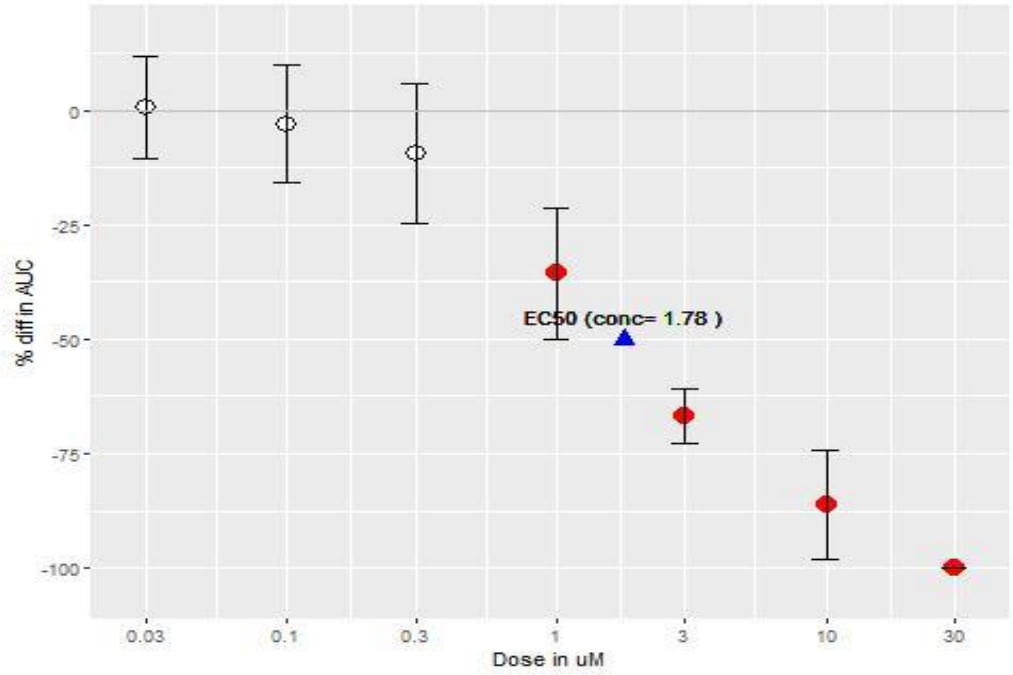
12. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Network Connectivity. Number of Network Spikes (network spike = 10 electrodes simultaneously active) (#NS) – from US EPA

ns.n_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3



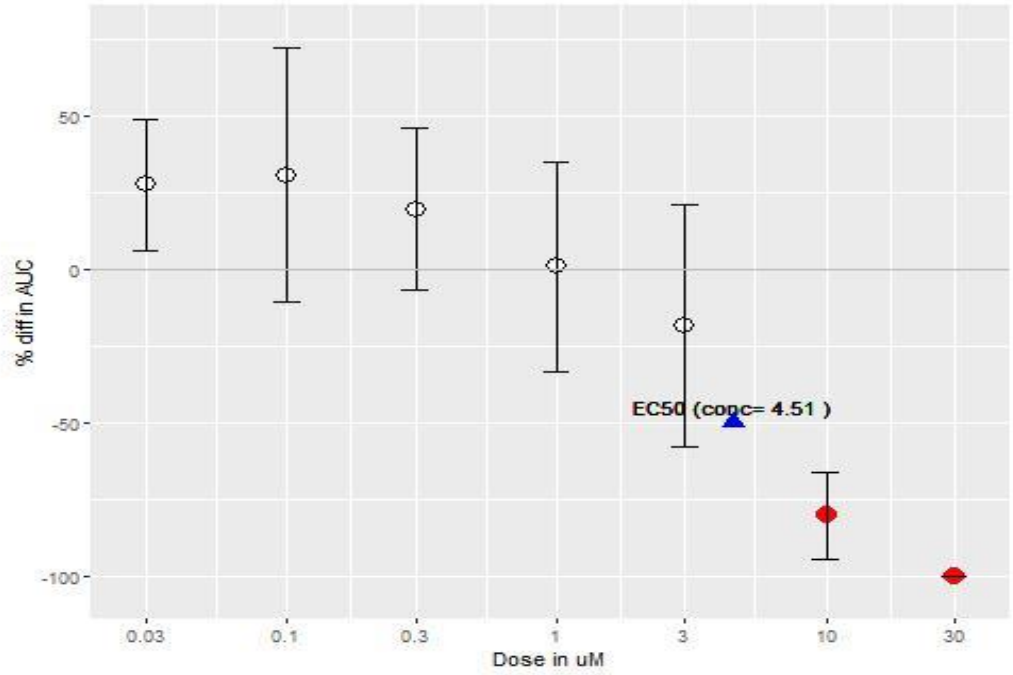
13. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Network Connectivity. % of spikes occurring in a network spike– from US EPA

ns.percent.of.spikes.in.ns_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3



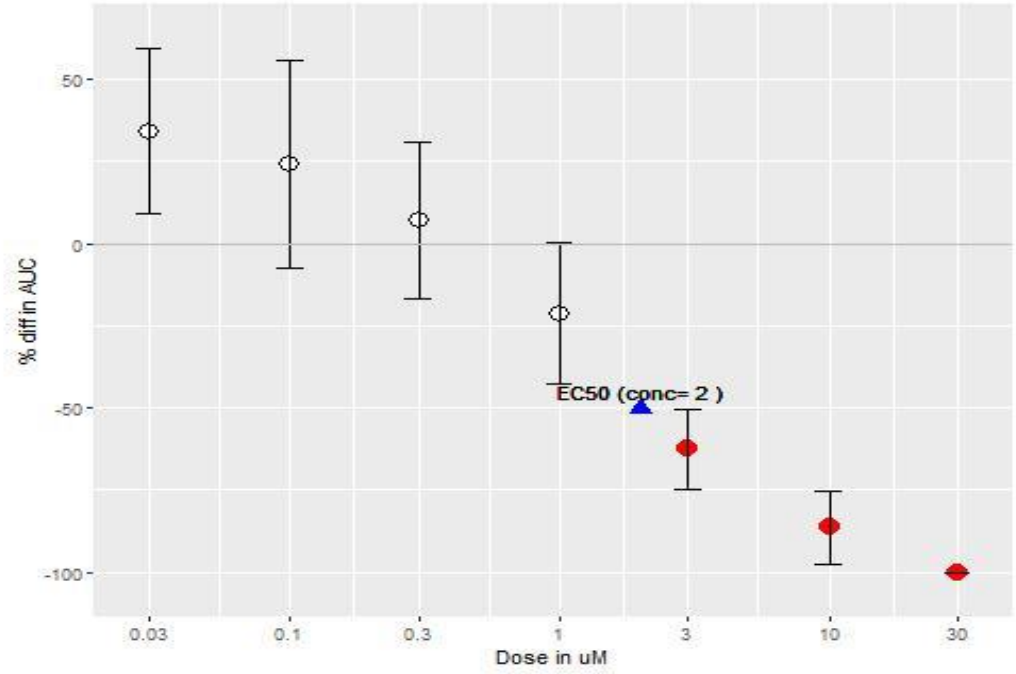
14. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Network Connectivity. Mean number of spikes in Network Spikes – from US EPA

ns.mean.spikes.in.ns_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3

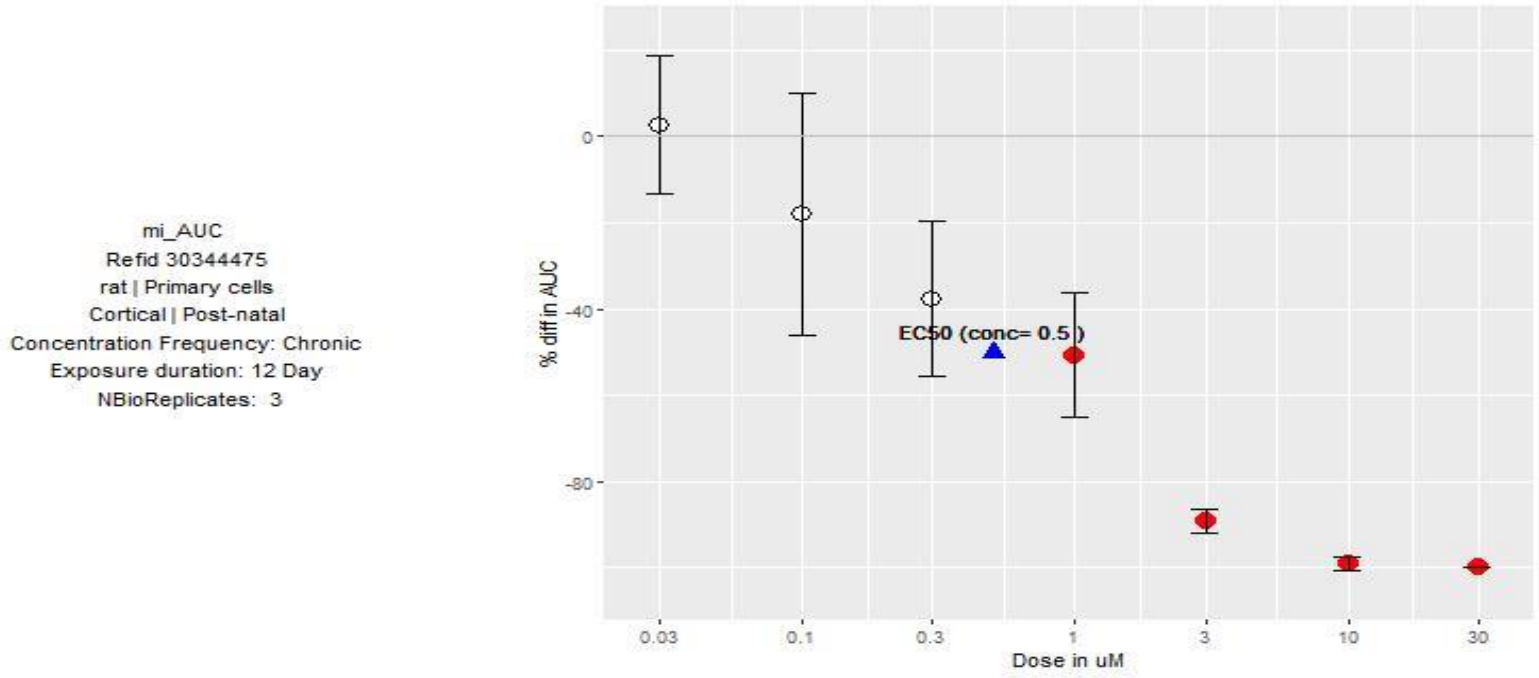


15. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Network Connectivity. Correlation Coefficient area under all electrodes, higher synchronicity) – from US EPA

r_AUC
Refid 30344475
rat | Primary cells
Cortical | Post-natal
Concentration Frequency: Chronic
Exposure duration: 12 Day
NBioReplicates: 3

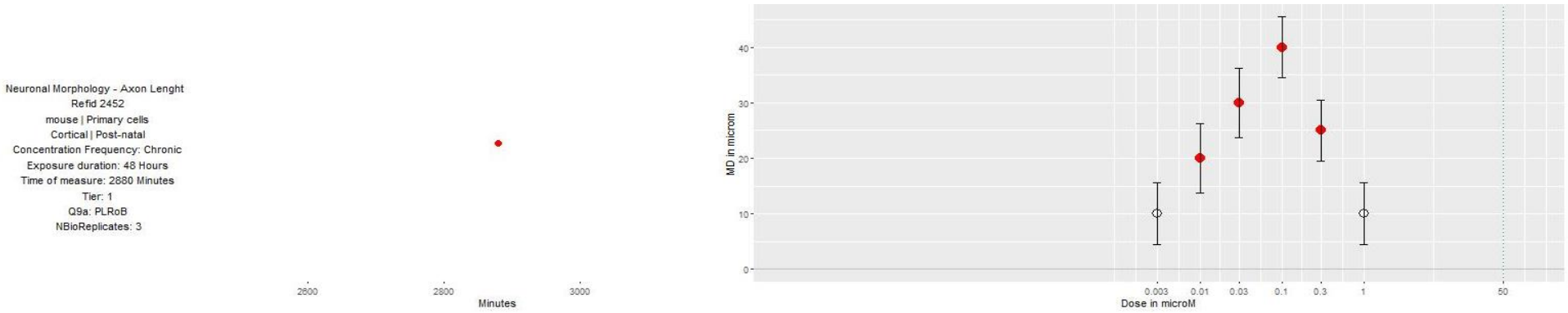


16. KE in vitro. KE4 – Altered Neuronal Network Function. MEA – Network Connectivity. Normalised Mutual Information – from US EPA

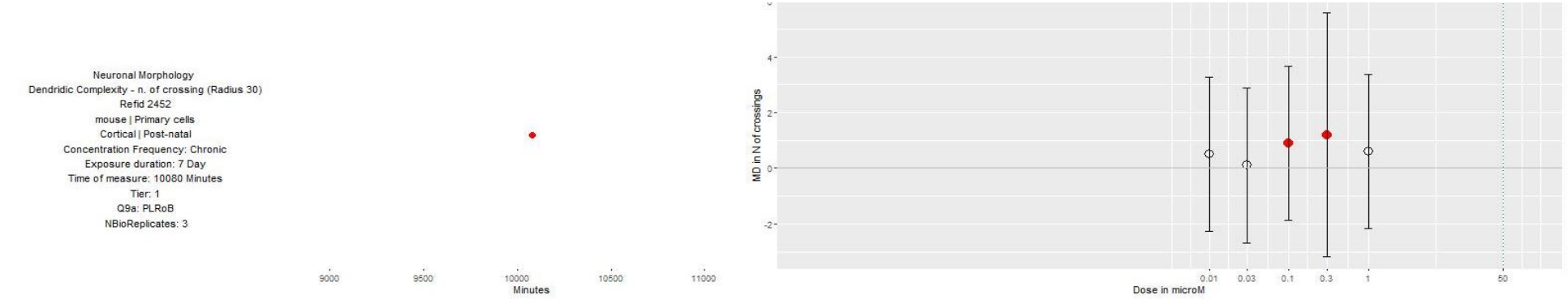


In vitro – KE

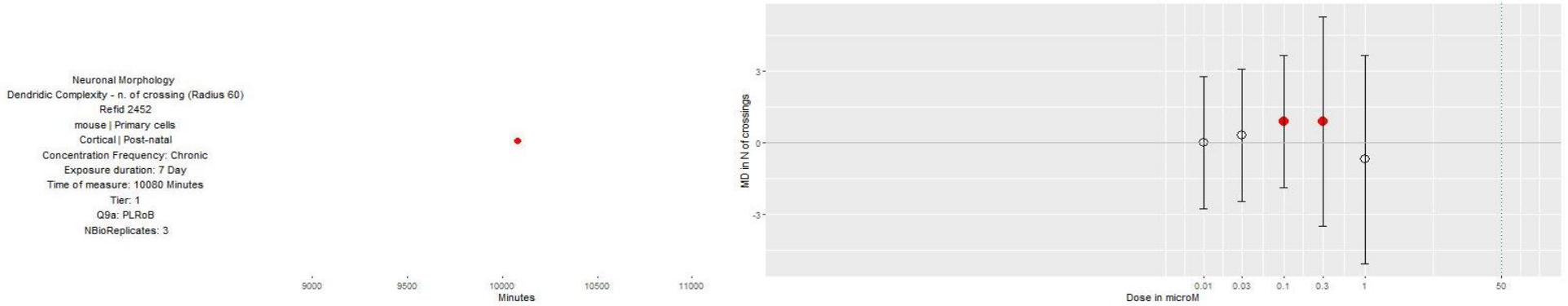
12. 1. KE in vitro. Neuronal Morphology. Axon length – from literature



12. 2. KE in vitro. Neuronal Morphology. Dendritic complexity – n of crossing – radius 30

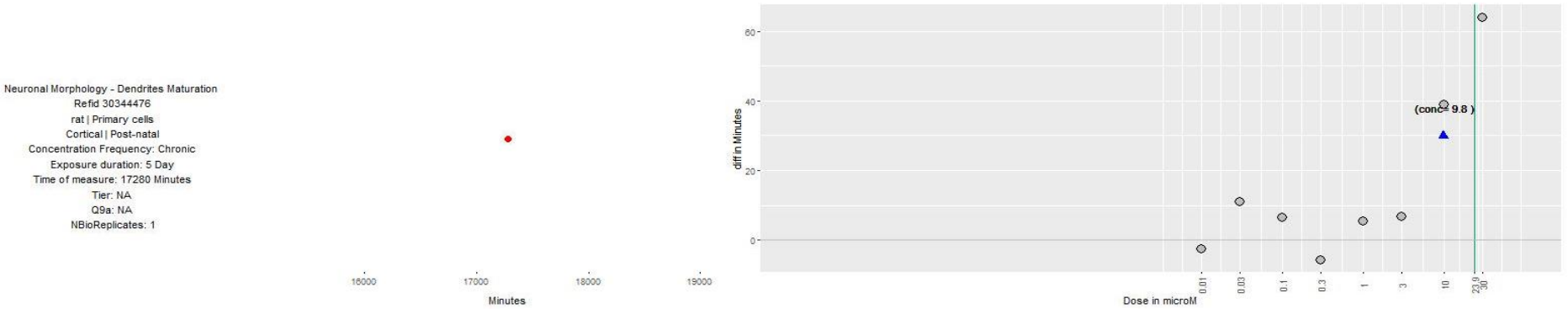


12. 3. KE in vitro. Neuronal Morphology. Dendritic complexity – n of crossing – radius 60



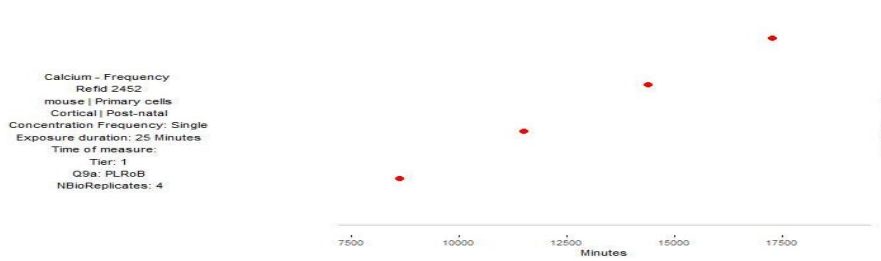
15. KE in vitro. Neuronal Morphology. Total neurite length per neuron

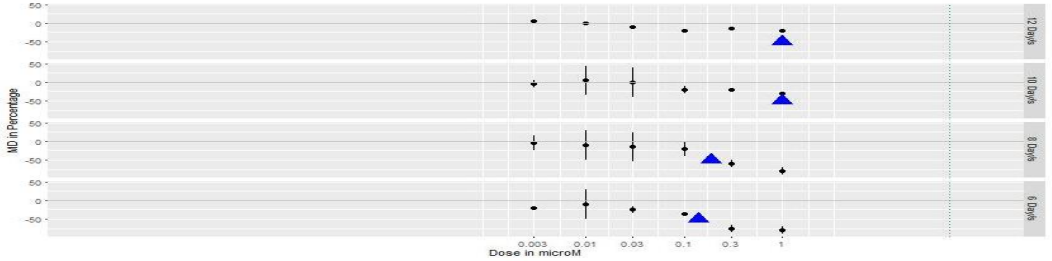
16. KE in vitro. Neuronal Morphology. Dendrite maturation (Dendrite total length per neuron)

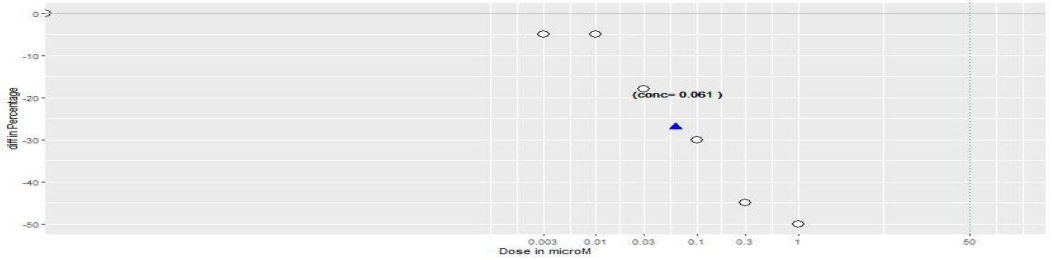


20. KE in vitro. Disruption of intracellular Calcium channel kinetic. Disruption of Channel Calcium Kinetics. Calcium Influx

21. KE in vitro. Disruption of intracellular Calcium channel kinetic. Disruption of Channel Calcium Kinetics. Calcium Frequency





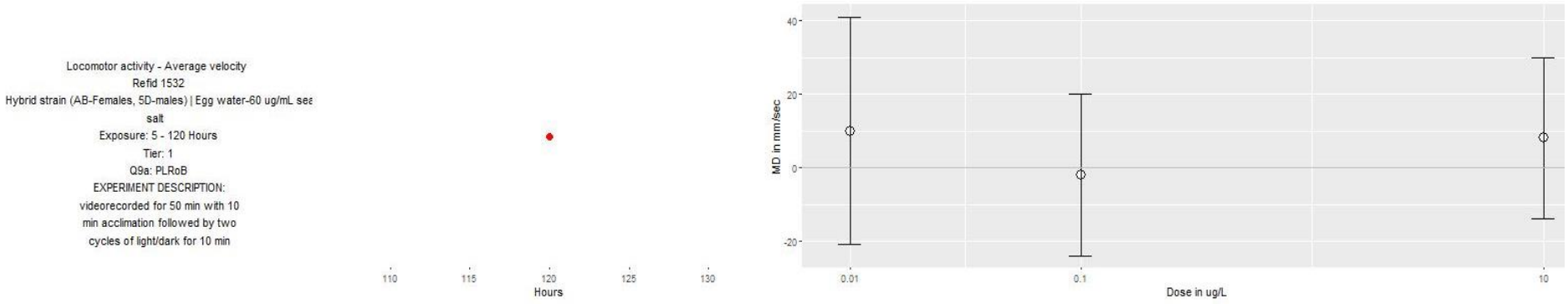


22. KE in vitro. Intracellular sodium in microglia cells. Intracellular sodium in microglia cells. Changes of intracellular sodium in Microglia cells

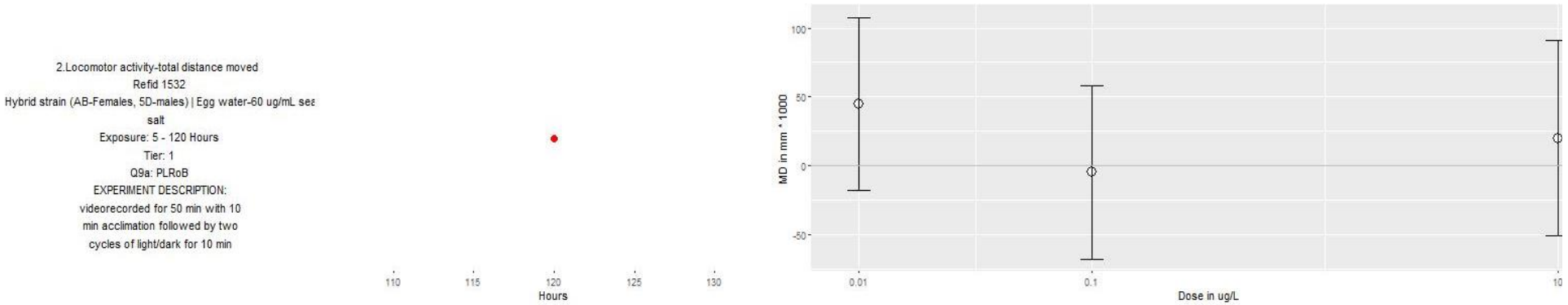
23. KE in vitro. Increase of Intracellular sodium in microglia cells. Microglia Activation. TNF-alpha secretion

AO Zebrafish

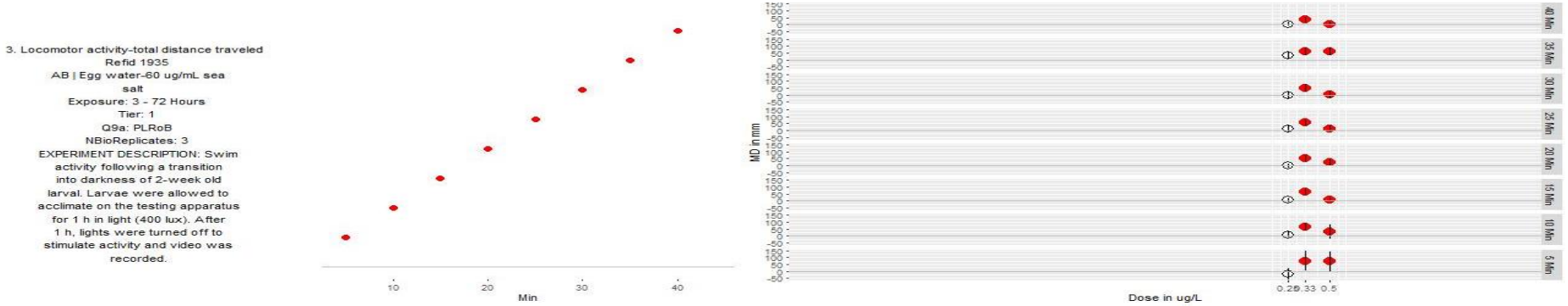
1. AO Zebrafish. Behaviour. Locomotor Activity – Average velocity



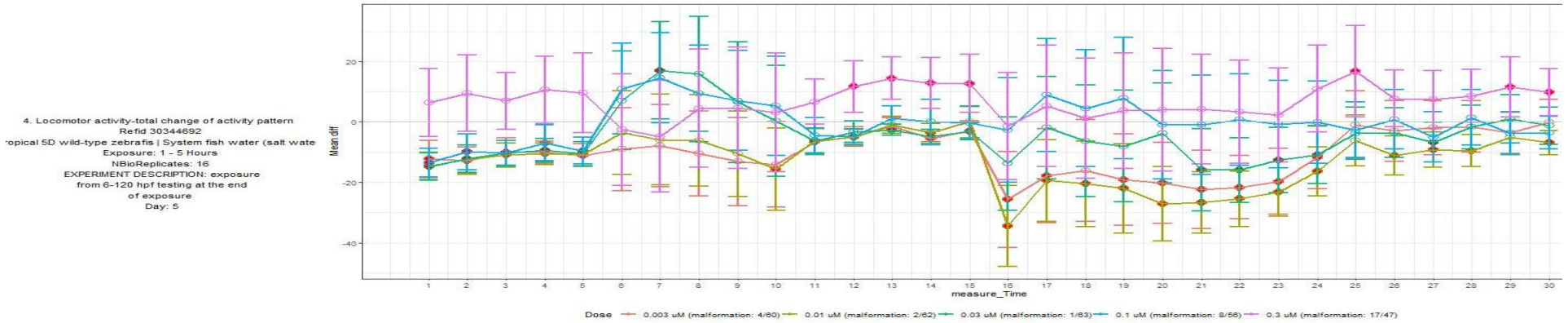
2. AO. Zebrafish. Behaviour. Locomotor Activity – Total distance moved



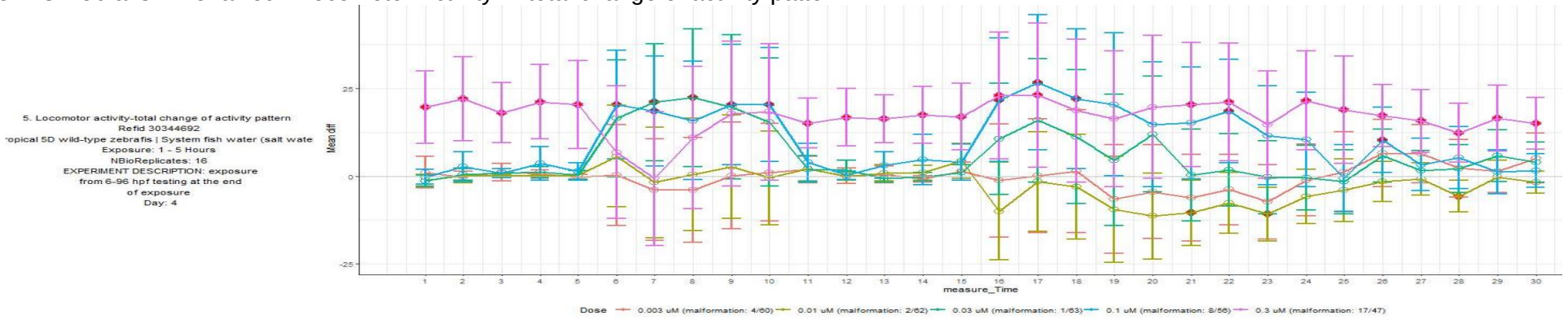
3. AO Zebrafish. Behaviour. Locomotor Activity – total distance travelled



4. AO Zebrafish. Behaviour. Locomotor activity – total change of activity pattern



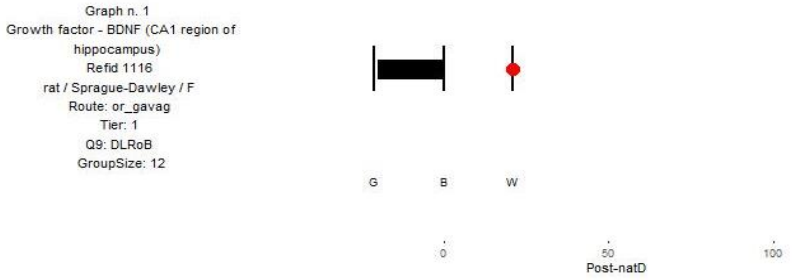
5. AO Zebrafish. Behaviour. Locomotor Activity – total change of activity pattern

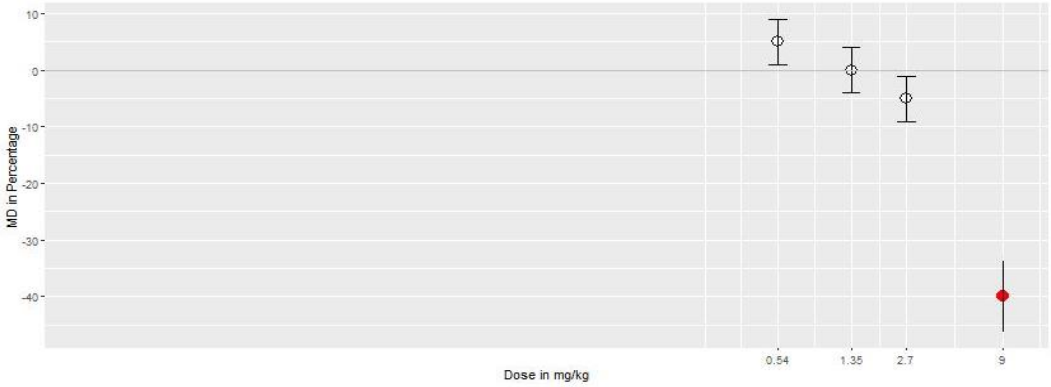


In vivo

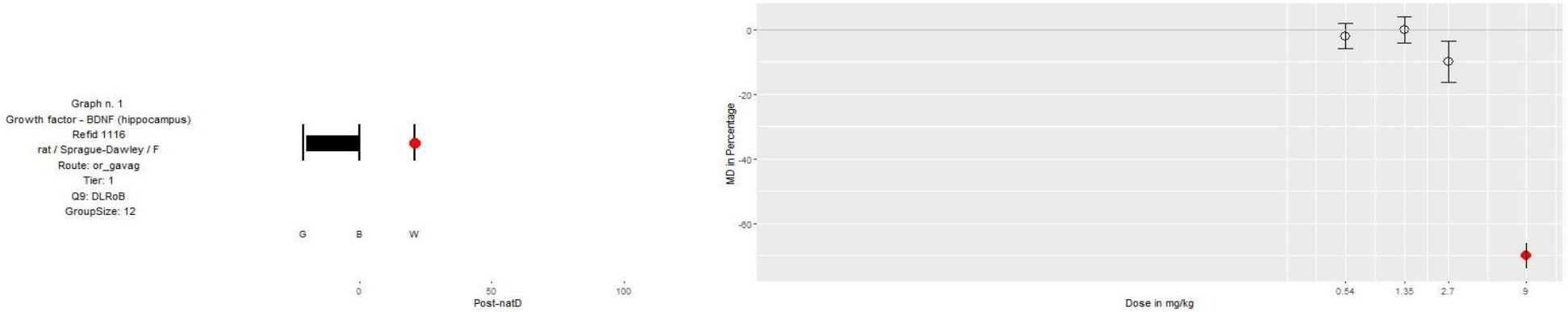
In vivo – KE

1. KE In vivo. Neurochemistry. Growth Factor. BDNF (CA1 region of hippocampus). Immunofluorescence

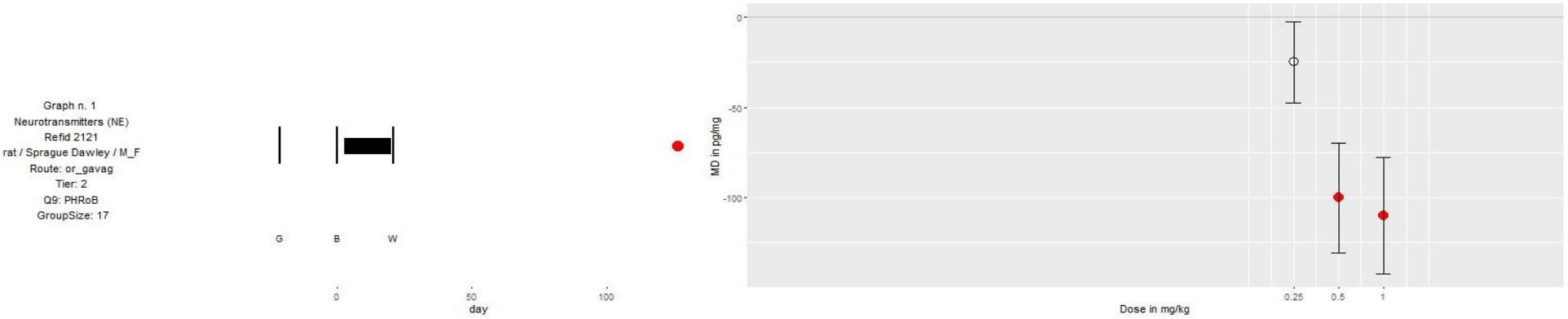




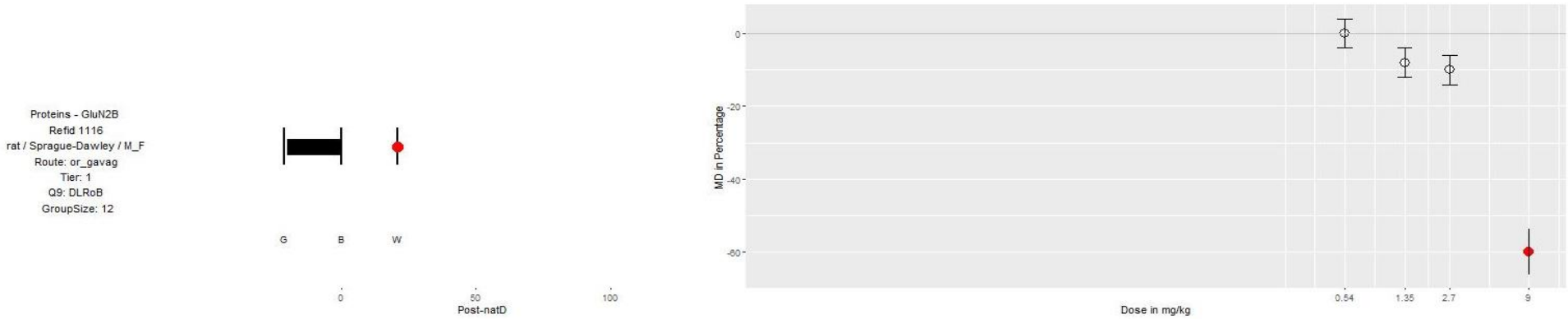
2. KE In vivo. Neurochemistry. Growth Factor. BDNF. (hippocampus) Western Blotting



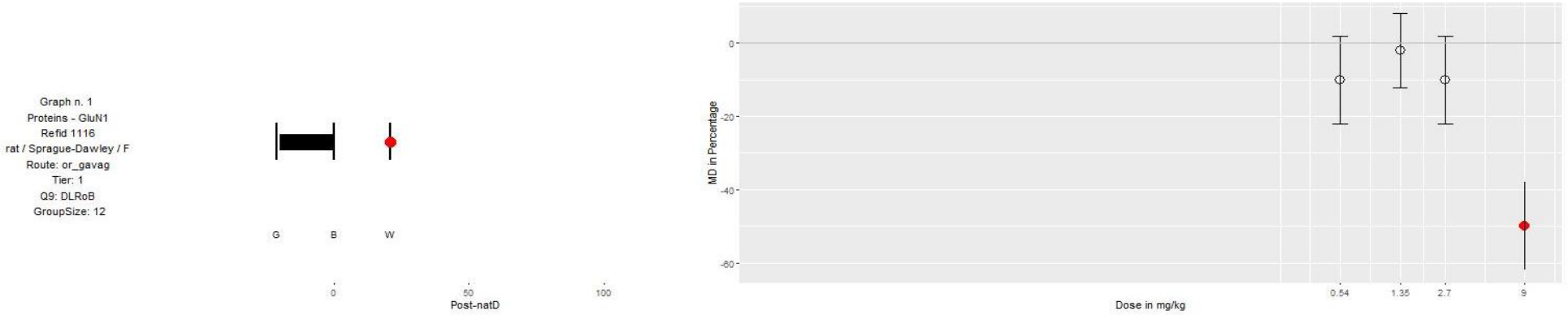
3. KE In vivo. Neurochemistry. Neurotransmitters – Norepinephrine (NE)



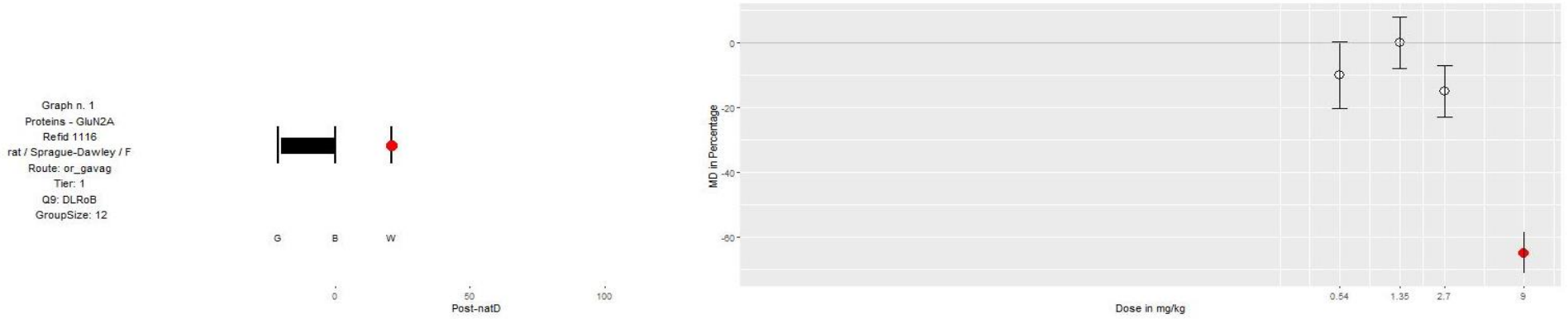
4. KE In vivo. Neurochemistry. Proteins. GluN2B



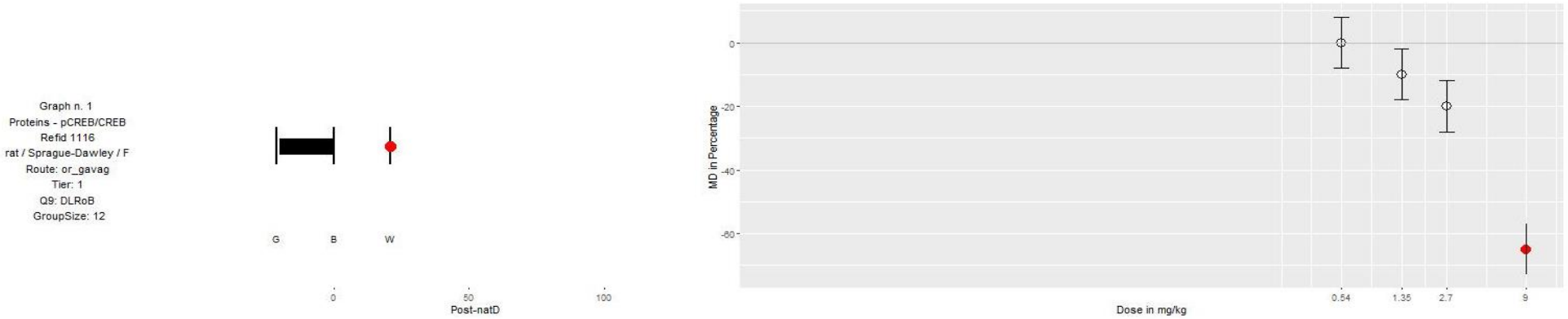
5. KE In vivo. Neurochemistry. Proteins – GluN1



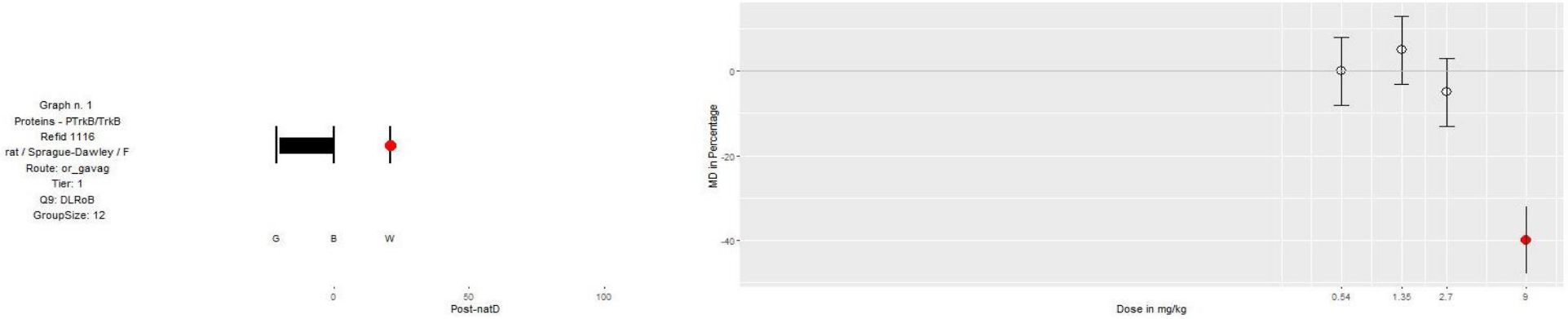
6. KE In vivo. Neurochemistry. Proteins – GluN2A



7. KE In vivo. Neurochemistry. Proteins – pCREB/CREB

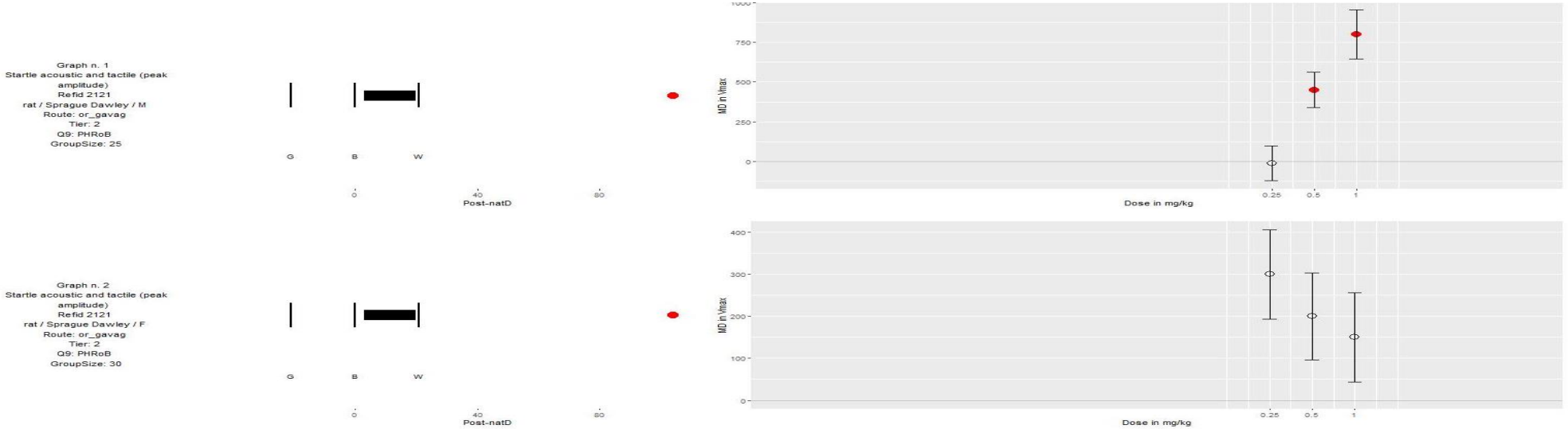


8. KE In vivo. Neurochemistry. Proteins – PTrkB/TrkB

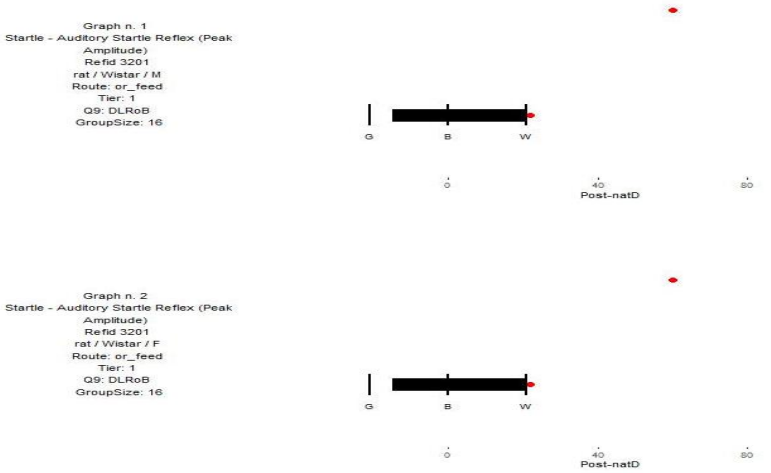


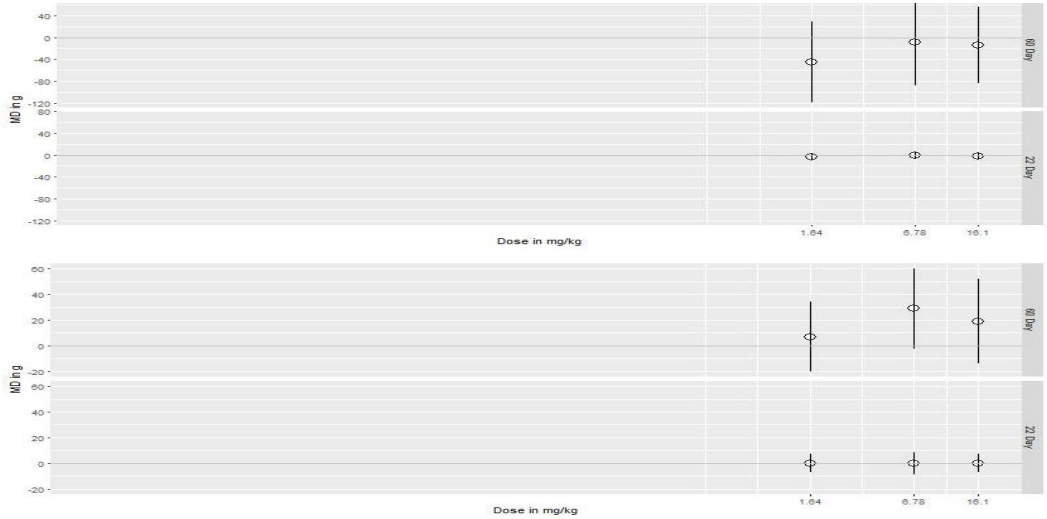
In vivo – AO Behaviour

1. AO In vivo. Impairment of behavioural function. Startle acoustic – Startle acoustic and tactile (peak amplitude)

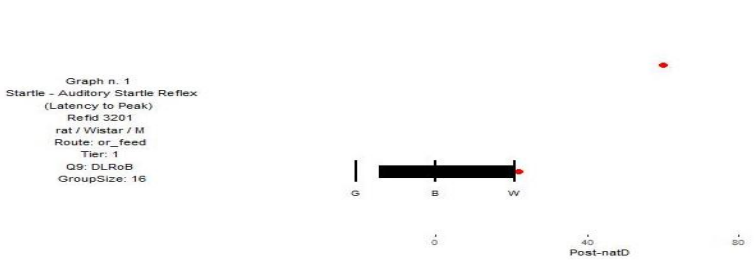


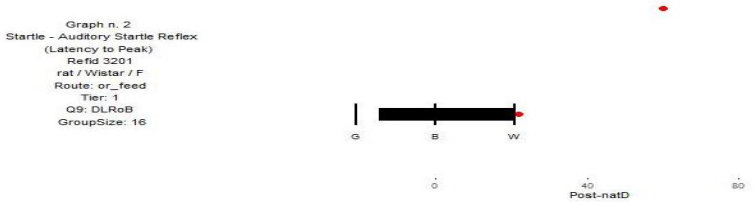
2. AO In vivo. Impairment of behavioural function. Startle acoustic – Auditory Startle Reflex (peak amplitude)

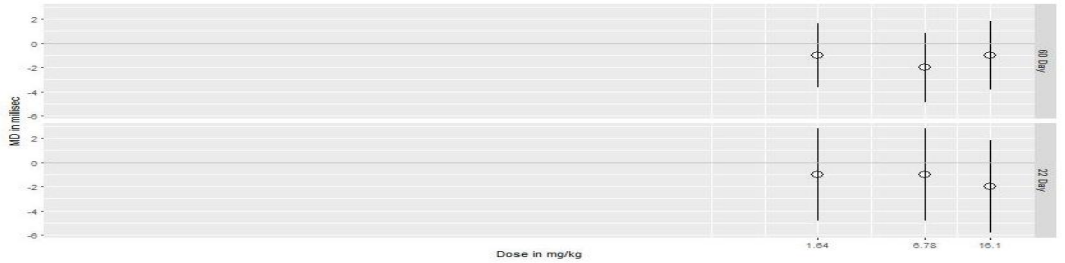




3. AO In vivo. Impairment of behavioural function. Startle acoustic – Auditory Startle Reflex (Latency to peak)



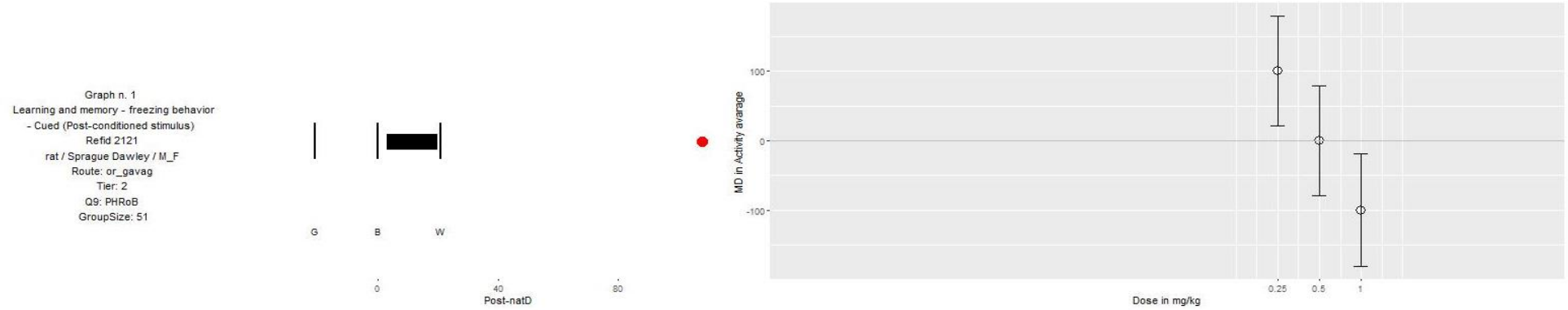




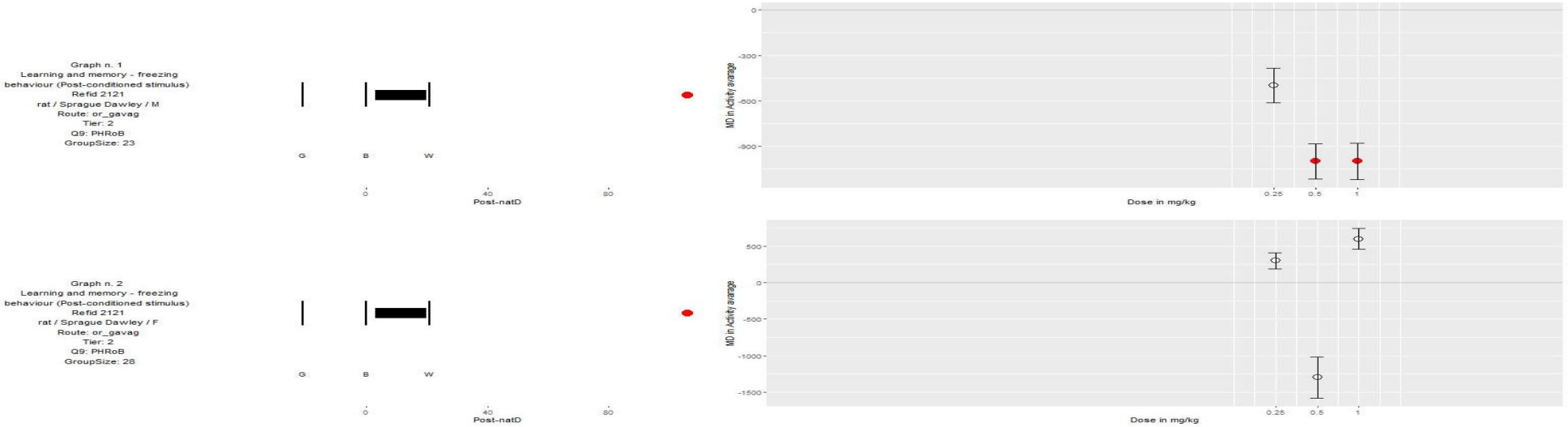
4. AO In vivo. Impairment of behavioural function. Startle acoustic – Auditory Startle Reflex (peak amplitude habituation)

5. AO In vivo. Impairment of behavioural function. Startle acoustic – Auditory Startle Reflex (Latency to peak habituation)

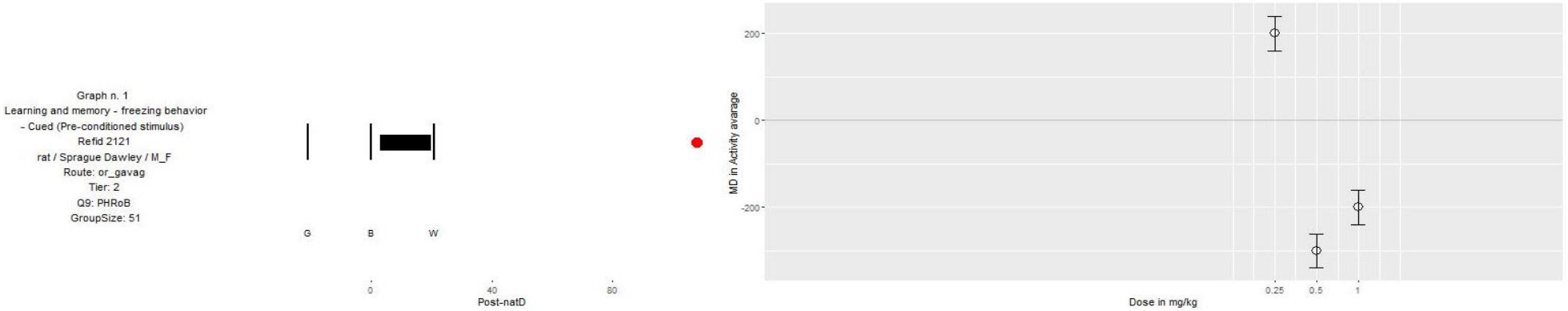
6. AO In vivo. Impairment of behavioural function. Freezing behaviour. Learning and memory – freezing behaviour – Cued – Post-conditioned



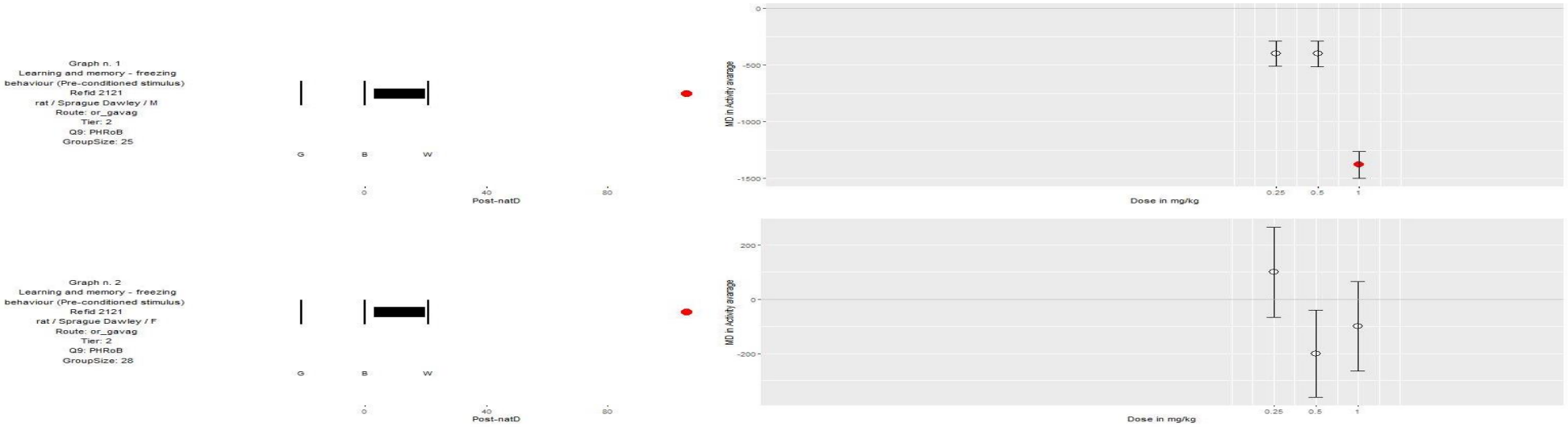
7. AO In vivo. Impairment of behavioural function. Freezing behaviour. Learning and memory – freezing behaviour – Post-conditioned



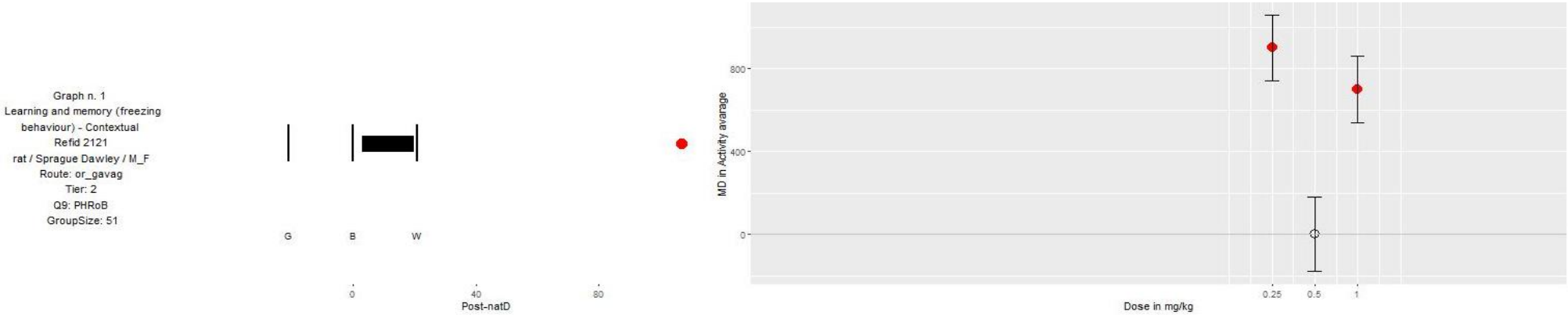
8. AO In vivo. Impairment of behavioural function. Freezing behaviour. Learning and memory – freezing behaviour – Cued – Pre-conditioned stimulus



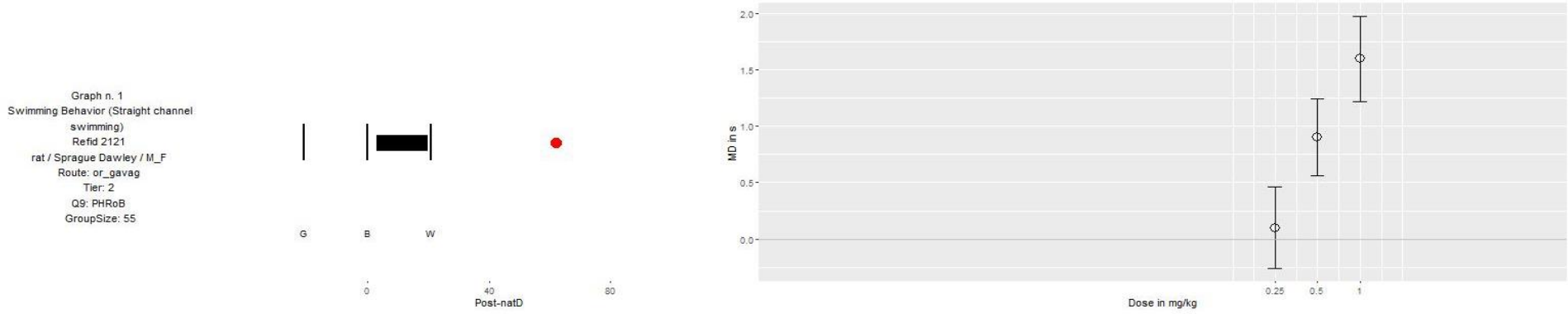
9. AO In vivo. Impairment of behavioural function. Freezing behaviour. Learning and memory – freezing behaviour – Pre-conditioned stimulus



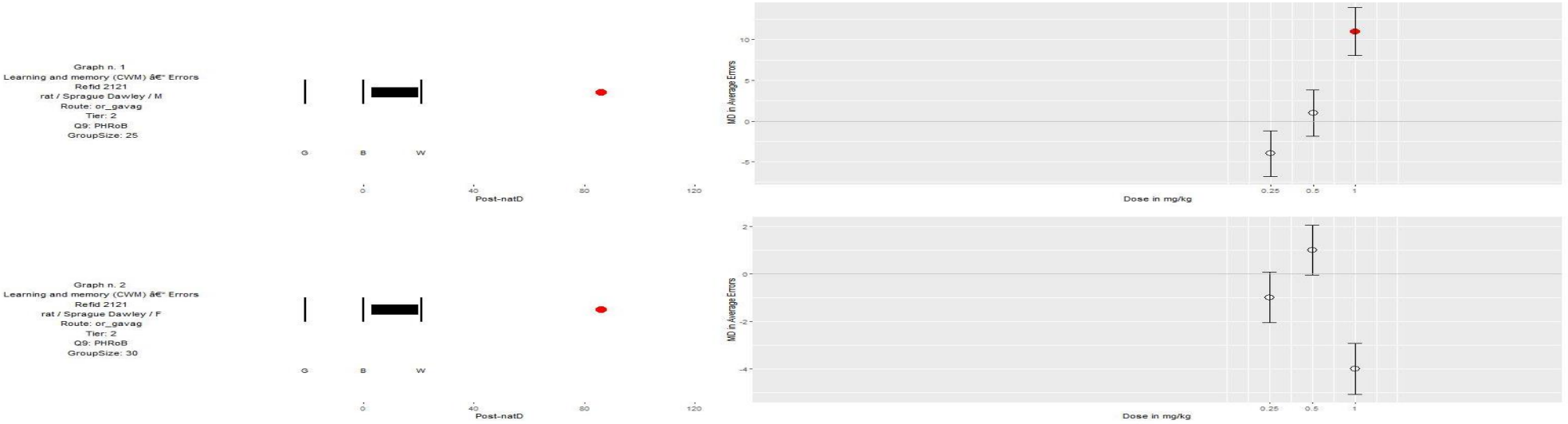
10. AO In vivo. Impairment of behavioural function. Freezing behaviour. Learning and memory – freezing behaviour – contextual



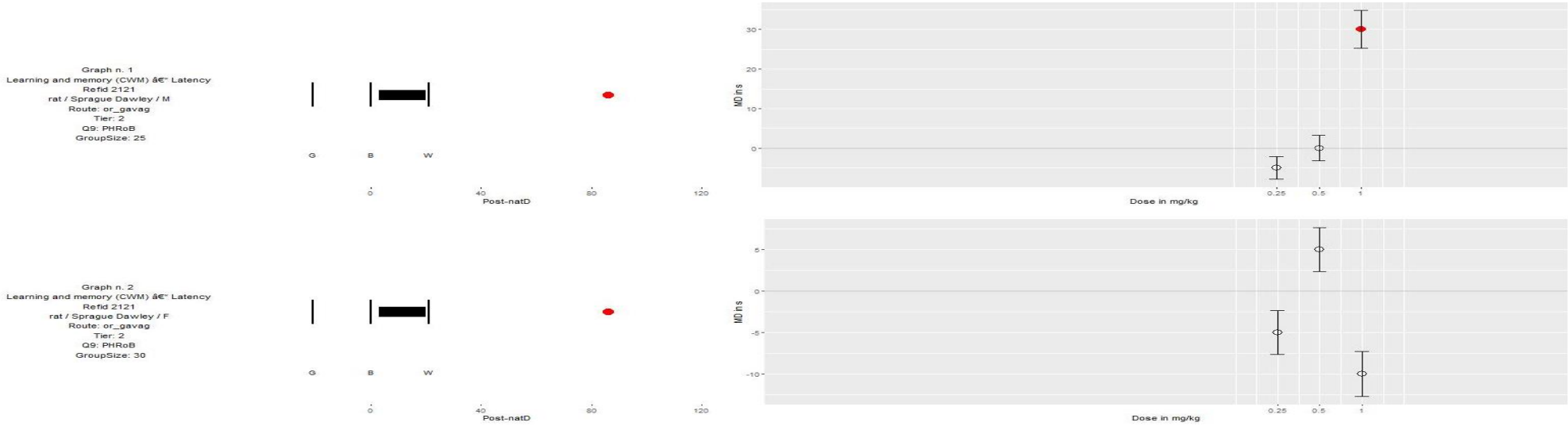
11. AO In vivo. Impairment of behavioural function. Swimming behaviour. Behaviour ontology Swimming Behaviour – Straight channel swimming



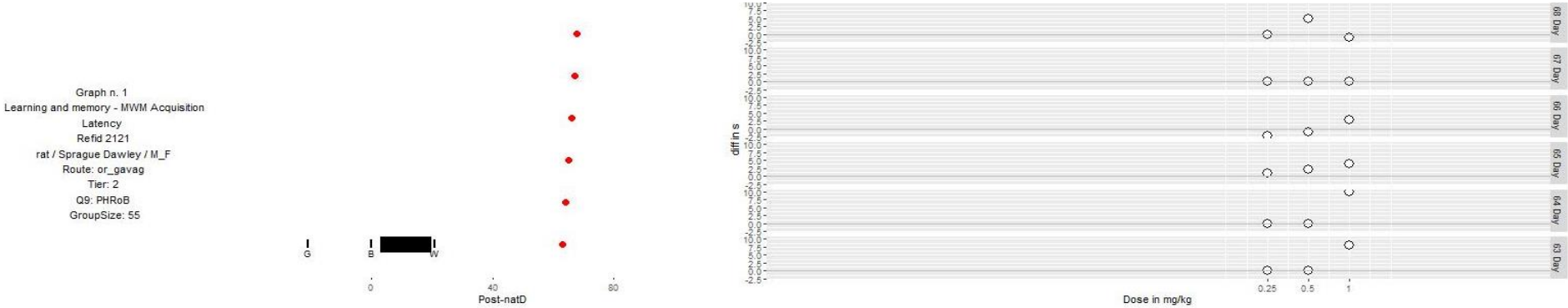
12. AO In vivo. Impairment of behavioural function. CWM. Learning and memory – CWM Errors



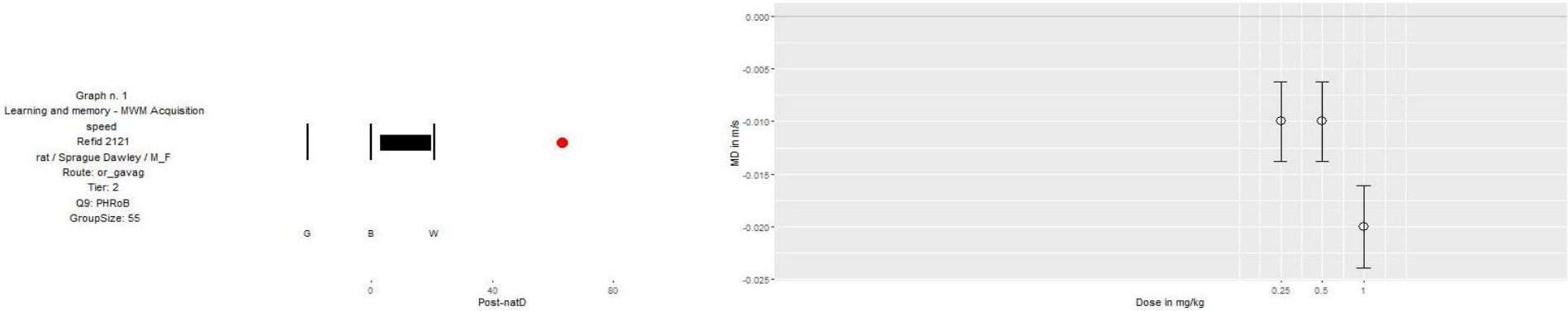
13. AO In vivo. Impairment of behavioural function. CWM. Learning and memory – CWM Latency



14. AO In vivo. Impairment of behavioural function. MWM. Learning and memory – MWM acquisition latency

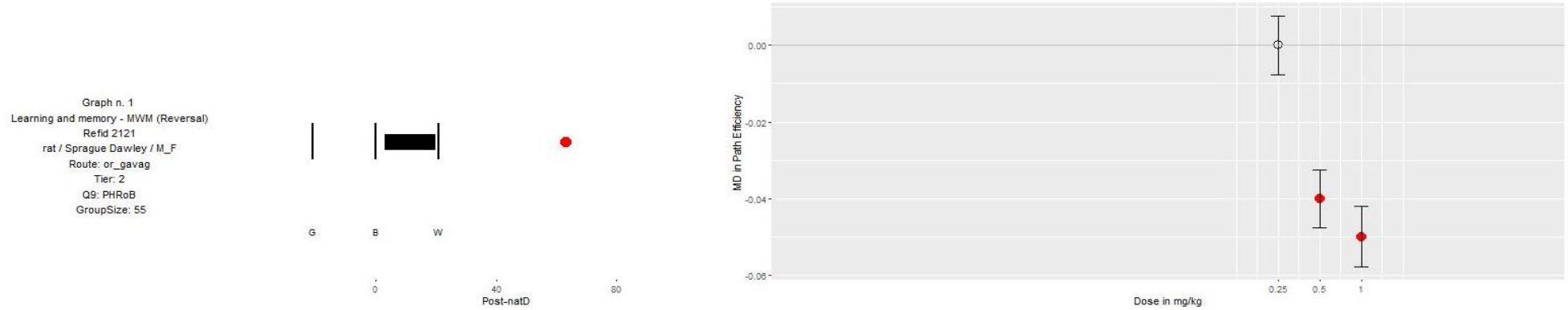


15. AO In vivo. Impairment of behavioural function. MWM. Learning and memory – MWM acquisition speed

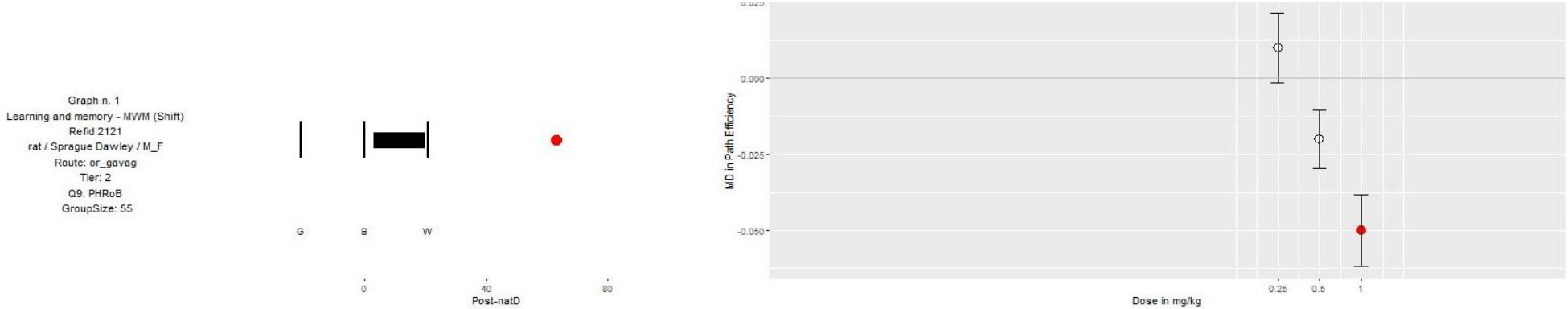


16. AO In vivo. Impairment of behavioural function. MWM. Learning and memory – MWM acquisition path efficiency

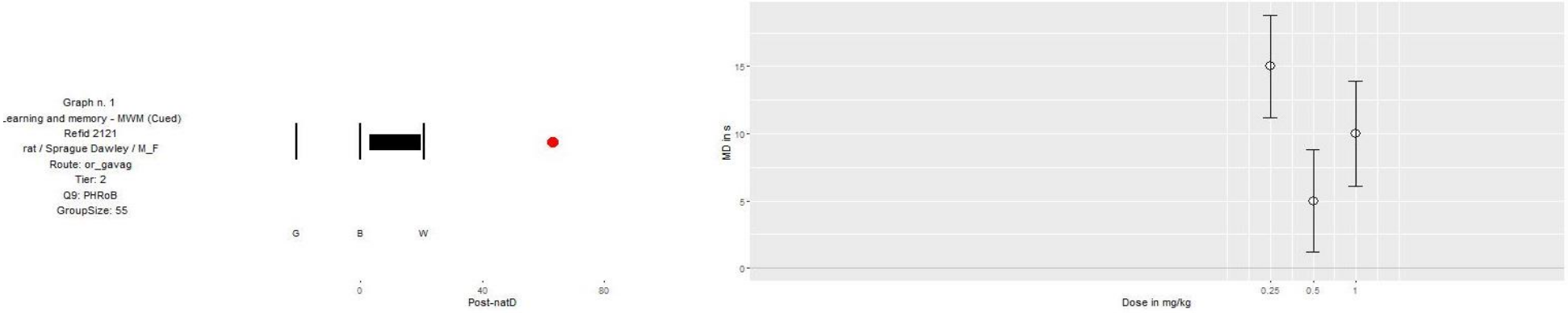
17. AO In vivo. Impairment of behavioural function. MWM. Learning and memory – MWM reversal path efficiency



18. AO In vivo. Impairment of behavioural function. MWM. Learning and memory – MWM shift path efficiency



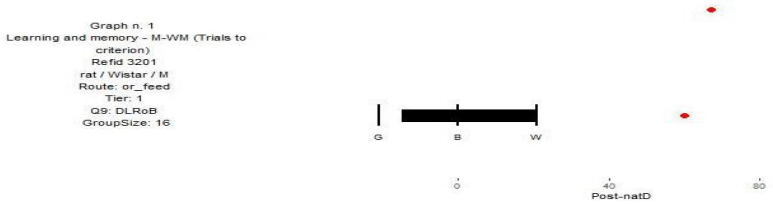
19. AO In vivo. Impairment of behavioural function. MWM. Learning and memory – MWM Cued Latency

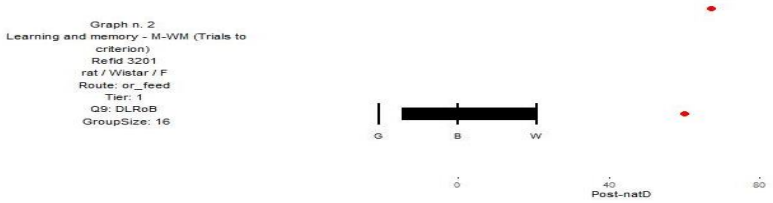


20. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – trial to criterium, learning phase (1–2 graphs)

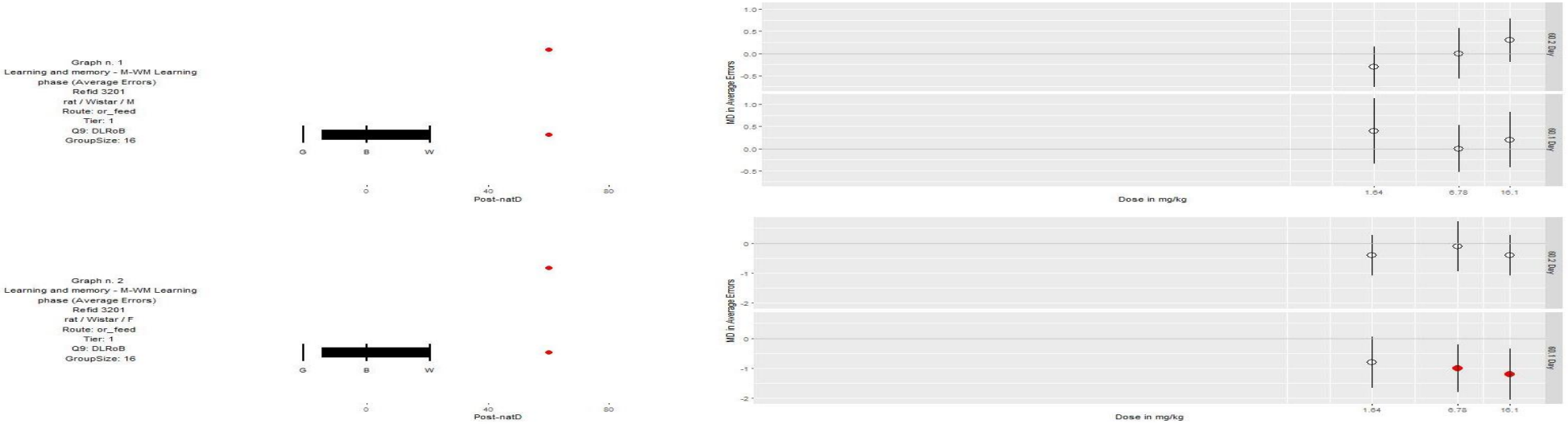
AND

21. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – trial to criterium, retention phase (1–2 graphs)





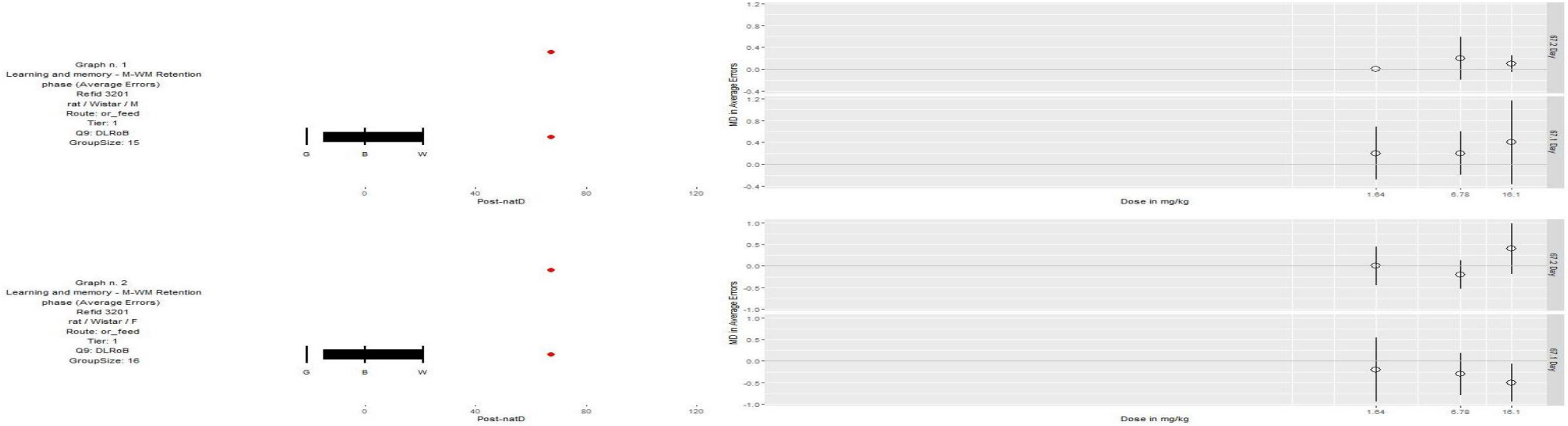
22. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – average errors learning phase (1–2 graphs)



23. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – duration retention phase (1–2 graphs)

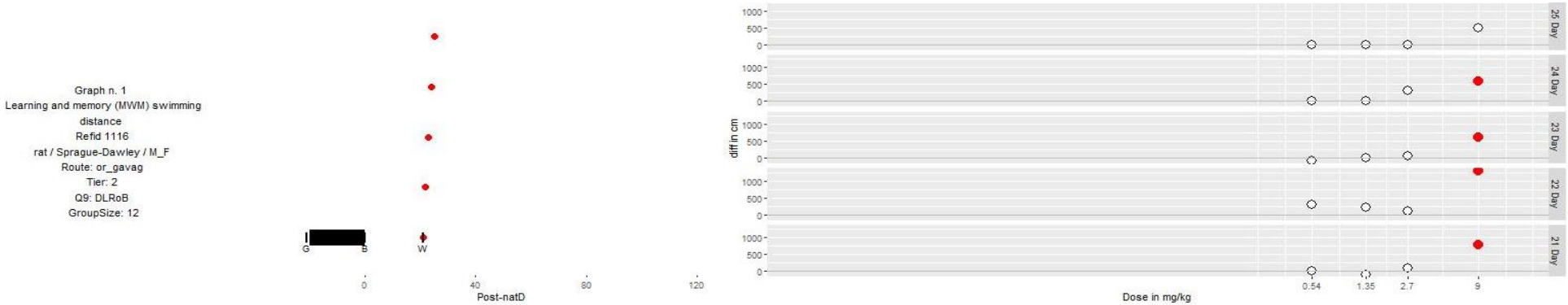
24. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – duration learning phase (1–2 graphs)

25. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – average errors retention phase (1–2 graphs) TBC

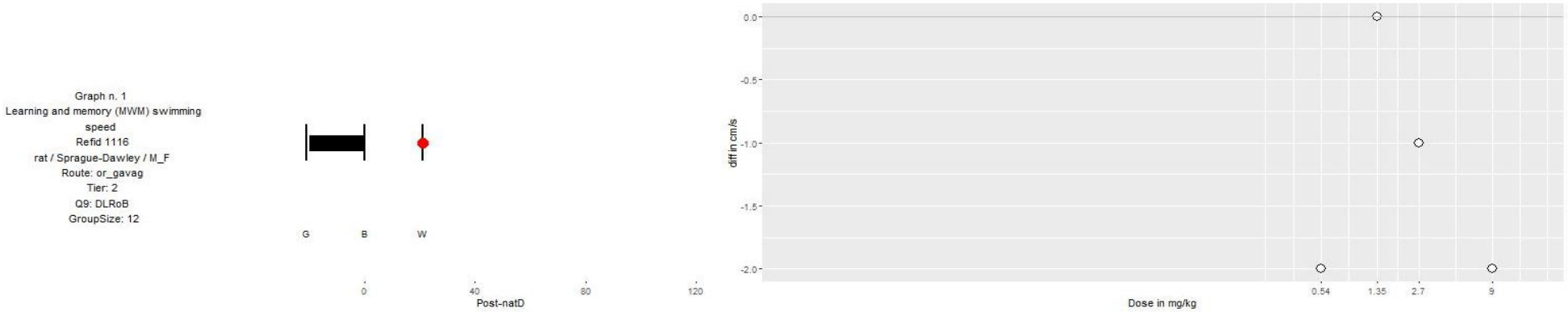


26. AO In vivo. Impairment of behavioural function. MWM. Learning and memory – (escape latency)

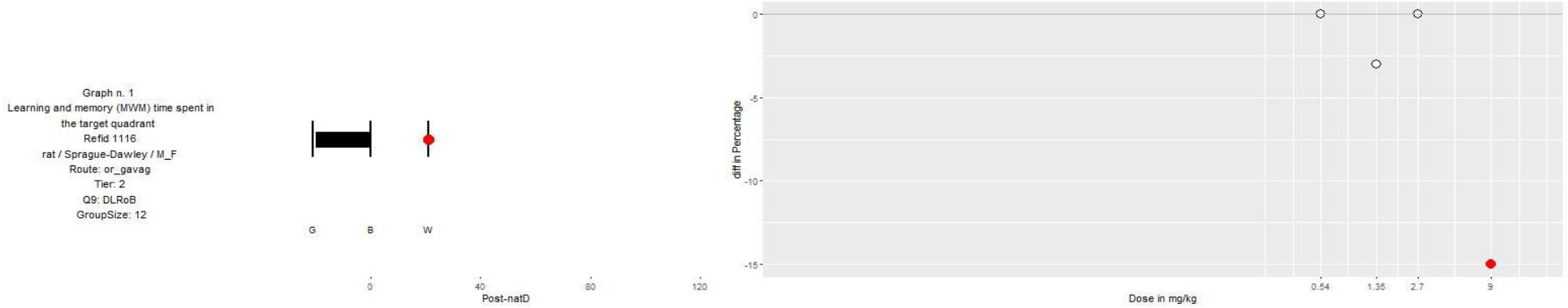
27. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – (swimming distance)



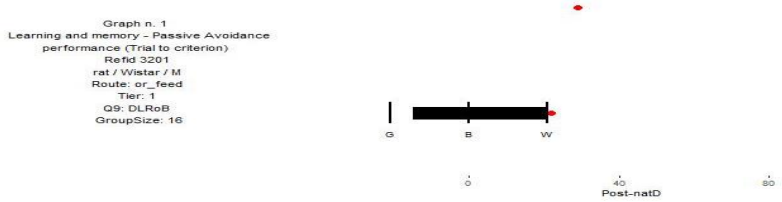
28. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – (swimming speed)

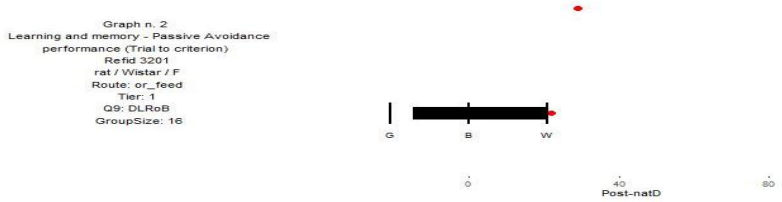


29. AO In vivo. Impairment of behavioural function. M-WM. Learning and memory – (time spent in the target quadrant)

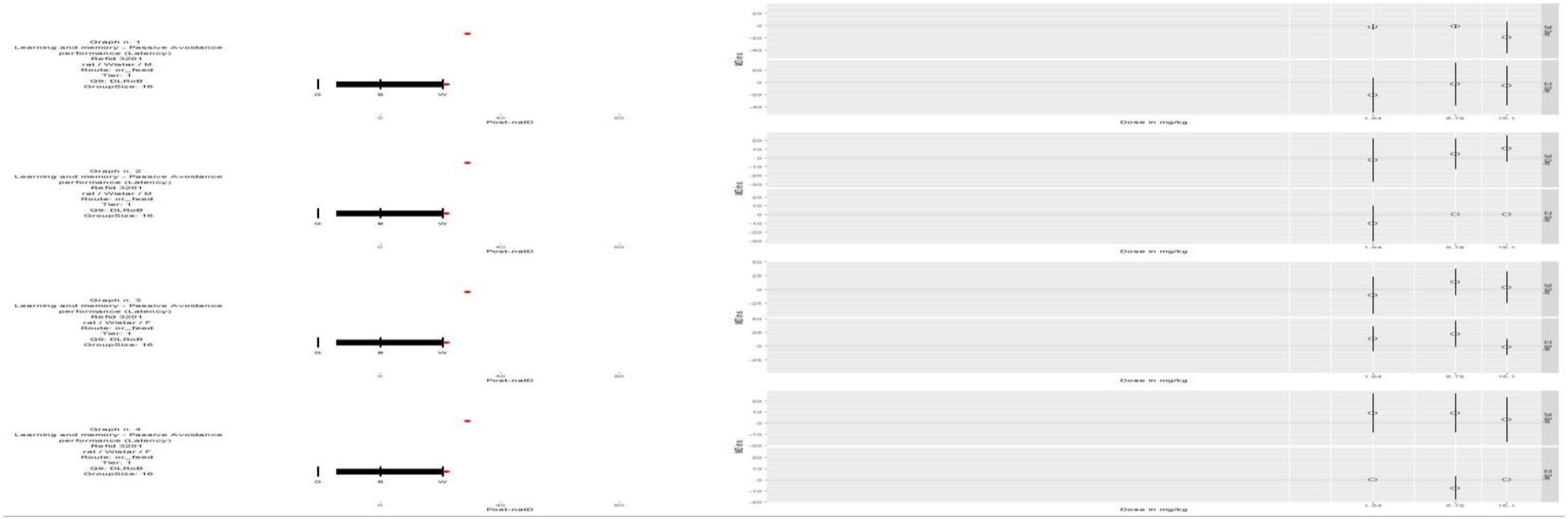


30. AO In vivo. Impairment of behavioural function. Passive avoidance. Learning and memory – Passive avoidance performance (trial to criterium) learning phase. AND 31. AO In vivo. Impairment of behavioural function. Passive avoidance. Learning and memory – Passive avoidance performance (trial to criterium) retention phase

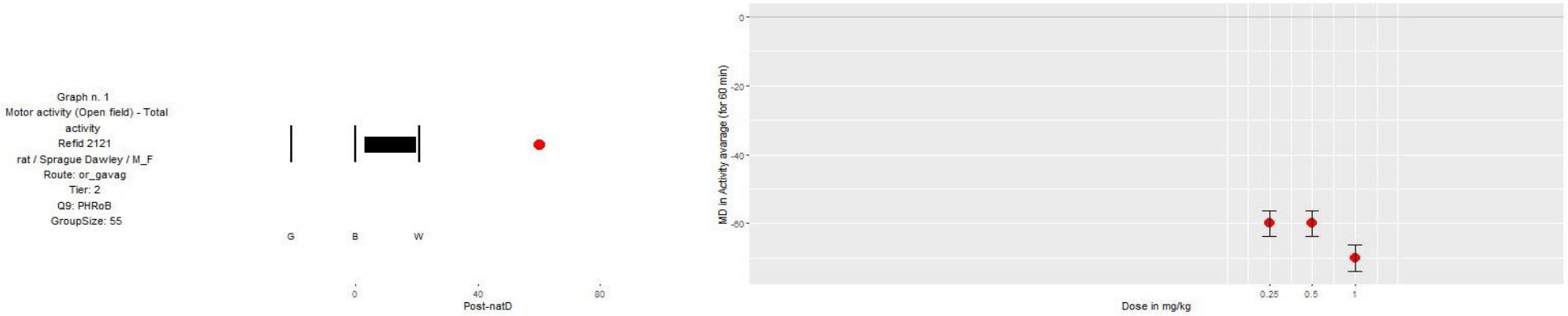




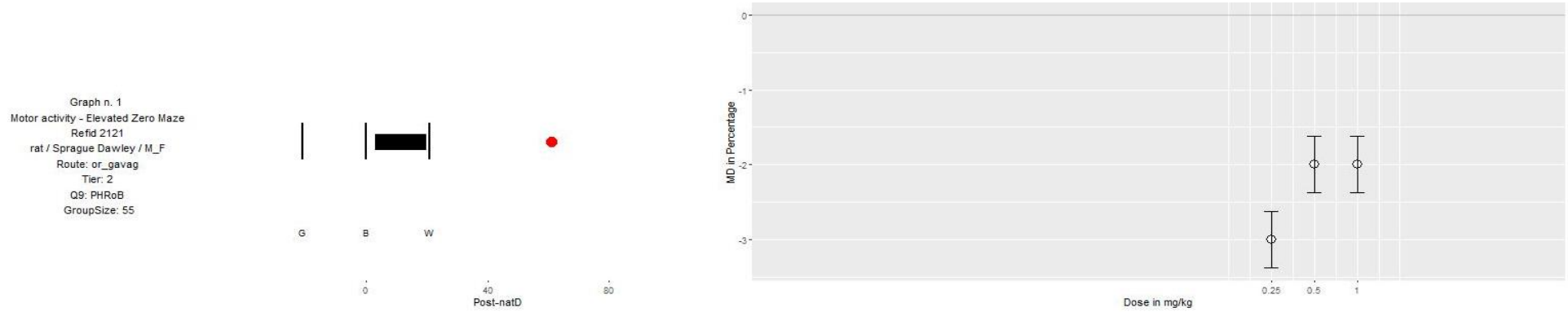
32. AO In vivo. Impairment of behavioural function. Passive avoidance. Learning and memory – Passive avoidance (Latency) learning phase AND 33. AO In vivo. Impairment of behavioural function. Passive avoidance. Learning and memory – Passive avoidance (Latency) retention phase



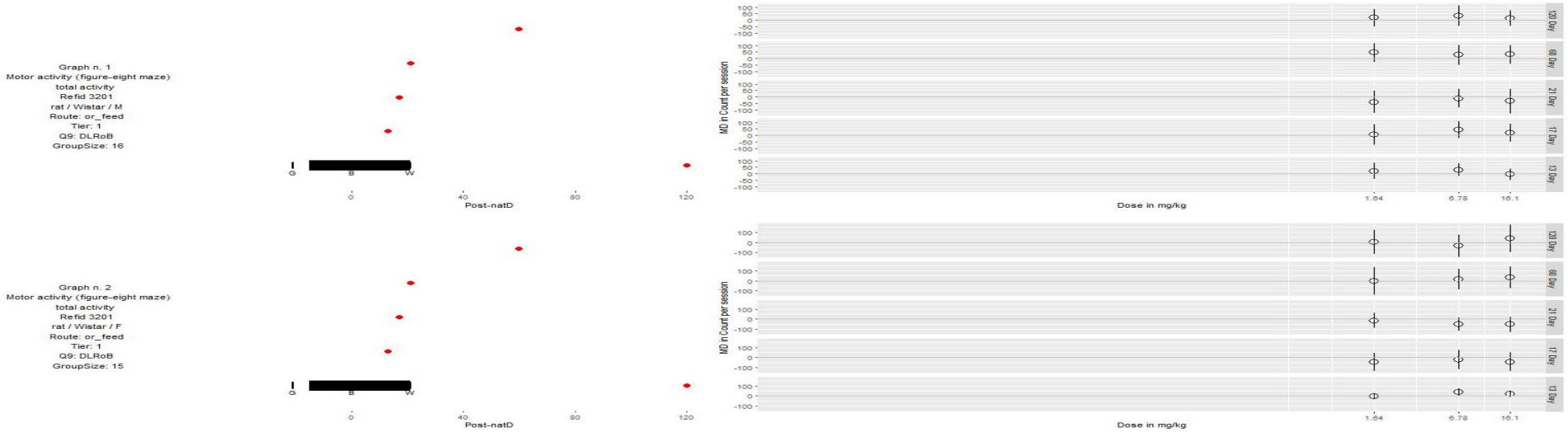
34. AO In vivo. Impairment of behavioural function. Motor activity – open field (total activity)



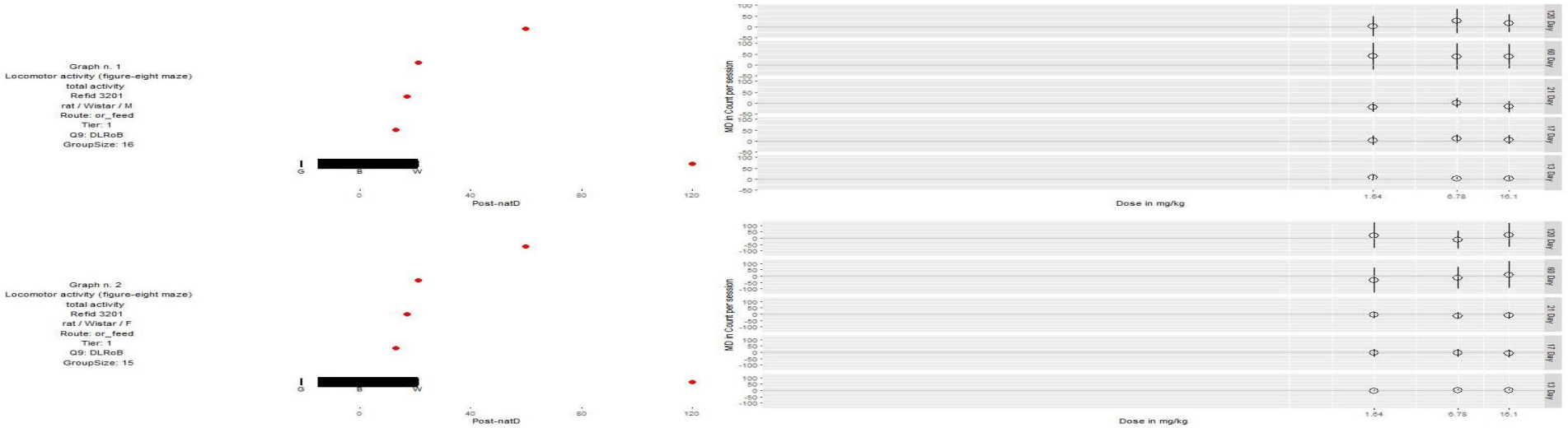
35. AO In vivo. Impairment of behavioural function. Motor activity – Elevated Zero Maze (% spent in the open arm)



36. AO In vivo. Impairment of behavioural function. Motor activity (figure eight maze) total activity



37. AO In vivo. Impairment of behavioural function. Motor activity – Locomotor activity (figure eight maze) total activity

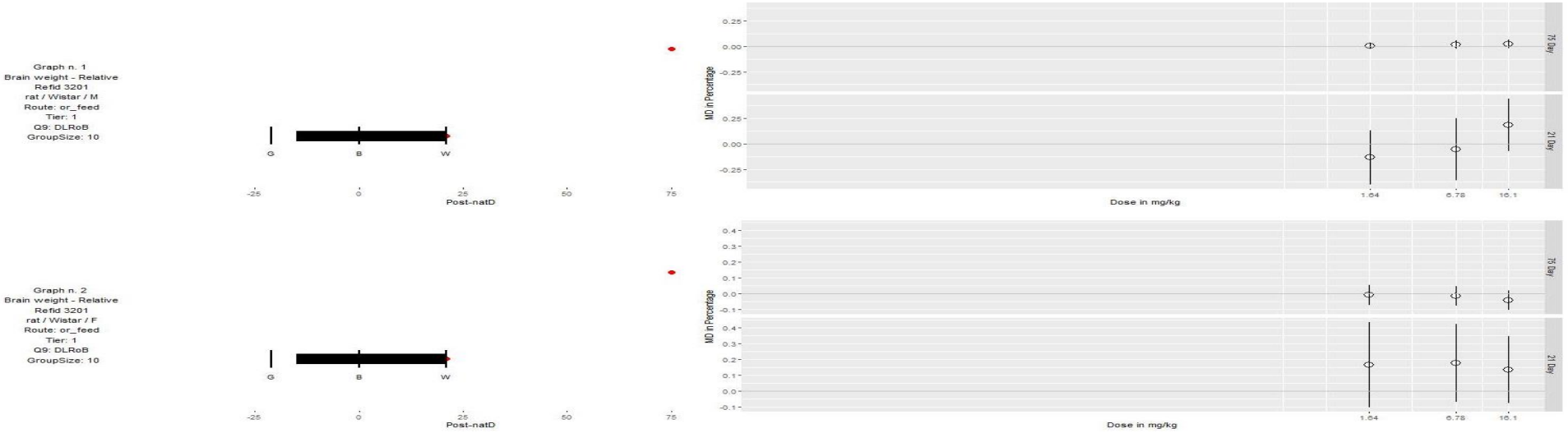


In vivo – AO Neuropathology

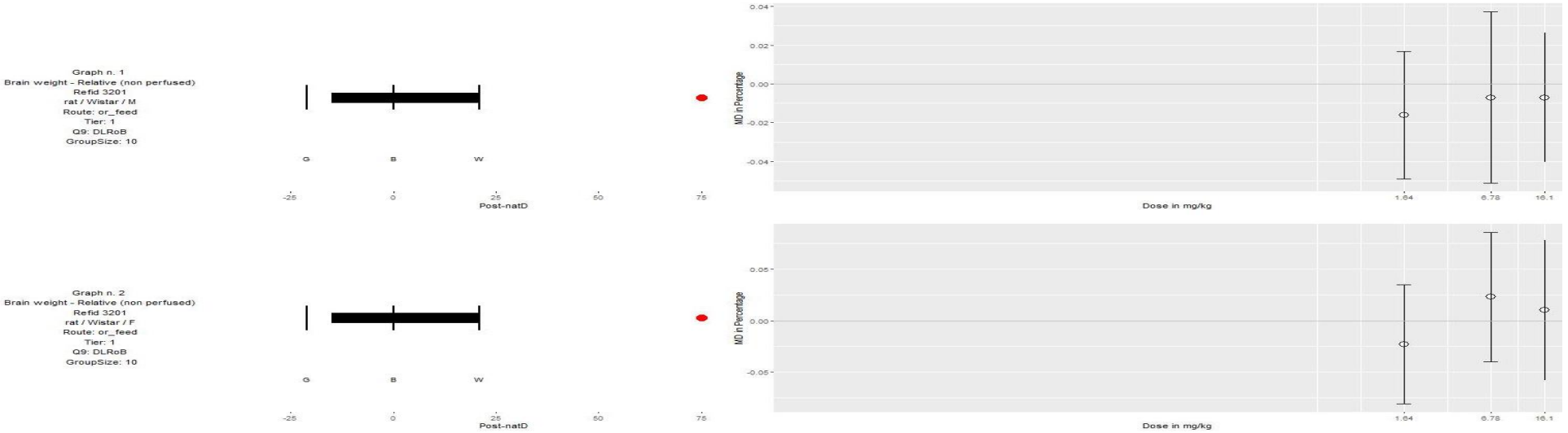
1. AO In vivo. Neuropathology. Brain weight absolute perfused

2. AO In vivo. Neuropathology. Brain weight absolute non-perfused

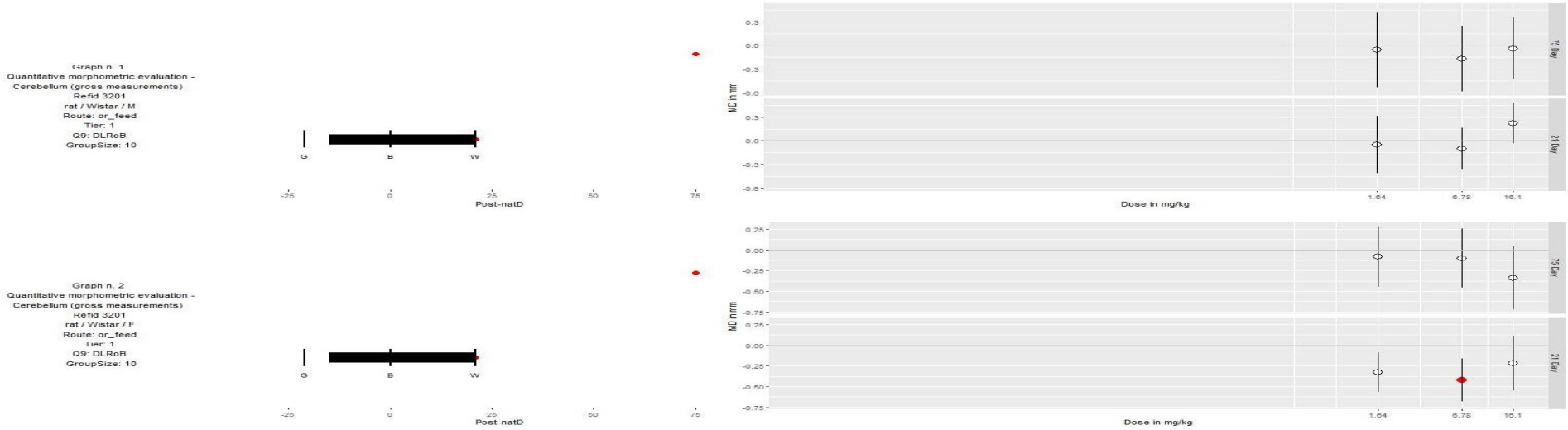
3. AO In vivo. Neuropathology. Brain weight relative perfused



4. AO In vivo. Neuropathology. Brain weight relative non-perfused



5. AO In vivo. Neuropathology. Quantitative morphometric evaluation – cerebellum (gross measurement)



6. AO In vivo. Neuropathology Quantitative morphometric evaluation – cerebellum

7. AO In vivo. Neuropathology Quantitative morphometric cerebrum length (gross measurement)

35. AO In vivo. Neuropathology Quantitative morphometric evaluation – frontal cortex, thignex

9. AO In vivo. Neuropathology Quantitative morphometric evaluation – Parietal Cortex

10. AO In vivo. Neuropathology Quantitative morphometric evaluation – Caudate Putamen

11. AO In vivo. Neuropathology Quantitative morphometric evaluation – Hippocampal Gyrus

Appendix B4.2 Human evidence table

Please refer to the separate publication for full Appendix B4.2.

ENV/CBC/MONO(2022)24/ANN3

Appendix B5.1. Uncertainty analysis tables for deltamethrin

Please refer to the separate publication for full Appendix B5.1.

ENV/CBC/MONO(2022)24/ANN4

Appendix C. Deltamethrin: Development of a putative Adverse Outcome Pathway (AOP)

1. Proposal for application of the Adverse Outcome Pathway conceptual framework

This network of putative AOPs has been developed in the context of the EFSA mandate. It has been derived for use in an IATA for the potential developmental neurotoxicity of deltamethrin. Therefore, the AOP was based on all available data on the DNT hazard of the stressor deltamethrin. For that, an evidence-based approach, through a systematic review was conducted and the data of the DNT in-vitro battery (IVB) included in a second step in the IATA iterative framework. The KERs has been constructed for each adjacent (continuous line in the graphic representation) and not adjacent (dash line in the graphic representation) upstream-downstream pair of KEs and evaluated using a quantitative probabilistic approach for its biological plausibility, empirical support, and quantitative understanding.

In order to develop a biological plausible, dose- and time-concordant AOP for the evidence on DLM causing DNT, all the available data in the scientific literature was first retrieved and appraised for risk of bias using a systematic review process (see Appendix A: Protocol of the IATA). All evidence was then considered in the UA and classified in a theoretical AOP conceptual framework. Evidence was classified as informing molecular initiating events (MIEs) from in vitro studies, key events (KEs) from mechanistic in vivo and in vitro studies, and adverse outcome (AO) from in vivo studies. This evidence was mapped into the AOP based on the biological plausibility and available knowledge for this class of chemicals.

After conducting the UA, only the endpoints assessed by the experts as being affected by deltamethrin with probabilities higher than 66% were mapped in the AOP (Figure C.1). The UA also aimed at estimating the lowest concentrations and doses, expressed as ranges or full probability distributions, triggering the causal relationship. A summary of the uncertainty assessments leading to the inclusion of the endpoints as KE into the AOP is provided in the Appendix B5.1. Uncertainty analysis tables for deltamethrin as supplementary material. For this stressor-based, putative AOP network, only MIEs and KEs were included if there was empirical data on DLT and after that the uncertainty analysis considered the events to be probably altered following deltamethrin exposure. Therefore, no potential biologically relevant additional intermediate theoretical KEs were postulated or included.

The Relationships between Key Events (KERs, also including the MIE and AO) were postulated based on: (1) expert knowledge for the biological plausibility; and (2) classification of the evidence as molecular, cellular, organ and organism responses (Figure C.2). These KERs were therefore assessed in terms of:(1) Biological Plausibility; (2) Empirical support (concentration/dose and temporal concordance); and (3) essentiality of the KE in the putative AOP. This was done according to the OECD AOP handbook for developing AOP (OECD, 2017). The KERs primarily use evidence from deltamethrin, however, in a second step of the analysis, evidence from other pyrethroids or drugs targeting the MIE

or evidence from human diseases or any other biology that is supportive, were used for the description of the KEs, support consistency of the data and to assess the overall AOP.

This document is intended to suggest a draft putative, stressor-based AOP network. Currently this putative network AOP is considered incomplete and additional biological plausible KEs can be considered. It is therefore recommended, following acceptance of a proposal to the OECD EAGMST, to complete this AOP according to the OECD guidelines and handbook, with the help of additional experts in the appropriate fields (e.g.,VGSCs, electrophysiology, oligodendrocyte development, microglia function in neuronal network formation).

The detailed assessment of the KERs and overall AOP network, including the quantitative assessment, is included in the Appendix B.

Figure C.1. DNT KEs assessed with a probability of higher than 66% to be affected by deltamethrin exposure in vitro or in vivo during the UA of the full body of evidence for DNT hazard of deltamethrin retrieved and appraised.

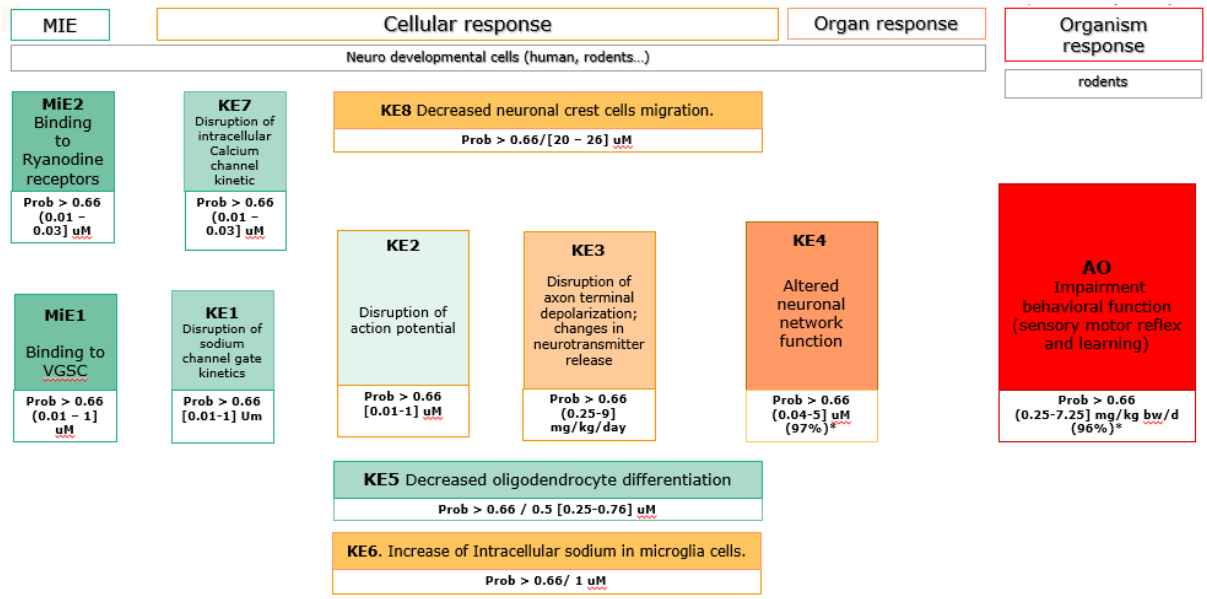
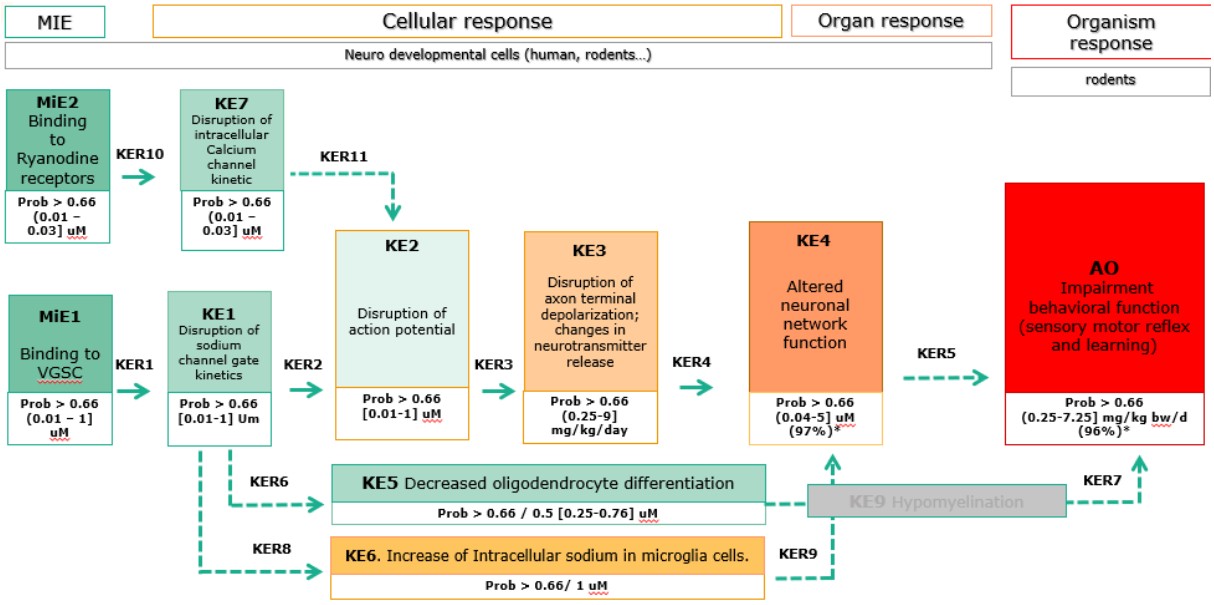


Figure C.2. The Relationships between Key Events (KERs, also including the MIE and AO) proposed when including the KE in Figure C.1 and built based on

(1) classification of the evidence as molecular, cellular, organ and organism responses and

(2) scientific knowledge for inferring the probable change in, or state of, a downstream KE from the known or measured state of an upstream KE.



2. KE descriptions (Probability >66%)

2.1. MIE1. Binding to Voltage Gate Sodium Channel

2.1.1. Short Title

Binding to Voltage Gate Sodium Channel

Molecular

2.1.3. Key Components and Biological Context

Biological Process: Voltage gate signalling; Objecte: Voltage gate sodium channel (VGSC); Action: decrease

2.1.4. MIE Description

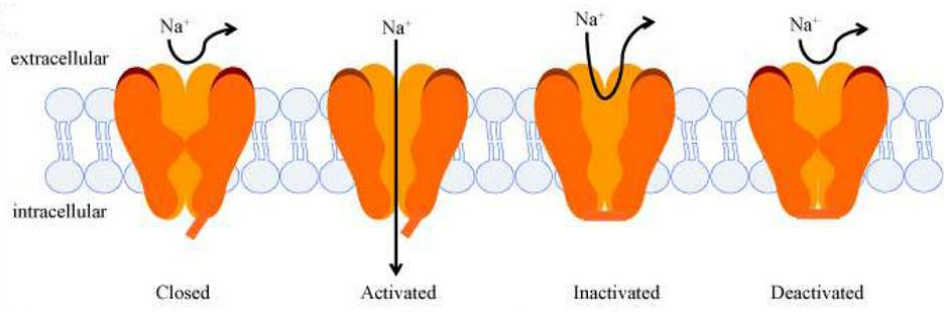
Ion channels are integral membrane proteins that are critical for neuronal function. They form pores in the plasma membrane that allow certain ions to travel with their concentration gradient across the membrane. Those that open in response to a change in membrane voltage potential are called voltage-gated ion channels. Channels that open in response to binding by a chemical signal or molecule are ligand-gated ion channels. In neurons, ion channels are essential for chemical communication between cells, or synaptic transmission. Ion channels also function to maintain membrane potential and initiate and propagate electrical impulses. Voltage-gated sodium channels are therefore responsible for action potential initiation and propagation in excitable cells, including nerve, muscle, and neuroendocrine cell

types. They are also expressed at low levels in non-excitable cells. Important to note is that functional voltage gate sodium channels (VGSC) are present in both gray and white matter in the brain and approximately 50% of white matter oligodendrocyte precursor cells producing trains of action potentials and receiving synaptic input (Fields, 2008). Voltage gated sodium channels (VGSC) are also present on microglia cells and contribute to release of major pro-inflammatory cytokines. (Hossain et al. 2016).

Mammalian VGSC are composed of one α and two β subunits. Ten separate α subunits (Ogata and Ohishi 2002) and four different β subunits (Isom 2002) have been identified and are expressed in a tissue, region and time specific manner. The diverse functional roles of VGSCs depend on the numerous potential combinations of α and β subunits (Ogata and Ohishi 2002). The type of VGSCs expressed in different cell types and regions, and their sensitivity and their functional role, may all contribute to the manifestation of toxicity and age dependent sensitivity, including the effects caused by pyrethroids.

Figure C.3. Four states of the VGSC.

At resting membrane potentials the channel is closed. During the rising phase of an action potential the channel activates or opens. Channel inactivation contributes to the falling phase. During the undershoot phase the channel deactivates prior to returning to the closed phase once resting membrane resting potential has been restored. Source: adapted from Motifolio Biomedical PowerPoint Toolkit Suite.



2.1.5. How it is measured or detected

The sodium channel protein has been discovered and characterized in biochemical and molecular detail, even to atomic resolution. The initial works performed to measure and detect the electrical signals in nerves were initiated by Hodgkin & Huxley in 1952, showing a voltage-dependent activation of sodium current that carries Na+ inward. The structure of voltage gate sodium channels is now days known in details and a number of seminal papers are available (Catterall, 2012).

Intracellular microelectrode recording using voltage or patch clamp are the common methods used for electrophysiological studies of VGSC. Channels and locations can also be measured by immunohistochemical methods, transcriptomics, and at protein levels.

Expression of different sodium channel isoforms can be measured using a panel of sodium channel subunit-specific antibodies. Quantification of immunocytochemical staining is difficult due to differences in equipment, tissue preparation, inter-assay variability and analysis methods. However, using a quantitative approach, it is possible to determine the localization and relative levels of sodium channel subunit protein expression (Westenbroek et al. 2013). PCR amplification and competitive PCR approach, real-time PCR, are used to isolate the mRNA levels of VGSC isoforms (Haufe et al. 2005)

2.1.6. Biological domain of applicability

2.1.6.1 Taxonomic Applicability

Vertebrates and Invertebrates.

2.1.6.2. Life stage applicability

All life stages. This AOP specifically refers to the developmental period in mammals.

2.1.6.3. Sex Applicability

Mixed

2.1.6.4. Evidence for Biological Domain of Applicability

Every cell within living organisms actively maintains an intracellular Na+ concentration that is 10–12 times lower than the extracellular concentration. The cells then utilize this transmembrane Na+ concentration gradient as a driving force to produce electrical signals, sometimes in the form of action potentials. The protein family comprising voltage-gated sodium channels (NaVs) is essential for such signaling and enables cells to change their status in a regenerative manner and to rapidly communicate with one another. VGSC were first predicted in squid and were later identified through molecular biology in the electric eel. Since then, these proteins have been discovered in organisms ranging from bacteria to humans (Chaihne 2012).

Sodium channels consist of a highly processed α subunit, which is approximately 260 kDa, associated with auxiliary β subunits of 33-39 kDa. Sodium channels in the adult central nervous system (CNS) and heart contain a mixture of β1 - β4 subunits, while sodium channels in adult skeletal muscle have only the β1 subunit. Nine different sodium channels have been identified by electrophysiological recording, biochemical purification, and cloning (Catterall, 2012).

Nomenclature of the different sodium channels utilises a numerical system to define subfamilies and sub-types based on similarities between the amino acid sequences of the channels. In this nomenclature system, the name of an individual channel consists of the chemical symbol of the principal permeating ion (Na) with the principal physiological regulator (voltage) indicated as a subscript (NaV). The number following the subscript indicates the gene subfamily (currently only NaV1), and the number following the full point identifies the specific channel isoform (e.g. NaV1.1). This last number has been assigned according to the approximate order in which each gene was identified. Splice variants of each family member are identified by lower-case letters following the numbers (e.g. NaV1.1a). Nine mammalian sodium channel isoforms have been identified and functionally expressed with all greater than 50% identical in amino acid sequence in the transmembrane and extracellular domains, where the amino acid sequence is similar enough for clear alignment (Catterall, 2012). In addition to these nine sodium channels that have been functionally expressed, closely related sodium channel-like proteins (Nax) have been cloned from mouse, rat, and human. They are approximately 50% identical to the NaV1 subfamily of channels but more than 80% identical to each other (Catterall, 2012).

In mammals, neuronal VGSC are expressed in the adult and developing brain. Evidence from mutation and knockout animal models demonstrates that perturbation of VGSC function during development impair nervous system structure and function, including muscle function, pain reception and cardia arrythmias (Chahine, 2018). VGSC show complex regional and temporal ontogeny in mammals (see Table C.1, from Shafer et al. 2005). In general, embryonically expressed forms of VGSCs are replaced by expression of adult forms as neurodevelopment proceeds.

Table C.1. Sodium channel αsubunit nomenclature and effects of pyrethroids.[a]

Table 1. Sodium channel α subunit nomenclature and effects of pyrethroids.[a]

α subunit	Older names	TTX sensitivity	Tissue expression	Developmental expression	Effect of pyrethroids
$Na_v1.1$	Rat I, HBSCI, GPBI, SCN1A	TTX-S	CNS, PNS, Purkinje, HP pyramidal cells, spinal motor neurons, somatic localization	Not detected in HP during development, detectable in CB Purkinje cells at PND15, detected at PND2 in SC; strong expression in motor neurons[b]	Not tested to date
$Na_v1.2$	Rat II, HBSCII, HBA	TTX-S	CNS, forebrain, substantia nigra, HP mossy fibers, CB molecular layer, axonal localization	In HP, increase between GD17 and PND30; in CB granule cells on PND15 and Purkinje cells on PND2; detected at all ages in SC[b] Splice variant expressed during development[c]	Cypermethrin-induced tail currents detectable at > 30 nM in rat 1.2 (adult splice variant) co-expressed with β_1 subunits; reported insensitive to permethrin or cismethrin[d]
$Na_v1.3$	Rat III	TTX-S	CNS and DRG	HP expression at GD17, increasing at PND2, then decreasing to barely detectable at PND30. Detected at GD17 in CB neuroepithelium, decreasing thereafter, similar in SC[b]; developmentally regulated splice variant[e]	Not tested to date
$Na_v1.4$	SkM1, μ1	TTX-S	Skeletal muscle	Increases with age[f]	Only slightly modified by 10 μM deltamethrin when expressed in HEK 293t cells[g]
$Na_v1.5$	SkM2, H1	TTX-R	Uninnervated skeletal muscle, heart, brain	mRNA expressed in rat PND0 limbic structures and medulla; expressed in fetal and adult human brain[h]	Not tested to date
$Na_v1.6$	NaCh6, PN4, Scn8a, CerIII	TTX-S	CNS, DRG (all diameter neurons), node of Ranvier–peripheral nerve	Truncated form expressed from GD12 to PND7, full-length mRNA expression is slight at GD14 and increases with age[i]	Not tested to date
$Na_v1.7$	NaS, hNE-NA, PN1	TTX-S	DRG (all diameter neurons) CNS, Schwann cells	All DRG neurons at PND2, increased during development[b]	Not tested to date
$Na_v1.8$	SNS, PN3, NaNG	TTX-R	DRG (small diameter neurons)	Expression beginning at GD15 with adult levels by PND7; largely in unmyelinated C-fibers[j]	Sensitive to both cismethrin and cypermethrin at thresholds of 500 nM and 30 nM, respectively[k]
$Na_v1.9$	NaN, SNS2, PN5, NaT, SCN12A	TTX-R	DRG (small diameter neurons)	Expression beginning at GD17 with adult levels by PND7; largely in unmyelinated C-fibers[j]	Not tested to date
Na_x	$Na_v2.1$, $Na_v2.3$ Na-G, SCL11	?	Heart, uterus, skeletal muscle, astrocytes, DRG	Transient between PND2 and 15 in HP; peak expression at PND2 in CB, SC; large DRG neurons, GD17 to PND30[b]	Not tested to date

Abbreviations: CB, cerebellum; CNS, central nervous system; DRG, dorsal root ganglion; GD, gestation day; HP, hippocampus; PND, postnatal day; PNS, peripheral nervous system; SC, spinal cord; TTX, tetrodotoxin; TTX-R, TTX resistant; TTX-S, sensitive to TTX.
[a]Data in the first four columns are based on information presented by Goldin et al. (2000) and Novakovic et al. (2001). [b]Felts et al. (1997). [c]Sarao et al. (1991). [d]Smith and Soderlund (1998). [e]Gustafson et al. (1993). [f]Kallen et al. (1990). [g]Wang et al. (2001). [h]Donahue et al. (2000). [i]Plummer et al. (1997). [j]Benn et al. (2001). [k]Smith and Soderlund (2001).

This complex ontogeny of VGSCs confounds any simple linkage of VGSCs to adverse outcomes and is an uncertainty in the development of this AOP. Since brain development in both humans and rodents extends from early gestation through lactation, it is not currently possible to state which VGSC subtype, or subtypes, may be responsible for the AOs.

Ion channels, including VGSCs, are also expressed in oligodendrocytes, Schwann cells (Baker, 2002) and microglia (Hossain et al. 2017). The expression and function of VGSS in cells of the oligodendrocyte lineage follow a time and regional ontongeny. While present and active in the early stages of oligodendrocyte maturation, VGSC function decreases over developmental time and is absent in mature oligodendrocytes (Paez et al. 2009, Berret et al. 2017). Knockdown of VGSC in rat oligodendrocyte precursor cells (OPCs) leads to reduced myelination suggesting a function of VGSC for axon myelination (Berret et al. 2017).

The physiological and anatomical ontogeny of Schwann cells is well known (Jessen & Mirsky, 2005). VGSCs are present in Schwann cells including the tetrodotoxin sensitive and Nav1.7 types (Ritche, 1993; Chiu et al., 1991, Baker, 2002) less is known about their developmental profile.

Microglia cells express several ion channels, including Cl-, K+, H+ and Ca 2+ and voltage gated sodium channels (VGSC) that are involved in several cellular functions such as maintaining the membrane potential, cellular volume and intracellular ion concentrations. VGSCs are demonstrated, to be present both in rodents and human microglia. Different isoforms are present in primary microglia (Nav 1.1. 1.2, 1.3, 1.5, 1.6, 1.7, 1.8, 1.9, and 2.1 isoforms) as compared to immortalized BV2 cells (Nav 1.2, 1.3, 1.4, 1.6, 1.8, 1.9, and 2.1 isoforms) (Jung et al, 2013; Black and Waxmann, 2012; reviewed by Hossain et al. 2017). Presence of sodium channel isoforms in immortalized BV2 cells and primary microglia were

detected by mRNA expression with standard PCR. BV2 cells express a number of sodium channel isoforms including Nav1.2, 1.3, 1.4, 1.6, 1.8, 1.9, and 2.1 whereas primary microglia from 1-2 days old mice express channel isoforms Nav 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7 1.8, 1.9, and 2.1. Primary microglia expressed higher levels of Nav 1.1. 1.2, 1.3, 1.6, 1.9, and 2.1 as compared with BV2 cells.

2.1.6.5. Evidence for Perturbation of MIE by Stressor

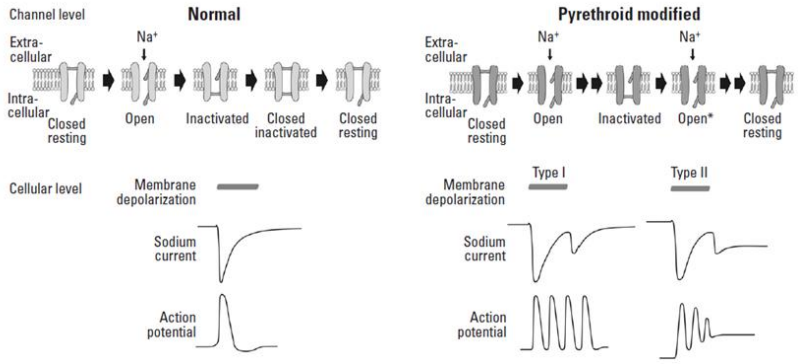
Due to their importance in neurons, sodium channels are known molecular targets of neurotoxins and neurotoxicants (Wakeling et al.2012). There is strong evidence implicating the voltage-sensitive sodium channel as the principal site of insecticidal action of pyrethroids, which has led to extensive studies of the action of pyrethroids on mammalian sodium channels. Binding studies using radioactive pyrethroid demonstrated specific binding of the pyrethroid to rat brain VGSC α subunits (Trainer et al. 1997).

Stressors

Natural toxins, produced by animal, plant, and microorganisms, target VGSCs through diverse strategies developed over millions of years of evolutions. The sodium transients can be antagonized by TTX (tetradotoxin) (Káradóttir et al 2008; Berrett et al. 2017) which is the classic stressor. A classic and well-studied stressor for VGSCs is pyrethroid insecticides. Indeed, it is well known and accepted that pyrethroids bind to the α subunit of the neuronal VGSC (Trainer et al 1997, Smith and Soderlund 1998 and Smith and Soderlund 2001, Catterall et al.2007; Cao and Shafer 2010). Mutations in the α subunit of both insects (Lee and Soderlund 2001, Smith et al. 1997) and mammals (Vais et al. 2000, 2001, Wang et al. 2001) alter the sensitivity of VGSCs to pyrethroids, supporting the conclusion that pyrethroid interact with the α subunit (Shafer et al. 2005). The β subunit has been observed to modulate the affinity of pyrethroid interaction with the channel (Smith and Soderlund 1998). However, the pyrethroid sensitivity of VGSCs subunits and splice variants expressed during development has yet to be examined (Shafer et al.2005). The actions of pyrethroid insecticides on sodium channels in invertebrate and vertebrate nerve preparation have been widely documented over the past decades and has been extensively and critically summarized in numerous reviews (Soderlund et al. 2002; Chahine, 2018).Based on their chemical structure and clinical symptoms of toxicity, pyrethroids are classified in Type I and Type II. Following the binding to a VGSC specific isoform/s, pyrethroid slow the activation, or opening, of VGSC. In addition, they slow the rate of VGSC inactivation (or closing) and shift to a more hyperpolarized potentials the membrane potentials at which VGSC activate (or open) (Narahashi, 1996). The result is that sodium channels open at more hyperpolarized potential and are held open longer, allowing more sodium ions to cross and depolarize the neuronal membrane. Type II pyrethroids delay the inactivation of VGSCs longer than do Type I pyrethroids, leading to a depolarization dependent block. These differences in prolongation of channel open times are considered to contribute to the different toxicological profile (Ray 2001). See Figure C.4 below from Shafer et al. 2005

Figure C.4. Pyrethroid effect son neuronal excitability.

The figure summarises the pyrethroid effects on individual channels, whole-cell sodium currents, and action potentials. Pyrethroid inhibit the function of two different "gates" that control sodium flux through VGSC, delaying inactivation (indicated by double arrow between states) of the channel and allowing continued sodium flux. Pyrethroid-mediated VGSC remain open when depolarization ends, resulting in a "tail" current. Type I pyrethroids action results in a series of action potentials, while type II pyrethroids cause greater membarane depolarization, leading to a depolarization-dependent block. Source: Shafer et al. 2005.



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2.2. MIE2. Binding to Ryanodine receptors

2.2.1. Short Title

Ryanodine binding

2.2.2. Level of Biological Organization

Molecular

2.2.3. KE Components and Biological Context

Process: release of Ca2+ from intracellular stores in the sarcoplasmic/endoplasmic reticulum.

Object: binding

Action: decrease

Biological Context: Molecular

2.2.4. MIE2 Description

Ryanodine receptors (RYRs) are a family of intracellular calcium channels located in the sarcoplasmic/endoplasmic reticulum responsible for the release of Ca2+ from intracellular stores during excitation-contraction coupling in both cardiac and skeletal muscle. RyRs are the largest known ion channels (homotetramers with a total molecular mass of about 2 MDa) with three known mammalian isoforms: RyR1, RyR2, and RyR3 which are classified as “skeketal muscle”, “heart” and “brain” types, respectively with respect to their major tissue distribution although all isoforms can be found in the brain (for a review on expression and molecular details see Lanner et al., 2010). RyR1 is expressed at low levels in cerebellum and Purkinje cells. Mutations of RyR1 are associated with skeletal muscle diseases (De Crescenzo et al., 2002). RyR2 is predominantly expressed in Purkinje cells of cerebellum and cerebral cortex, and in dentate gyrus of the hippocampus, and mutations are associated with cardiac

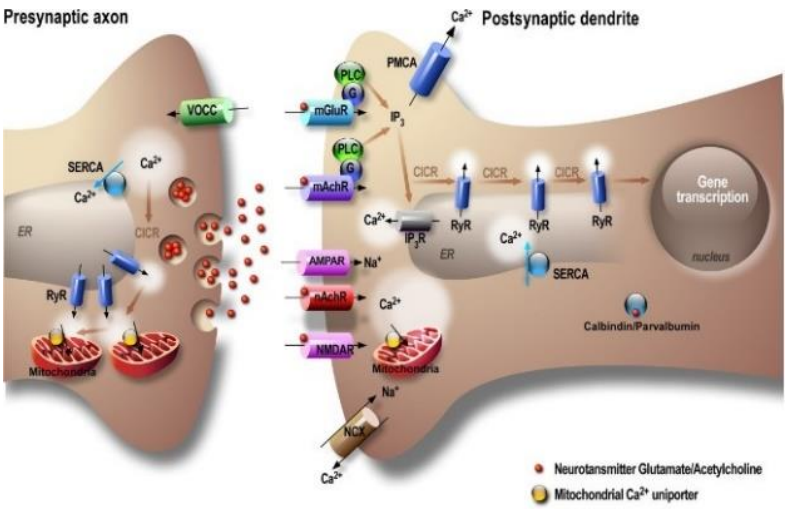
arythmias (Benkusky et al., 2004). RyR3 has been detected in hippocampal CA1 pyramidal cell layer, the basal ganglia, and olfactory bulbs (Lanner et al., 2010; Giannini et al., 1995; Del Prete et al., 2014).

All are homotetrameric proteins that are regulated by phosphorylation and interact with, redox modifications, and a variety of small proteins and ions. Each subunit of the receptor has a molecular mass of about 565 kDa, with the 4/5 of the channel comprising an huge N-terminal cytoplasmic domain that serves as a scaffold for channel regulators while the remaining domain is in the ER (endoplasmic reticulum) lumen (Zalk et al., 2007).

Intracellular Ca 2+ signalling in many tissues depends on Ca 2+ ions cycling between the bulk of the cytoplasm and specialised intracellular Ca 2+ stores in endoplasmic reticulum (ER). Ca 2+ is released from the stores through two classes of ion channel: the inositol 1,4,5-trisphosphate receptor (IP3R) and the ryanodine receptor (Bers., 2013). The RyR opens in response to an action potential during excitation-contraction (EC) coupling. The action potential travels along the fibre surface and throughout the muscle fibre along transverse tubule extensions of the surface membrane, which forms multiple junctions with the SR membrane (Franzini-Armstrong., 1972).

Figure C.5. Neuronal Ca2+signaling. Cytosolic [Ca2+]i rises are the result of an influx across the plasma membrane via voltage-gated Ca2+channels (VGCCs), ionotropic glutamate receptors (N-Methyl-D-Aspartic acid receptors, NMDARs; and alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate, AMPARs), and the release from the ER through the inositol 1,4,5-trisphosphate (IP3R) and the ryanodine (RyR) receptors.

Intraneuronal Ca2+ compartmentalization, is also maintained by the activity of Ca2+-binding buffering proteins (e.g., calbindin and parvalbumin), and regulated within signaling microdomains which involve, ATP-dependent Ca2+ pumps SERCA (Sarco-Endoplasmic Reticulum Ca2+ ATPase) accumulating Ca2+ from the cytosol to ER, and the sodium-Ca2+ exchanger (Na+/Ca2+), which act together with PMCA (Plasma Membrane Ca2+ ATPase) to restore [Ca2+]i back to resting levels by extruding Ca2+ from the cytosol to the extracellular space. Although much of the Ca2+ entry into neuron is predominantly mediated by plasma membrane channels, IP3R- and/or RyR-mediated Ca2+ release can be subsequently recruited via the phenomenon of Ca2+-induced Ca2+ release (CICR), a regenerative process in which Ca2+ enhances its own release from IP3R and RyR. Source: Del Prete et al., 2014.



2.2.5. How is measured or detected

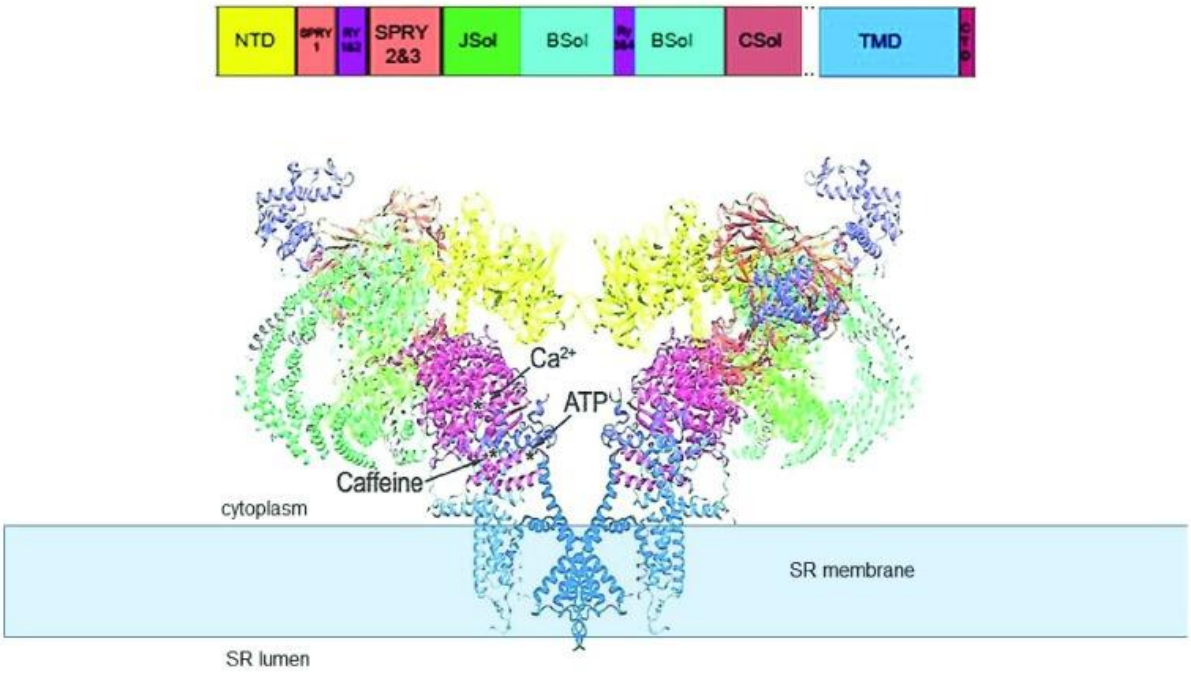
Ryanodine can be measured by [^{3}H] ryanodine binding analysis in the tissues (Brooks andStorey1992). In Zheng et al., 2019 equilibrium binding analyses of Ryanodine in skeletal muscle, cardiac muscle and brain was recently measured using homogenates (0:5 mg=mL) at 37°C for 3h in a binding buffer and [^{3}H] ryanodine with specific activity of 56.6 Ci/mmol.

RyR has been studied by transcriptomics and protein expression in nervous system, skeletal muscle and myocardium (Takeshima et al. 1989; Zorzato et al. 1990). RyR1 is the most thoroughly examined isoform because of its high expression levels and ease of purification from skeletal muscle. In humans, the gene encoding RyR1 is located on chromosome 19q13.2 and spans 104 exons. The gene encoding RyR2 is located on chromosome 1q43 and spans 102 exons, whereas the RyR3 gene with 103 exons is on chromosome 15q13.3-14. RyR1, 2, and 3 are located in chromosomes 7A3, 13A2, and 2E4 in mice (Mattei et al. 1994). In nonmammalian vertebrates RyRα and RyRβ are highly homologous to the three mammalian isoforms (Oyamada et al. 1994; Ottini et al. 1996). RyRs have been identified in *Drosophila (D) melanigaster*, *Caenorhabditis*

The first crystal structures of RyRs are from the amino-terminal domain. The first 210 amino acids of the RyR1 structure (rabbit) was at 2.5 Å resolution (PDB ID code 3HSM) (Amador 2009). Most cryo-EM studies on RyRs, (Radermacher et al. 1992; Radermacher et al. 1994; Serysheva et al. 1995; Orlova et al. 1996; Sharma et al. 1998; Serysheva et al. 1999; Benacquista et al. 2000; Sharma et al. 2000; Ludtke et al. 2005; Samsó et al. 2005; Serysheva et al. 2005; Serysheva et al. 2008; Samsó et al. 2009) and all the subnanometer resolution analysis (Serysheva et al. 2008; Samsó et al. 2009) have focused on the RyR1, however, some progress has been made with RyR2 (Sharma et al. 1998; Liu et al. 2002) and RyR3 (Sharma et al. 2000; Liu et al. 2001). Overall, the structures of all three isoforms are similar, consistent with the high sequence homology (~65%). Over the past years high-resolution cryo-electron microscopy has helped in the structural interpretation of RyR physiology and the binding sites to Ca2, ATP and caffeine (see Figure C.6 from Dulhunty., 2018)

Figure C.6. Open RyR1 channel structure.

The structure (PDB code 5TAL) reveals the major domains of the protein and the location of Ca 2+, ATP, and caffeine binding sites identified by 13. Adapted from 17; the structural domain nomenclature is as given in 10. The transmembrane domain (TMD) containing the permeation pathway is shown embedded in the sarcoplasmic reticulum (SR) membrane, which is depicted as a solid pale blue rectangle. NTD, N-terminal domain; RyR, ryanodine receptor. Source: Dulhunty et al., 2018



2.2.6. Biological domain of applicability

2.2.6.1. Taxonomic Applicability

Vertebrates and Invertebrates

2.2.6.1. Life stage applicability

All life stages. This AOP specifically refers to the mammalian developmental period.

2.2.6.1. Sex Applicability

Mixed

2.2.6.1. Evidence of Biological Domain of Applicability

RyR are expressed in the adult and developing brain. Sensitization of RyR in the developing brain has been observed to alters synaptic connectivity leading to neurobehavioral perturbations (Bal-Price et al., 2017). Some evidence from mutation and knockout models demonstrates that RyR expression levels may have a direct role in behaviour and cognitive traits (See review Del Prete et al., 2014).

Evidence for Perturbation of MIE by Stressor

RyRs are named after the plant alkaloid ryanodine, which binds to RyRs with high affinity and specificity and displays preferential interactions with the open state of the channel allowing its usage to evaluate the functional state of the channel (Imagawa et al. 1987; Inui et al. 1987; Lai et al. 1988; Chu et al.

1990). Ryanodine at nanomole concentrations locks the channel in an open subconductance state and inhibits the channel at high concentrations (>100 µM) (Meissner et al. 1986; Lai et al. 1989; McGrew et al. 1989).

Stressors

RyRs are modulated directly or indirectly by the dihydropyridine receptor (DHPR; also known as L-type Ca^{2+} channel, $Ca_V1.1/1.2$) and by various ions, small molecules and proteins, e.g., Ca^{2+}, Mg^{2+}, protein kinase A (PKA), FK506 binding proteins (FKBP12 and 12.6), calmodulin (CaM), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), calsequestrin (CSQ), triadin, junctin (Smith e al., 1986; Tanabe et al. 1990; Ikemoto et al. 1991; Sabbadini et al. 1992; Wang and Best 1992; Brillantes et al. 1994; Chen and MacLennan 1994; Yang et al. 1994; Ma et al. 1995; Mayrleitner et al. 1995; Tripathy et al. 1995; Timerman et al. 1996; Nakai et al. 1998; Moore et al. 1999b; Rodney et al. 2000)

Caffeine is a pharmacological modulator of RyRs. Caffeine induces a [Ca2+]i rise without a requirement for extracellular Ca2+, and the [Ca2+]i elevation is not associated with plasmalemmal Ca2+ movements. Caffeine-evoked [Ca2+]i elevations are sensitive to pharmacological modulators interacting with RyRs or with SERCA pumps (Usachev et al., 1993).

Initials screenings showed that the Type I pyrethroid, deltamethrin, at 5uM increased [^{3}H] ryanodine binding to membrane vesicles, suggesting its direct activation of RyRs (Morisseau et al., 2009), regulating Ca2+ dynamics and activity dependent dendritic growth (Wayman et al., 2012a, 2012b). Binding of [^{3}H] Ry to RyR1 and RyR2 was higher at DM concentrations >3 uM than with vehicle with a maximal increment of 239.5% in cortex preparations (Zheng et al., 2019).

2.2.7. References MiE2

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2.3. KE1. Disruption of sodium channel gate kinetics

2.3.1. Short Title

Disruption of sodium channel gate kinetics

2.3.2. Level of Biological Organization

Cellular

2.3.3. KE Components and Biological Context

Process: changes in the opening time of the VGSC

Object: binding

Action: decrease

Biological Context: Molecular

2.3.4. Key Event description

Action potentials are a temporary shift (from negative to positive) in the neuron's membrane potential caused by ions flowing in and out of the neuron. During the resting state, before an action potential occurs, all the gated sodium and potassium channels are closed. These gated channels only open once when action potential has been triggered. They are called "voltage-gated" because they are open and close depends on the voltage difference across the cell membrane. Voltage-gated sodium channels have two gates (gate m and gate h), while the potassium channel only has one (gate n). Gate m (the activation gate) is normally closed and opens when the cell starts to get more positive. Gate h (the deactivation gate) is normally open, and swings shut when the cells gets too positive. Gate n is normally closed, but slowly opens when the cell is depolarized (very positive). Voltage gated sodium channels exist in one of three states: Deactivated (closed)- at rest, channels are deactivated. The m gate is closed and does not let sodium ions through. Activated (open) - when a current pass through and changes the voltage difference across a membrane, the channel will activate, and the m gate will open. Inactivated (closed) - as the neuron depolarizes, the h gate swings shut and blocks sodium ions from entering the cell. Voltage-gated potassium channels are either open or closed.

Slowed VGSC activation leads to a decrease in peak Na+ current. By slowing VGSC inactivation and deactivation leads to a prolonged VGSC open time. The longer channel open time results in more Na+ entering the cell and this leads to hyperexcitability, membrane depolarization, increase in firing rate and conduction block. Prolongation of the channel opening time for a short period cause repetitive firing of action potential (repetitive discharge). However, if the channel is hold opened for a sufficient long period, the membrane potential eventually becomes depolarized to the point at which generation of action potentials is not possible (depolarization dependent block). Modification of a small percentage of VGSCs can increase Na+ current substantially (Narahashi, 1996).

2.3.5. How is measured/ Detected

The modifications of the sodium channel gating have been studies on voltage and patch clamp experiments in different models (Ruigt et al. 1987), showing that the prolongation of the sodium current is mainly due to the reduced rate of closure of a fraction of sodium channels affected by pyrethroids. In neuroblastoma cell preparation, deltamethrin and other Type II pyrethroids induced slow tail current with a relatively rapid time constant. The rate at which sodium channels close during the pyrethroid-induced slow tail current depends not only on pyrethroid structure, but also on the time of exposure, temperature and membrane potential (Ruigt et al.1987).

The voltage-clamp technique typically uses two microelectrodes, allowing control of the membrane potential and record transmembrane currents that result from ion channel opening and closing (Guan et al. 2013).

Patch-clamp is a highly sensitive version of the voltage-clamp technique in which currents flowing through a single ion channel can be measured. A single electrode serves both to measures voltage and pass current (Molleman, 2003).

2.3.6. Biological domain of applicability

2.3.6.1. Taxonomic Applicability

Vertebrates and Invertebrates

2.3.6.2. Life stage applicability

All life stages. This AOP specifically refers to the developmental period

2.3.6.3. Sex Applicability

Mixed

2.3.6.4. Evidence of Biological Domain of Applicability

Ion channels are essential for the initiation and propagation of action potential in excitable cells from both vertebrate and invertebrate species. In neurons, ion channels are essential for chemical communication between cells, or synaptic transmission. Ion channels also function to maintain membrane potential and initiate and propagate electrical impulses. VGSC are therefore a target of natural and synthetic chemicals and disruption of the gate kinetics has been characterized in insects and mammalian cells (Soderlund et al., 2002).

2.3.7. References KE1

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2.4. KE2. Disruption in action potential generation

2.4.1. Key Event description

An action potential is defined as a sudden, fast, transitory, and propagating change of the resting membrane potential. Neurons and muscle cells can generate an action potential; that property is called the excitability. The initial signal comes from other cells connecting to the neuron, and it causes positively charged ions to flow into the cell body. These ions pass through channels that open when a specific neurotransmitter binds to the channel, leading to opening. For example, when acetylcholine is

released at the synapse between a nerve and muscle (called the neuromuscular junction) by a presynaptic neuron, it causes postsynaptic Na+ channels to open. Na+ enters the postsynaptic cell and causes the postsynaptic membrane to depolarize. This depolarization is called an excitatory postsynaptic potential (EPSP) and makes the postsynaptic neuron more likely to fire an action potential. These incoming ions change the membrane potential closer to 0, a process known as depolarization. When positive ions flow into the negative cell, the cell's polarity decreases. If gets positive enough, it can trigger the voltage-gated sodium channels found in the axon, then the action potential will be sent.

This process lets positively charged sodium ions to flow into the negatively charged axon and depolarize the surrounding axon. Once one channel opens and lets positive ions in, it sets the stage for the channels down the axon to do the same thing in a domino-like process. This stage is known as depolarization, the neuron becomes positively charged as the action potential passes through. When the inactivation gates of the sodium channels close, they stop the inward rush of positive ions. At the same time, the potassium channels open. As there is much more potassium inside the cell than out, so when these channels open, more potassium exits than comes in. The cell therefore loses positively charged ions and returns back toward its resting state. This step is called repolarization. As the action potential passes through, potassium channels stay open a little bit longer, and continue to let positive ions exit the neuron. This means that the cell temporarily hyperpolarizes or gets even more negative than its resting state. As the potassium channels close, the sodium-potassium pump works to re-establish the resting state. This step is called hyperpolarization and makes the cell more negative than its typical resting membrane potential.

Therefore, sodium channel gating is a well-regulated process that is critical to normal neuronal function, activation and propagation of the action potential. Shape, speed of conduction, and fidelity in propagation of the action potential are essential to the timing, synchrony, and efficacy of neuronal communication. Waveform, timing, and fidelity of the axonal action potential can be modulated, which leads to changes in presynaptic neurotransmitter release. Action potential normally develops first in the initial segment of the axon. During axonal action potential initiation, the active depolarization propagates both towards the soma (antidromic) and down the axon (orthodromic). The conduction velocity of the antidromic action potential may have a significant impact on dendritic backpropagation. This in turn will affect spike-timing dependent plasticity i.e. the synaptic plasticity sensitive to the timing of dendritic action potentials relative to incoming synaptic information. The orthodromic velocity will affect the degree of synchrony of arrival of information at different postsynaptic targets of the same axon. In neurons, voltage-gated sodium conductances play an essential role in action potential initiation and propagation. Voltage-gated sodium channels activate and inactivate within milliseconds. As the cell membrane is depolarized, sodium channels activate, resulting in the influx of sodium ions to further depolarize the membrane. This inward current produces the upstroke of the action potential. Along with the gating of potassium channels, sodium channel inactivation participates in the action potential downstroke. Although variations in many ion channels likely participate in the diversity of action potential waveforms observed in neurons, differences in sodium channel subunit composition, localization, and modulation may participate in shaping a neuron's action potential. Sodium channel subunit composition at the axon initial segment contributes to the firing properties of neurons, particularly the characteristic maximum firing frequency of a particular cell class. Thus, at nodes of Ranvier the sodium channel subunit composition may contribute to action potential propagation fidelity. Steady-state persistent sodium currents can contribute to excitability and to the shape of an action potential. These sodium channels are active near rest (−65 mV) and do not inactivate even with quite strong depolarization. Therefore, these currents can participate in cellular excitability and in setting action potential threshold (Kress et a. 2009). Alterations in VGSCs can result in changes in membrane polarization and propagation of neuronal action potentials. Changes in neuronal excitability in glutamatergic networks are described following treatment to deltamethrin and permethrin on neuronal activity in hippocampal neuronal cultures using patch clamp and microelectrode array (MEA) recordings (Mayer et al., 2008).Cao et al. (Cao et al.2011) demonstrated that VGSC responses of a neuronal network to pyrethroids with an increase of

intracellular calcium concentration and these responses are secondary to activation of VGSCs. The effect of pyrethroids on neurotransmitters release and neuronal excitability in glutamatergic networks are described following treatment to deltamethrin and permethrin on neuronal activity in hippocampal neuronal cultures using patch clamp and microelectrode array (MEA) recordings (Mayer et al., 2008). The distinct abilities of pyrethroids to elevate BDNF mRNA expression are consistent with the demonstration of a range of pyrethroid efficacies in the stimulation of calcium influx. In vivo, deltamethrin has been reported to increase BDNF in the cortex and hippocampus (Imamura et al., 2006; Cao et al., 2011), and both deltamethrin and permethrin alter transcription profiles of activity dependent genes in the cortex including c-fos, Egr1, and Camk1g (Harrill et al., 2008; Cao et al.,2011). Thus, activity-dependent changes in gene transcription after pyrethroid exposure can occur both in vitro and in vivo (Cao et al.2011, Pitzer et al. 2019, Zhang et al. 2018).

2.4.2. How is measured or Detected

The action potential is a cycle of membrane depolarization, hyperpolarization, and return to the resting value. To measure action potential, the patch clamp or the intracellular recording (impale a sharp electrode into the cell cytosol) technique are generally used. For eithers, a glass-made microelectrode is sufficient to measure action potential. The measurement of Na+ ion concentration would not detect single action potentials but a change in bulk ion concentration over a longer time which might depend mainly on the firing rate of the cells and the activity of Na+/K+-pumps. Patch clamp is the preferred technique for the qualification and quantification of the altered firing rate (Meyer et al., 2008).

Neurotransmitter release can be evaluated in vivo by western blotting quantification or by micro dialysis and analytical quantification.

2.4.3. Biological domain of applicability

2.4.3.1. Taxonomic Applicability

Vertebrates and Invertebrates

2.4.3.2. Life stage applicability

All life stages. In this AOP specifically refers to the developmental period

2.4.3.3. Sex Applicability

Mixed

2.4.3.4. Evidence of Biological Domain of Applicability

Action potentials or nerve impulses are rapid and transient electrical activity that is propagated in the membrane of excitable such as neurons and muscle cells. Action potentials allow long-distance signalling in the nervous system.

An action potential results from the sequential opening and closing of voltage-gated cation channels. First, opening of Na+ channels permits influx of Na+ ions for about 1 ms, causing a sudden large depolarization of a segment of the membrane. The channel then closes and becomes unable to open (refractory) for several milliseconds, preventing further Na+ flow. Opening of K+ channels as the action potential reaches its peak permits efflux of K+ ions, which initially hyperpolarizes the membrane. As these channels close, the membrane returns to its resting potential. The same basic mechanism is used by all neurons. Myelination produced by oligodendrocytes increases the Velocity of Impulse Conduction

(Lodish et al., 2000, “The Action Potential and Conduction of Electric Impulses” in Molecular Cell Biology section 21.2, New York)

2.4.4 References KE2

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2.5. KE3 Disruption of axon terminal depolarization; changes in neurotransmitter release.

2.5.1. Short Title.

Release of neurotrasmitters

2.5.2. Level of Biological Organization.

Cellular

2.5.3. KE Components and Biological Context.

Process: alteration of the excitable cell process that synapses onto another cell.

Object: lack of fidelity in axonal digital signal

Action: decrease

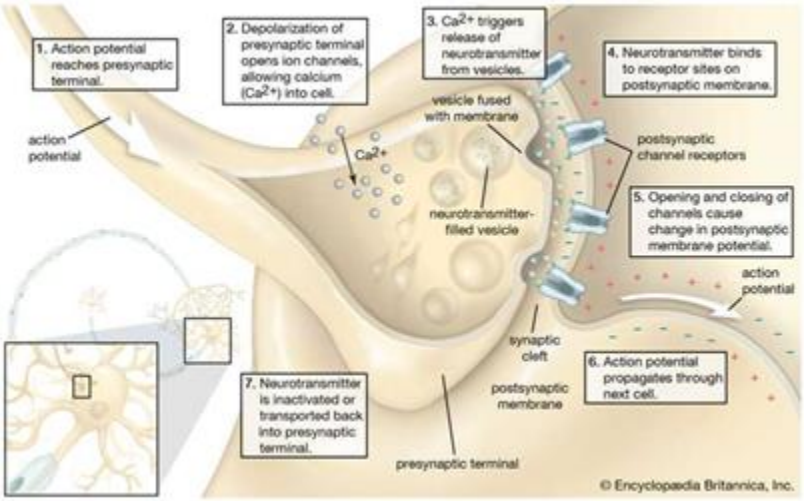
Biological Context: Cellular

2.5.4. Key Event Description:

The arrival of the nerve impulse at the presynaptic terminal stimulates the release of neurotransmitter into the synaptic gap. The binding of the neurotransmitter to receptors on the postsynaptic membrane stimulates the regeneration of the action potential in the postsynaptic neuron. Neurotransmitters are released by cells near the dendrites, often as the end result of their own action potential.

Figure C. 13. Mechanism involved in synaptic neurotransmitter release

(Source: Encyclopædia Britannica, Inc. https://www.britannica.com/science/synapse, accessed on December 2020)



When an action potential reaches the axon terminal it depolarizes the membrane and opens VGSC. Sodium ions enter the cell, further depolarizing the presynaptic membrane. This depolarization causes voltage-gated Ca2+ channels to open. Calcium ions entering the cell initiate a signalling cascade that causes small membrane-bound vesicles, called synaptic vesicles, containing neurotransmitter molecules to fuse with the presynaptic membrane. Fusion of a vesicle with the presynaptic membrane causes neurotransmitter to be released into the synaptic cleft, the extracellular space between the presynaptic and postsynaptic membranes. The neurotransmitter diffuses across the synaptic cleft and binds to receptor proteins on the postsynaptic membrane. Once neurotransmission has occurred, the neurotransmitter is removed from the synaptic cleft and the postsynaptic membrane can “reset” and be ready to receive another signal. This can be accomplished in three ways: the neurotransmitter can diffuse away from the synaptic cleft, it can be degraded by enzymes in the synaptic cleft, or it can be recycled (sometimes called reuptake) by the presynaptic neuron.

There is enough evidence to suggest that by modulating sodium channels it is possible to alter neurotransmitter release in a qualitative and quantitative manner. Neurotoxins acting on sodium channels have similar effects, at the steady state, on increase of Na influx, depolarization, Ca2 increase,

and exocytosis. Depending on the toxin binding site on sodium channels, the release of neurotransmitter can be modified qualitatively and/or quantitatively (Messensini et al. 2003).

2.5.5. How is measured

Neurotransmitter release can be evaluated in vivo by western blotting quantification or by micro dialysis and analytical quantification.

One way to estimate neurotransmitter release is to measure the postsynaptic response that it evokes. It is an indirect measure since it includes events following release, such as neurotransmitter diffusion from the pre- to the postsynaptic element, binding of neurotransmitter molecules to postsynaptic receptors and induction of the postsynaptic current. In addition, to be a reliable detector of release events the postsynaptic responses (EPSP or IPSP) should not activate postsynaptic voltage-dependent currents that would amplify or decrease them.

Neurotransmitter release can also be examined using the genetically encoded synaptic transmission reporter synapto-pHluorin, which uses pH-sensitive mutants of GFP. The interiors of synaptic vesicles are acidified to a pH of approximately 5.7, an environment that keeps the pHluorins in an off state. When the vesicle fuses with the plasma membrane during exocytosis, the pH rises to extracellular levels, switching the pHluorin on and causing it to fluoresce. One of the advantages of using synapto-pHluorins is that the signal regenerates through multiple rounds of vesicle release and recycling, which permits vesicle recycling to be imaged in addition to synaptic transmission (Carter and Shieh, 2015).

2.5.6. Biological domain of applicability

2.5.6.1. Taxonomic Applicability

Vertebrates and Invertebrates

2.5.6.2. Life stage applicability

All life stages. This AOP specifically refers to the developmental period

2.5.6.3. Sex Applicability

Mixed

2.5.6.4. Evidence of Biological Domain of Applicability

The connections between neurons, and between neurons and downstream effector cells, occur at specialized cell junctions called synapses. Synapses can occur by direct electrical coupling between two cells, but chemical synapses, in which communication is via the release of a neurotransmitter, are more common and are involved in more complex information processing. The proteins involved in chemical synaptic transmission are much more numerous and diverse than those involved in electrical conduction In vertebrates, synaptic transmission usually travels in one direction, but ctenophore and cnidarian synapses are often bidirectional.

We divide the synapse into (a) a presynaptic module, in which calcium signals are transduced into chemical secretions (known as excitation–secretion coupling); (b) a postsynaptic module (postsynaptic density), which comprises the proteins that support the specialized postsynaptic membrane and the signaling that goes on there; and (c) a module that determines the specific wiring diagram of neurons during development (axonogenesis). Despite its apparent specialization for neuronal signalling, the

excitation–secretion system in neurons comprises many ancient gene families. However, like the transduction module, these gene families are often used differently in the various animal lineages. The proteins involved in docking and in recycling are, for the most part, conserved across eukaryotes (Liebenskind et al. 2017).

Several neurotransmitters have been found not only in animals, but also in plants and microorganisms. Thus, the presence of neurotransmitter compounds has been shown in organisms lacking a nervous system and even in unicellular organisms. Today, we have evidence that neurotransmitters, which participate in synaptic neurotransmission, are multifunctional substances participating in developmental processes of microorganisms, plants, and animals (Roshchina, 2010).

The neurotransmission wiring code, which includes Excitation–Secretion Coupling, Postsynaptic Density and Axonogenesis is present across multiple taxa and is representing a fundamental brain developmental process.

2.5.7. References KE3

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2.6. KE4. Decrease of neuronal network function

2.6.1. Short Title.

Network formation

2.6.2. Level of Biological Organization.

Organ

2.6.3. KE Components and Biological Context.

Process: neural network formation, synaptogenesis, synchronous electrical signal in a neural network

Object: lack of fidelity in a neural network

Action: decrease

Biological Context: organ

2.6.4. Key Event Description

The nervous system can be considered as a network of electrically excitable neurons that underlies animal behaviour. Other cell types beside neurons are electrically excitable and exist in other systems, such as pancreatic or muscle cells or in the nervous system i.e. glial cells. Plants and unicellular organisms also make use of electrical excitability to mediate behaviour, although they are without a nervous system. It is indeed the presence of neurons that really distinguishes the animal nervous systems. To encode information, neurons are organized in a complex network of synapses connecting excitable cells (i.e. neurons and glia). The neural network system encodes information in two principal ways: by establishing an electrical code within neurons and, second, in establishing a wiring code based on neurotrasmitters. Plasticity in these signaling modes is responsible for learning, memory, development, and behavioural complexity (Liebenskind et al. 2017). The electrical network is based on three key elements; the creation of an action potential based on ion homeostasis, the sensory and intracellular transduction into an electrical signal, and the propagation of the electrical signals along neurons. Therefore, a correct maintenance and homeostasis of the electrical network is a necessary step to eventually code the downstream behavioural event (Liebenskind et al. 2017).

There are striking differences in neuronal network formation and function among the developing and mature brain. The developing brain shows a slow maturation and a transient passage from spontaneous, long-duration action potentials to synaptically-triggered, short-duration action potentials.

Furthermore, at this precise developmental stage the neuronal network is characterised by "hyperexcitability", which is related to the increased number of local circuit recurrent excitatory synapses and the lack of γ-amino-butyric acid A (GABAA)-mediated inhibitory function that appears much later. This "hyperexcitability" disappears with maturation when pairing of the pre- and postsynaptic partners occurs and synapses are formed generating population of postsynaptic potentials and population of spikes followed by developmental GABA switch. Glutamatergic neurotransmission is dominant at early stages of development and NMDA receptor-mediated synaptic currents are far more times longer than those in maturation, allowing more calcium to enter the neurons. The processes that are involved in increased calcium influx and the subsequent intracellular events seem to play a critical role in establishment of wiring of neural circuits and strengthening of synaptic connections during development (reviewed in Erecinska et al., 2004). Neurons that do not receive glutaminergic stimulation are undergoing developmental apoptosis.

During the neonatal period, the brain is subject to profound alterations in neuronal circuitry due to high levels of synaptogenesis and gliogenesis. For example, in neuroendocrine regions such as the preoptic area-anterior hypothalamus (POA-AH), the site of gonadotropin-releasing hormone (GnRH) system is developmentally regulated by glutamatergic neurons. The changes in the expression of the N-methyl-D-aspartate (NMDA) receptor subunits NR1 and NR2B system begin early in postnatal development, before the onset of puberty, thereby playing a role in establishing the appropriate environment for the subsequent maturation of GnRH neurons (Adams et al., 1999).

Neural network formation and function happens in all brain regions, but it appears to onset at different time points of development (reviewed in Erecinska et al., 2004). Glutamatergic neurotransmission in hippocampus is poorly developed at birth. Initially, NMDA receptors play important role but the vast majority of these premature glutamatergic synapses are "silent" possibly due to delayed development of hippocampal AMPA receptors. In contrast, in the cerebral cortex the maturation of excitatory glutamatergic neurotransmission happens much earlier. The "silent" synapses disappear by PND 7-8 in both brain regions mentioned above.

There is strong evidence suggesting that NMDA receptor subunit composition controls synaptogenesis and synapse stabilization (Gambrill and Barria, 2011). It is established fact that during early postnatal development in the rat hippocampus, synaptogenesis occurs in parallel with a developmental switch in

the subunit composition of NMDA receptors from NR2B to NR2A. It is suggested that early expression of NR2A in organotypic hippocampal slices reduces the number of synapses and the volume and dynamics of spines. In contrast, overexpression of NR2B does not affect the normal number and growth of synapses. However, it does increase spine motility, adding and retracting spines at a higher rate. The C terminus of NR2B, and specifically its ability to bind CaMKII, is sufficient to allow proper synapse formation and maturation. Conversely, the C terminus of NR2A was sufficient to stop the development of synapse number and spine growth. These results indicate that the ratio of synaptic NR2B over NR2A controls spine motility and synaptogenesis, and suggest a structural role for the intracellular C terminus of NR2 in recruiting the signalling and scaffolding molecules necessary for proper synaptogenesis. Interestingly, it was found that genetic deletion of NR3A accelerates glutamatergic synaptic transmission, as measured by AMPAR-mediated postsynaptic currents recorded in hippocampal CA1. Consistent, the deletion of NR3A accelerates the expression of the glutamate receptor subunits NR1, NR2A, and GluR1 sugesting that glutamatergic synapse maturation is critically dependent upon activation of NMDA-type glutamate receptors (Henson et al., 2012).

The development of neuronal networks can be distinguished into two phases: an early ‘establishment’ phase of neuronal connections, where activity-dependent and independent mechanisms could operate, and a later ‘maintenance’ phase, which appears to be controlled by neuronal activity (Yuste and Sur, 1999). These neuronal networks facilitate information flow that is necessary to produce complex behaviors, including learning and memory (Mayford et al., 2012).

The recent observations suggest that neuronal activity also regulate the targeting of myelin to specific axons (Almeida, 2018) , in addition to long-standing observations that activity also influences OPC proliferation (Li et al., 2010; Gibson et al., 2014), oligodendrocyte differentiation and survival (Hill et al., 2014; McKenzie et al., 2014; Hughes et al., 2018) and myelin formation itself (Makinodan et al., 2012; Liu et al., 2012).

2.6.5. How is measured or detected

In vivo: The recording of brain activity by using electroencephalography (EEG), electrocorticography (ECoG) and local field potentials (LFP) assists towards the collection of signals generated by multiple neuronal cell networks. Advances in computer technology have allowed quantification of the EEG and expansion of quantitative EEG (qEEG) analysis providing a sensitive tool for time-course studies of different compounds acting on neuronal networks' function (K Binienda et al., 2011).

In vitro: Microelectrode array (MEA) recordings are also used to measure electrical activity in cultured neurons (Keefer et al., 2001, Gramowski et al., 2000; Gopal, 2003; Johnstone et al., 2010). MEAs can be applied in high throughput platforms to facilitate screening of numerous chemical compounds (McConnell et al., 2012). Using selective agonists and antagonists of different classes of receptors their response can be evaluated in a quantitative manner (Novellino et al., 2011; Hogberg et al., 2011). The MEA allow examination of general network activity, bursting activity and network connectivity and using at least 16 measures it has been demonstrated to identify negative and positive control compounds, to identify concentrations at which network failure begins and to identify selective network perturbations versus nonspecific cytotoxic effects when coupled with terminal cell death assays when using different test systems from different species (human, rats, mouse; Brown et al., 2016; Brown et al., 2017; Frank et al., 2017; Masjosthusmann et al., 2020; Vassallo et al. 2017).

Patch clamping technique can also be used to measure neuronal network activity. In some cases, if required, planar patch clamping technique can also be used to measure activity in specific neuron in a neuronal networks activity (e.g., Bosca et al., 2014, Kosnik et al. 2020; Strickland et al. 2018).

The number of excitatory or inhibitory synapses can be functionally studied at an electrophysiological level by examining the contribution of glutamatergic and GABAergic synaptic inputs. The number of

them can be determined by variably clamping the membrane potential and recording excitatory and inhibitory postsynaptic currents (EPSCs or IPSCs) (Liu, 2004).

2.6.6. Biological domain of applicability

2.6.6.1. Taxonomic Applicability

Vertebrates and Invertebrates

2.6.6.2. Life stage applicability

All life stages. This AOP specifically refers to the developmental period

2.6.6.3. Sex Applicability

Mixed

2.6.6.4. Evidence of Biological Domain of Applicability

The flow of ions that creates an action potential is powered by actively maintained electrochemical gradients. The maintenance of these gradients is a common feature of all cells, but in neurons the energetic cost is much steeper (Liebenskind et al. 2017). In vitro studies in brain slices applying electrophysiological techniques showed significant variability among species (immature rats, rabbits and kittens) related to synaptic latency, duration, amplitude and efficacy in spike initiation (reviewed in Erecinska et al., 2004). A number of studies applying MEA-based network formation assay (NFA) using primary cortical cells from new born rats, indicate that by measuring the integrated neural function over a period assays, is possible to reproduce a temporal change in the electric signal mimicking neural network development (Shafer et al. 2019).

2.6.7. References KE4

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2.7. KE5. Decreased oligodendrocyte differentiation

2.7.1. Short name

Decreased oligodendrocyte differentiation.

2.7.2. Level of Biological organization

Cellular. Olygodendrocytes lineage cells

2.7.3. KE Components and Biological Context

Biological Process: Oligodendrocytes Differentiation; Object: Oligodendrocytes; Action: decrease

2.7.4. KE description

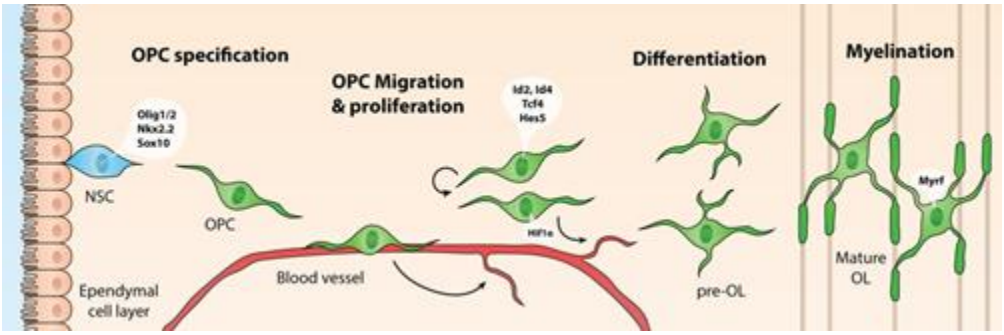
2.7.4.1. Biological state

Glial cells, i.e. astrocytes, microglia and oligodendrocytes, constitute the majority of cells in the nervous system. Oligodendrocytes (OLs) are responsible for axon myelination facilitating rapid saltatory conduction of action potentials within the central nervous system. The development of OLs involves different, highly regulated, sub sequential steps of proliferation, migration and differentiation.

The origin of these cells has been studied in the rodents CNS, particularly in the forebrain, the cerebellum and the spinal cord. These cells are generated by neuroepithelial cells of the ventricular

zones and by radial glial cells that, under the influence of transcription factors such as Olig1, Olig2, Nkx2.2, and Sox10, give rise to committed oligodendrocytes progenitor cells (OPCs; Figure C. 14). OPCs can then terminally differentiate into postmitotic, pre-myelinating oligodendrocytes (pre-OLs) which will mature and myelinate nearby receptive axons (Emery et al 2010).

Figure C. 14. OLs developmental stages from specification to differentiation; (Source: Van Tilborg et al. 2017).



More in details, the subventricular zone (SVZ) is a germinal matrix of the forebrain that first appears during the later third of murine embryonic development, enlarges during the peak of gliogenesis, between PND 5 and PND 20, and then shrinks but persists into adulthood.

In rodents, OPCs development takes place in three temporal waves starting in the ventral ventricular zone after neural tube closure from embryonic day E9.5. This first wave of OPC generation in the forebrain is followed by a smaller second wave from the dorsal ventricular zone and by a third postnatal wave originating in the cortex (Kessaris et al. 2006). While rat OPCs first appear at around embryonic day E14, first human OPC emerge at embryonic day E45 (gestational week 6.5, reviewed in Kuhn et al. 2019).

OPCs contribute to 5%–8% of total glial cells, which distribute across white (WM) and grey matter (GM), with a marginally higher abundance in WM. For OPC-neuronal interaction and finally axon myelination, OPC migrate from their site of origin into the developing WM tracts of the CNS. A wide variety of signalling molecules have been implicated in regulating OPCs migration for instance: spatial gradients of bone morphogenic proteins, local cues such as growth factors, extracellular matrix components such as laminin and fibronectin, neural activation, glutamate. Also, a proper brain vascularization is demonstrated to have a crucial role for OPCs migration. As a matter of facts, OPCs migrate in a jumping or crawling mode along blood vessels within the CNS (reviewed by Van Tilborg et al. 2018).

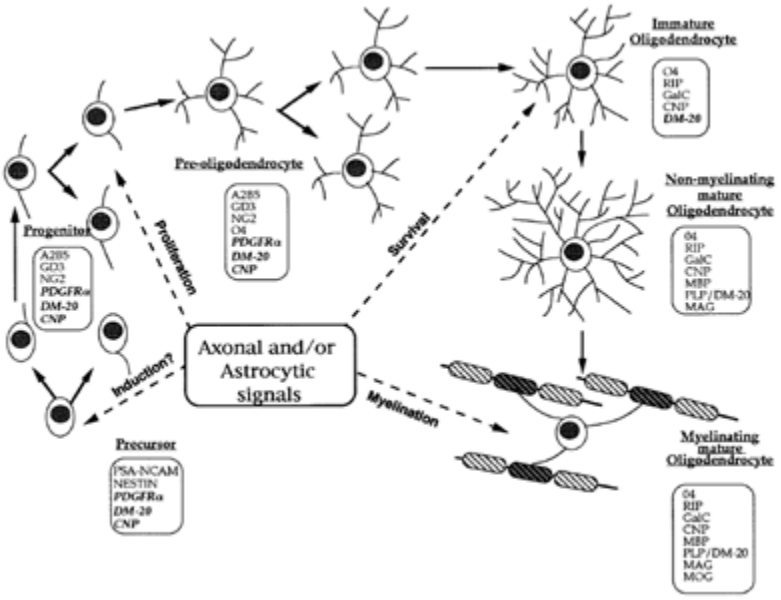
Succeeding excessive proliferation, especially in the WM, leads to an abundant pool of progenitors throughout the brain and spinal cord. OPCs matured to pre-OLs, multiprocessed cells, which keep the property of cell division and acquire the marker 04, then engage with target axons, thereby losing their bipolarity. They start building plasma membrane extensions, filamentous myelin outgrowths, that eventually wrap around nerve axons in a concentric fashion. This is the complex and tightly regulated process called myelination. Besides myelin production, there is increasing evidence that OPC possess immunomodulatory capacities. Similar to microglia, OPCs can migrate to sites of injury responding to inflammatory cues by presenting antigens to cytotoxic T-cells and causing cytotoxic cell death (reviewed in Kuhn et al. 2019).

Oligodendrocytes development is accompanied by the expression of specific markers via the pre-oligodendrocyte (pre-OL) stage to mature oligodendrocytes (OL; Figure C. 15). The sequential

expression of developmental markers, identified by a panel of cell specific antibodies, divide the lineage into distinct phenotypic stages characterized by specific features. Indeed, the differentiation involves the loss of certain surface or intracellular antigens and the acquisition of new ones.

Figure C. 15. Schematic representation of developmental stages of cells of the oligodendrocyte lineage.

(Source: Baumann et al. 2001).



Concerning their electrophysiological properties, OPCs express a wide variety of channels and receptors previously thought to be exclusively attributed to neurons (reviewed in Paez et al. 2009, Marinelli et al. 2016). These include AMPA, NMDA (Craven, R., 2006) and voltage gated sodium channels (VGSC) that are expressed and functional in cells of the oligodendrocyte lineage in rats (Káradóttier et al. 2008) and mice (Tripathi et al. 2011) in vivo as well as in a variety of species including rodents, zebrafish and humans in vitro (Sontheimer et al. 1989, Barres et al. 1990, Williamson et al. 1997, Livesey et al. 2016, Tsata et al. 2019, Marton et al. 2019).

2.7.4.2. Biological compartment

In rodents, OPCs originate from neuroepithelial or radial glia cells of the ventricular zones, at very early stages during embryonic life. In humans, there is an increased need for myelinating cells in the enlarged gyrencephalic brains due to not only grey, but also white matter expansion. This demand is contended by OPC generation from outer radial glia cells via recently identified epidermal growth factor-expressing pre-OPSs, which seem to enlarge the OPC pool by epidermal growth factor-dependent proliferation (Huang et al. 2020).

2.7.4.3. Role in biology

Oligodendrocytes are the myelinating cells of the central nervous system (CNS). They are generated from OPCs following tightly orchestrated processes of migration, proliferation and differentiation.

Oligodendrocytes are fundamental to myelin formation in the developing CNS and critical for myelin regeneration (reviewed in Kuhn et al. 2019).

The biological role of oligodendrocyte VGSC during rat postnatal development was recently studied via shRNA-mediated Nav1.2 knockdown. Pre-OLs devoid of this channel displayed significant morphological and structural changes including smaller cell volumes, reduction of process number and length and altered alignment of pre-OL with surrounding axons. These are critical for successful myelination and thus resulted in disturbed elaboration of connecting structures between pre-OLs and the axons and ultimately reduced (compacted) myelination (Berret et al. 2017). Furthermore, action potential generating OPCs can form axo-oligo synapses indicating that these progenitor OLs are capable of receiving and responding to electrical activity in nearby axons possibly guiding migration/differentiation of OPCs (reviewed in Paez et al. 2009).

2.7.5. How is measured or detected

Ex vivo: Due to distinct molecular marker expression during development, maturation stages of OPC, pre-OL and mature OL can be individually studied e.g. by immunocytochemistry or single cell RNAseq.

In vitro: Similar to in vivo, expression of different oligodendrocyte lineage markers can be detected via immunocytochemistry *in vitro*.

The human neural progenitor cell (NPC), oligodendrocyte differentiation (NPC5) and maturation (NPC6) test methods (Bal-Price et al. 2018) were recently put into assay formats (Dach et al. 2017). Species-overarching assays were developed for mouse (Dach et al. 2017), rat (Klose et al., 2020) and rabbit NPC (Barenys et al. 2020). The number of identified 04+ oligodendrocytes divided by the number of total nuclei in the migration area reveals the % of differentiated oligodendrocytes. Quantification of mRNA expression of myelin basic protein divided by the % of oligodendrocytes allows assessment of oligodendrocyte maturation (Dach et al. 2017). Electrical activity can be measured via patch clamping of live stained oligodendrocyte lineage cells (Livesey et al. 2016, Marton et al. 2019).

2.7.6. Biological Domain of Applicability

2.7.6.1. Taxonomic Applicability

Vertebrates and Invertebrates.

2.7.6.2. Life Stage Applicability

This AOP specifically refers to the developmental period.

2.7.6.3. Sex Applicability

Mixed.

2.7.6.4. Evidence of biological domain of applicability

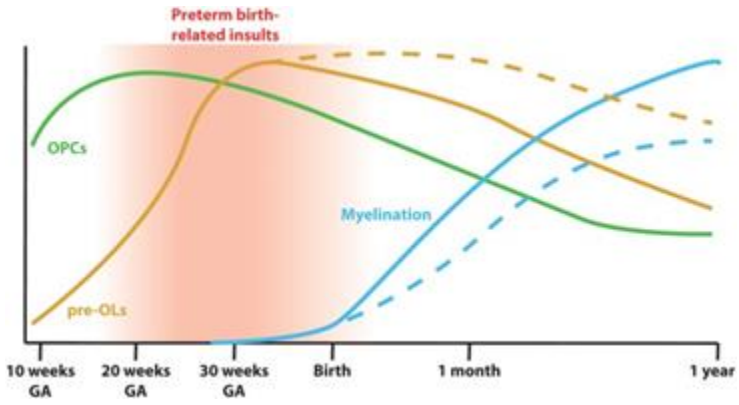
Oligodendrogliogenesis has been described in vertebrate species, such as rodents, human, chick, and Xenopus indicating a strong conservation of the process throughout myelinated vertebrates.

In birds and rodents, the first oligodendrocytes originate in the ventral region of the spinal cord. At embryonic day E14 in rat or mouse and at embryonic day E6 in chick, two clusters of cells stained with 04 antibody were found on each side of the ventricular zone (VZ) (Hajihosseini, et al 1996).

An analysis of human fetal brain tissue revealed that the first OPCs emerge in the developing brain around 10 weeks gestational age (GA), followed by an expansion of population before mid-gestation (15-20 weeks). Similarly to rodents, OPCs in human forebrain are derived from the lateral-medial

ganglionic eminence and the SVZ from where they migrate toward the cortical plate (Figure C. 10). It has been observed that in contrast with humans, rodents have a high turnover of OLs that is crucial for myelin maintenance (reviewed by Van Tilborg et al. 2018).

Figure C. 10. Developmental progression of OPCs, pre-OLs, and myelination in human. (source: Van Tilborg et al. 2017).



By using proteolipid protein (PLP) antibody and confocal microscopy the detailed three-dimensional morphology of oligodendrocytes was observed throughout early and late Xenopus Laevis tadpole development. The distinct phenotypes of oligodendrocytes (premyelinating, transitional, and mature oligodendrocytes) observed in rodents during development are resembled in Xenopus, indicating a strong morphological conservation of oligrodendrocytes shape (Yoshida, 1997).

2.7.7 References KE5

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2.8. KE6. Increase of Intracellular sodium in microglia cells

2.8.1. Short name

Sodium Kinetics in Microglia.

2.8.2. Level of biological organization.

Cellular. Cell type: Microglia

2.8.3. KE Components and Biological Context

Biological Process: Sodium Kinetic; Object: Microglia; Action: alteration

2.8.4. KE description

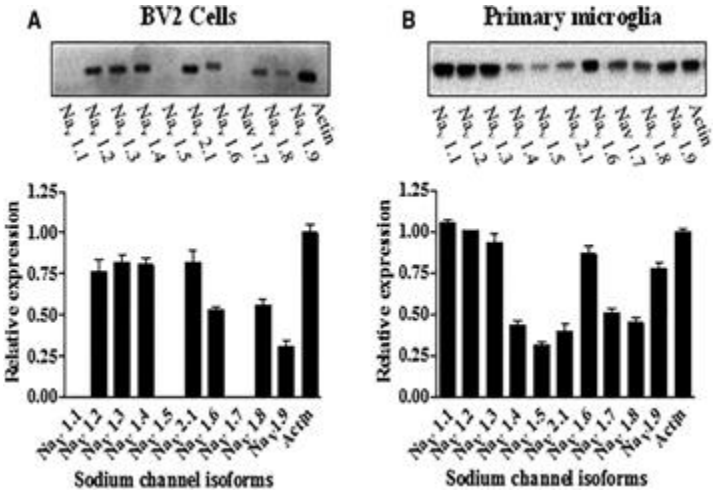
2.8.4.1. Biological state

Microglia are immune cells in the central nervous system, deriving from erythromyeloid progenitors, subsequent proliferate to colonize brain and spinal cord contributing significantly to ongoing neuroinflammation in a variety of neurodegenerative diseases. There are also a more limited number of other immune cells in the CNS, including myeloid cells, monocytes/macrophages, dendritic cells, T cells, B cells and natural killer (NK) cells (Korin et al., 2017).

Microglia cells express on their surface a number of receptors and ion channels which have a pivotal role in the transduction of external stimuli to intra-cellular responses, regulating a number of cellular functions such as morphological transformation, migration, proliferation, and phagocytosis eventually triggering or amplifying the innate immune response. Indeed, to sense injury in the CNS, microglia monitor constantly the parenchyma of the healthy brain through the continuously extension and retraction of their processes. In addition, microglia play pivotal role during neuronal development, as they model the developing brain not only through phagocytosis of apoptotic cells, but also by controlling the fate of neurons and of their progenitors (review by Wake and Fields, 2011).

The presence of Voltage Gated Sodium Channels (VGSCs) isoforms in microglia has been characterized in immortalized mouse (C57Bl/6) microglia cells (BV2) and in primary microglia cells (isolated from neonatal mice) by mRNA expression with standard PCR and agarose gel electrophoresis. There are some differences in isoform expression between the two cell types: BV2 cells expressed Nav 1.2, 1.3, 1.4, 1.6, 1.8, 1.9, and 2.1 whereas primary microglia cells expressed channel isoforms Nav 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7 1.8, 1.9, and 2.1 (Figure C. 11) with higher expression levels of Nav 1.1. 1.2, 1.3, 1.6, 1.9, and 2.1 as compared with BV2 cells. Evidence suggests that Nav 1.6 plays a predominant role in modulating microglia function, as in mouse, rat and human this isoform produce a persistent sodium current visible at the end of depolarization (Hossain et al. 2017).

Figure C. 11. Sodium channel isoforms in microglia cells.
Source: Hossain et al. 2017.



A number of *in vitro* and *in vivo* studies demonstrated that the interaction with VGSCs on microglia initiated a series of cellular events such accumulation of intracellular sodium, activation of NADPH oxidase and secretion of pro-inflammatory cytokines. Microglia activation is accompanied also by changes in cells morphology as they become bigger, with less processes and assume an amoeboid shape typical of phagocytic cells (Black et al. 2009; Hossain et al. 2020; Jung et al. 2013).

In details, it has been observed that, when microglia are activated with lipopolysaccharide (LPS), the sodium channel blockers, phenytoin and TTX, significantly reduced the activity of microglia (e.g. phagocytosis) and attenuated the release of pro-inflammatory cytokines (Black et al. 2009). Similar, evidence has been observed with pyrethroids (e.g. deltamethrin and permethrin). These chemicals caused a rapid sodium influx and increased accumulation of intracellular sodium, significantly reduced by TTX. The increase of intracellular sodium and its accumulation inside cells eventually leads to the release of TNF-α.

2.8.4.2. Biological compartment

The origin of microglia is controversial. Initially, they have been reported to originate from mesodermal sources such as embryonic mesenchymal cells in pia mater. However, later observation of murine fetal brain at embryonic day E 9.5 leads to the hypothesis that microglia can originate also from yolk sac macrophages (Kaur et al. 2017; Ginhoux and Prinz, 2015).

2.8.4.3. Role in Biology

Microglia are immune cells in the central nervous system. They are active phagocytes help to eliminate degenerating axons and cells during different conditions (e.g. CNS development, injuries and infections) (Kaur et al. 2017).

2.8.5. How is measured or detected

In vitro. Sodium influx and intracellular sodium accumulation can be determined by incubating cells with fluorescence indicator and measuring the fluorescence intensity with excitation at 485 nm and emission at 515 nm (Hossain et al. 2017).

Electrophysiological recording from primary microglia can be carried using the whole patch clamp technique (Jung et al. 2013).

Ex vivo. Sodium channel presence and expression can be determined ex vivo with immunocytochemistry and in situ hybridization cytochemistry techniques (Craner et la. 2005).

2.8.6. Biological domain of applicability

2.8.6.1. Taxonomic Applicability

Vertebrates and Invertebrates.

2.8.6.2. Life Stage Applicability

This AOP specifically refers to the developmental period.

2.8.6.3. Sex Applicability

Mixed.

2.8.6.4. Evidence of biological domain of applicability

Electrophysiological studies demonstrated the presence of VGSCs with properties indistinguishable from sodium channels of neurons in rodents and human microglia (Craner et la. 2005).

Neonatal rat microglia in vitro express tetrodoxin TTX-sensitive sodium channels Nav 1.1 and Nav 1.6 and tetrodoxin resistant channel Nav 1.5, but not detectable levels of Nav1.2, Nav1.3, Nav1.7, Nav1.8, and Nav1.9 (Black and Waxmann, 2012; Eder , 1998).

2.8.7. References KE6

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2.9. KE7. Disruption of intracellular Calcium channel kinetic

2.9.1. Short Title

Intracellular calcium regulation

2.9.2. Level of Biological Organization

cellular

2.9.3. KE Components and Biological Context

Process: regulation of intracellular Calcium homeostasis by the endoplasmic reticulum Ryanodine receptors

Object: lack of fidelity in a neural network

Action: decrease

Biological Context:

Maintenance of the intracellular Calcium concentration

2.9.4. KE description

Developing neuronal networks display spontaneous synchronized Calcium oscillations (SCOs) (Cao etal.2012a), whose patterns depend on the balance of excitatory and inhibitory neurotransmission within the neuronal network (Pacico and Mingorance-LeMeur2014). SCOs are orchestrated by multiple Calcium signalling pathways that are highly integrated and regulated (Dravid and Murray2004). SCOs depend on electrical spike activity (ESA) (Spitzeretal.1995), and both are crucial for neuronal development, activity-dependent neurite outgrowth, and network plasticity (Dolmetsch etal.1998; Spitzer etal. 1995; Wayman et al.2006).

Calcium regulation is a critical process in neurons, which have developed extensive and intricate Calcium signalling pathways. The ER is a Calcium store within the cell with a typical intraluminal Calcium concentration of about 0.5 mM. Several proteins are involved in Calcium homeostasis in the ER; their elaborate kinetics and regulatory mechanisms make the regulation of Calcium homeostasis in the ER very complex. Nevertheless, there are two main aspects to ER Calcium homeostasis, namely, ‘‘calcium-induced calcium release’’ (CICR) and ‘‘storage-operated calcium entry’’ (SOCE). CICR is seen where a comparatively small increase in cytoplasmatic Calcium concentration induces the opening of RyRs (RyR1 can also be activated by depolarizing membrane potential in skeletal muscle cells and possibly also in neurons), which will then release more Calcium from the ER. If the increase in the Calcium concentration in the ER is accompanied by an increase in inositol-1,4,5-trisphosphate (IP3), typically via activation of phospholipase C, then IP3-Rs will also release Calcium from the ER. Ryanodine receptors (RyRs), together with inositol-1,4,5-trisphosphate receptors (IP3R) influence Calcium release from sarcoplasmic reticulum/endoplasmic reticulum (SR/ER) stores, and thus mediate a myriad of Calcium-regulated cellular processes (Lanner et al. 2010; Pessah et al. 2010). Altered activities of RyRs channel will therefore influence Calcium dynamics, patterns of synchronous Calcium oscillations (SCOs), and electrical spike activity (ESA) functions.

2.9.5. How it is measured or detected

The Fluorescent Plate Reader technique (FLIPR) permits simultaneous measurements of intracellular Calcium transients in intact neurons in a 96-well format. This method permitted a high temporal resolution (every half second) recording of internal Calcium concentration to reveal SCO dynamics in parallel across 96 wells before and after exposure to chemicals (Cao et al. 2017). However, this measurement was assessed as uncertaint since has a limited sensitivity compared to single-cell fluorescence microscopy and to confirm theis KE in an evidence based postulated AOP further single-cell fluorescence microscopy studies are recommended.

2.9.6. Biological domain of applicability

2.9.6.1. Taxonomic Applicability

Vertebrates and invertebrate

2.9.6.2. Life stage applicability

All life stages. This AOP specifically refers to the developmental period

2.9.6.3. Sex Applicability

Mixed

2.9.6.4. Evidence of Biological Domain of Applicability

Multiple biological processes are involved in the maintenance of ion homeostasis eventually maintaining a low cytoplasmic calcium level to protect the fidelity of calcium signaling and maintaining a negative voltage across the membrane to power action potentials. The maintenance of this ions gradient is a feature of all cells. Proteins that maintain low cytoplasmic calcium levels are found across eukaryotes as part of a pan-eukaryotic calcium-signaling toolkit. The ion homeostasis and the processes that are involved in their regulation are evolutionarily ancient and pleiotropically expressed in multiple cell types (Liebenskind et al. 2017).

2.9.7. References KE7

Cao, Z., Hammock, B. D., McCoy, M., Rogawski, M. A., Lein, P. J., & Pessah, I. N. (2012a). Tetramethylenedisulfotetramine alters Ca2+ dynamics in cultured hippocampal neurons: mitigation by NMDA receptor blockade and GABAA receptor-positive modulation. Toxicological Sciences, 130(2), 362-372.

Cao, Z., Xu, J., Hulsizer, S., Cui, Y., Dong, Y., & Pessah, I. N. (2017). Influence of tetramethylenedisulfotetramine on synchronous calcium oscillations at distinct developmental stages of hippocampal neuronal cultures. Neurotoxicology, 58, 11-22.

Dolmetsch, R. E., Xu, K., & Lewis, R. S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. Nature, 392(6679), 933-936.

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Lanner, J. T., Georgiou, D. K., Joshi, A. D., & Hamilton, S. L. (2010). Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harbor perspectives in biology, 2(11), a003996.

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Pessah, I. N., Cherednichenko, G., & Lein, P. J. (2010). Minding the calcium store: Ryanodine receptor activation as a convergent mechanism of PCB toxicity. Pharmacology & therapeutics, 125(2), 260-285.

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2.10. KE8 Decreased neuronal crest cells migration

2.10.1 Short Title

Decreased neuronal crest cells migration

2.10.2 Level of Biological Organization

cellular

2.10.3. KE Components and Biological Context

Process: decrease of neuronal crest cell migration in the brain of developing embryo

Object: neuronal crest cell

Action: decrease

Biological Context: Cellular

2.10.4. KE Description

Neural crest cells (NCC) differentiate during neurulation from the cells of the neural roof plate. These cells eventually give rise to over 100 different cell types in the human body, including the peripheral nervous system, melanocytes, cardiomyocytes or facial connective tissue (Huang & Saint-Jeannet, 2004). One ma-jor feature of NCC is their migration to the different parts of the developing embryo. Once they arrive at their final destination, they differentiate into the according cell type. A large percentage of developmental disorders (e.g. congenital heart defects, orofacial clefts, Hirschsprung's disease) are caused by inhibition of neural crest cell migration. These kinds of alterations can be induced by genetic factors (Lee et al, 2009) or exposure to pharmaceuticals (e.g. valproic acid, Fuller et al, 2002) and pesticides (e.g. triadimefon, Menegola et al, 2005). For the migration inhibition of NCC (cMINC assay, UKN2), hiPSC are differenti-ated into NCC, which are subsequently used for the migration assay (Nyffeler et al, 2017). As endpoint-specific controls, Cytochalasin D, a known inhibitor of actin polymerisation and taxol, which inhibits the breakdown of microtubules are used. As cell migration requires a dynamic variability of the cytoskeleton these two compounds lead to migration inhibition.

2.10.5. How it is measured or detected

Migration across the battery was tested as NCC migration, radial glial migration, neuronal migration and oligodendrocyte migration. The test systems represent the different cell type of the developing brain which have the ability to differentiate in e.g. neurons, astrocytes and oligodendrocytes (Masjosthusmann et al., 2020)

2.10.6. Biological domain of applicability

2.10.6.1. Taxonomic applicability

Vertebrates and invertebrates

2.10.6.2. Life stage applicability

Developmental phase

2.10.6.3. Sex applicability

Mixed

2.10.6.4. Evidence of Biological Domain of applicability

The KE effect of neuronal crest cell migration was considered to be effected within the above mentioned specific assay (Masjosthusmann et al., 2020) with a probability of ≥ 66% based on the result of an Experts-Knowledge-Elicitation assessment

Table C.2. Evidence for Type II pyrethroid DLM on neuronal crest cell migration

Reference	Result
Masjosthusmann et al., 2020 UKN2 (hiPSC derived neural crest cells): 2D assay, 24h crest cell migration	neuronal crest drop in 25% (BMC25 of 26 uM) up to 30% at 100uM.
1. NPC2a (primary human NPCs from fetus): 3D assay, 72h radial glia migration 2. NPC2a (primary human NPCs from fetus): 3D assay, 120h radial glia migration 3. NPC2b (primary human NPCs from fetus): 3D assay, 120h migration distance neurons 5. NPC2c (primary human NPCs from fetus): 3D assay, 120h oligodendrocyte migration 6. Percent mean migration distance all oligodendrocyte (NPC 3-5/IUF) 120 hrs *Number of nuclei (NPC 3-5/IUF) 120 hrs – (no migration endpoint, reference endpoint covering radial glia proliferation	No decrease in the other specific cell migration endpoints.

The same assay has been applied for testing other pyrethroids. Some of them were not identified as hits, i.e. λ-Cyhalothrin, beta-Cypermethrin, beta-Cyfluthrin, Cypermethrin. However, in addition to DLM just other 10 hits were identified within 117 chemicals. The potency among these 10 hits ranged between 0.6 – 20 uM and DLM ranked at position 9.

Considering that (1) the test battery cell migration was tested in multiple test system and only NCC cells were affected, (2) a dose concordance could not be established for this AOP because of the high concentration affecting this KE and (3) based on the evidence that other pyrethroids were not affecting this KE. It is not plausible to link this KE with MIE1 or MiE2 or with any downstream KE or AO. Therefore, this KE would be not consider it in the probabilistic calculation and in the AOP postulation.

2.10.7. References for KE8

Fuller, L. C., Cornelius, S. K., Murphy, C. W., & Wiens, D. J. (2002). Neural crest cell motility in valproic acid. Reproductive Toxicology, 16(6), 825-839.

Huang, X., & Saint-Jeannet, J. P. (2004). Induction of the neural crest and the opportunities of life on the edge. Developmental biology, 275(1), 1-11.

Lee, G., Papapetrou, E. P., Kim, H., Chambers, S. M., Tomishima, M. J., Fasano, C. A., ... & Tabar, V. (2009). Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. Nature, 461(7262), 402-406.

Masjosthusmann, S, Blum, J, Bartmann, K, Dolde, X, Holzer, A-K, Stürzl, L-C, Hagen, Keßel E, Förster, N, Dönmez, A, Klose, J, Pahl, M, Waldmann, T, Bendt, F, Kisitu, J, Suciu, I, Hübenthal, U, Mosig, A, Leist, M, Fritsche, E, (2020). Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity, *EFSA Supporting Publications 2020:* 17(10), 1938E, 152 pp, doi: 10.2903/sp.efsa.2020.EN-1938

Menegola, E., Broccia, M. L., Di Renzo, F., Massa, V., & Giavini, E. (2005). Craniofacial and axial skeletal defects induced by the fungicide triadimefon in the mouse. Birth Defects Research Part B: Developmental and Reproductive Toxicology, 74(2), 185-195.

Nyffeler, J., Karreman, C., Leisner, H., Kim, Y. J., Lee, G., Waldmann, T., & Leist, M. (2017). Design of a high-throughput human neural crest cell migration assay to indicate potential developmental toxicants. Alternatives to Animal Experimentation: ALTEX, 34(1), 75-94.

2.11. KE9 Hypomyelination

2.11.1. Short name

Hypomyelination.

2.11.2. Level of biological organization.

Organ.

2.11.3. KE Components and Biological Context

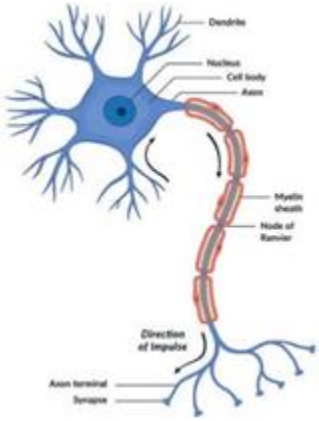
Biological Process: Myelination; Object: Neuronal Axons; Action: Decrease.

2.11.4. KE description

2.11.4.1. Biological state

Hypomyelination, consist in a permanent deficit in myelin deposition in brain. Hypomyelination is different form demyelination and dysmielination, conditions that respectively refer to disruption of myelin and abnormal myelin deposition.

Figure C. 12. Nerve cells insulated by multiple myelin sheaths
(available from https://medlineplus.gov/genetics/condition/pol-iii-related-leukodystrophy/).



Given the importance of the process for the functioning of central nervous system (CNS), the myelination of individual axons is highly regulated by several mechanisms. The simplest one is the expression on axons surface of inhibitory or permissive cues for myelination (Emery,2020).

It is well documented that a number of factors can impair the myelination in the developing brain leading to cognitive, attentional, behavioural, and social deficits in developing child. For instance, due to the importance of thyroid hormones for oligodendrocyte maturation, conditions like congenital hypothyroidism, maternal hypothyroidism and Allan-Herndon-Dudley syndrome (AHDS) may lead to hypomyelination and mild to severe mental retardations in children. Also, genetic mouse models with impaired expression of proteins regulating cholesterol clearance show reduced OPC maturation, hypomyelination and developmental delays (Klose et al. 2020). In addition to this, it has been observed that the stressful environment that preterm infants reside during the first weeks of life negatively affect the development of oligodendrocytes. The dominant lesion found in infant born prematurely, is a form of cerebral white matter injury termed periventricular leukomalacia (PVL) characterized by pre-OLs injuries, thereby causing deficits in myelination and subsequent loss of mature myelinating cells, which lead to cerebral hypomyelination and cognitive deficits (reviewed by Volpe et al. 2011).

2.11.4.2. Biological Compartment

Hypomyelination can affect different parts of the nervous system such as cerebrum, cerebellum and spinal cords. Indeed, myelinations starts in the cerebrum where sizeable white matter tracts and commissures are the first to be insulated followed by thalamic and basal nuclei and other subventricular areas. In cerebral cortex myelination occurs first in projection zones and continues in association areas. In spinal cords, lumbar regions are myelinated only after the cervical tracts (Bolon et al. 2018; “Chapter 21-Nervous System”, in Fundamentals of Toxicologic Pathology (Third Edition) 2018, edited by Wanda M. Haschek et al., Elsevier Science & Technology, 2018).

2.11.4.3. Role in biology

Hypomyelination of nerve fibres can impair and reduce the transmission of nerve impulses, leading to a series of different behaviour alterations (e.g. cognitive, attentional, behavioural, and social deficits) in developing child.

2.11.5. How it is measured or detected

In humans and in vivo. Hypomyelination can be detected through brain Magnetic Resonance Imaging (MRI).

2.11.6. Biological Domain of applicability

2.11.6.1. Taxonomic Applicability

Vertebrates and Invertebrates.

2.11.6.2. Life Stage Applicability

This AOP specifically refers to the developmental period. Since OL development begins during foetal development and continuous throughout the first two years of life.

2.11.6.3. Sex Applicability

Mixed.

2.11.6.4. Evidence of biological domain of applicability

Hypomyelination has been observed in human, rodent and rabbits' brains (Baumann et al. 2001).

Social isolation of juvenile or adult animals negatively affected myelination of the prefrontal cortex in mice. Similarly, in humans, social deprivation at early ages was related with reduced white matter integrity (Van Tilborg et al 2018).

Myelination is highly conserved among the different species. In mouse it starts at birth in the spinal cord and is almost completed around 45-60 days postnatally. In humans the peak of myelin formation occurs postnatally, although it starts during the second half of foetal life in the spinal cord (Volpe et al. 2011). The number of oligodendrocytes, established in childhood, increases until 5 years and remains stable after this age. In human adulthood, differently from rodents, only 1/300 oligodendrocytes are exchanged annually, therefore injuries affecting oligodendrocytes cell type can have important consequences for the entire myelination maintenance (Yeung et al. 2014).

2.11.7. References KE9

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2.12. AO. Impairment behavioural function (sensory motor reflex and learning)

2.12.1 Short Title

Impairment of the behavioural function

2.12.2. Level of Biological Organization

Organism

2.12.3. Key Components and Biological Context.

Process:

Object:

Action:

Biological Context:

2.12.4. AO Description

For the purposes of this AO, impaired behavioural function (sensory motor reflex and associative learning) is defined as an organism's inability or impairment in the capability to carry out new behaviours in the following processes: associative learning (that can be measured in the acquisition phase during classical conditioning) and/or startle behavior (that can be measured as startle reflex in behavioural studies). Currently, there is an existing AO in the AOP Wiki (number 341) for Impairment, Learning and memory some sections were adapted from there and reference (Sachana et al., 2016)

Learning can be defined as the process by which new information is acquired to establish knowledge by systematic study or by trial and error. There are many types of learning including simple and complex ones. Simple forms of learning consist of habituation and sensitization. Habituation refers to a process that an individual becomes accustomed with a stimulus after repeated presentation of it. Sensitization refers to the increase of response to stimuli of an individual after exposure to an intense stimulus. There are complex forms of learning, classical conditioning, animals or people learn to associate two stimuli occurring in sequence or operant conditioning, learn to forma an association between a behaviour and its reward or punishment. These two types of learning are considered in neurobehavioral studies: a) associative learning and b) non-associative learning. (Ono, 2009'). Associative learning is based on making associations between different events. In associative learning, a subject learns the relationship among two different stimuli or between the stimulus and the subject's behaviour (Sachana et al., 2016). The acquisition phase is the initial step for conditioning an animal in classical conditioning which includes also extinction, generalization and discrimination.

The startle response is an unconditional reflex, characterized by the rapid contraction of facial and skeletal muscles, elucidated by a sudden and intense startling stimulus. Startle in humans consists of the following set of muscle movements: blinking of the eyes, forward head movement, a characteristic facial expression that includes a widening of the mouth and occasional baring of the teeth, raising and drawing forward of the shoulders, abduction of the upper arms, bending of the elbows, pronation of the lower arms, flexion of the fingers, forward movement of the trunk, contraction of the abdomen, and bending of the knees .

The startle reflex can be initiated by a number of different sensory stimuli including, an burst of noise, an electric shock (Ison, 1984), a light flash, and tactile stimuli such as a puff of air (Ison, 1984).

Work by Davis and colleagues (Davis et al., 1982), as well as Koch and colleagues (Koch and *Schnitzler, 1997)* have well documented the physiological pathway of the acoustic startle response. It starts in the inner ear, where a sudden and intense acoustic stimulus activates hairs cells in the cochlea that in tern activate neurons in the cochlear root nucleus. The pathway continues to the nucleus reticularis pontis caudalis which then activates the reticulospinal tract neurons that connent to ventral horn motor neuron that activate neuromuscular junctions. Pathways involved in tactile startle and prepulse inhibition has not been as well defined.

Despite the varying forms of the movement during the startle response, its behavioural role is similar among the different species. The response serves as protective function that can move the animal rapidly out of the way of an attacking predator or protect vital parts of the body (Ono., 2009). An appropriately timed, rapid, and powerful startle response can mean the difference between life and death as a consequence the neuronal networks underlying the startle response are well designed for both rapid response and a powerful activation of muscle. The startle response is tightly linked to maturation of the cochlea with the hearing and musculature with responses beginning within the second postnatal week (Brunjes and Alberts, 1981; Parisi and Ison, 1979; Sheets et al., 1988; Shnerson and Willott, 1980). The startle reflex is evolutionarily conserved across mammals and intense acoustic stimulation is particularly effective in eliciting startle, more intense stimuli producing larger responses (Davis., 1982; Braff et al. 2001). The startle reflex has served as a tool for studying fundamental properties of nervous function ranging from neurophysiological and anatomical relationships within the pons and reticular formation to forebrain regulation of complex behavioral states and cognitive processes. The primary startle circuit is located at or below the pons, while the forebrain modulates several forms of startle plasticity, including habituation and prepulse inhibition (Crofton, 1992; Davis, 1980; Davis et al., 1982; Fechter et al., 1986; Geyer and Swerdlow, 1998; Hoffman and Ison, 1980).

Learning and memory depend upon the coordinated action of different brain regions and neurotransmitter systems constituting functionally integrated neural networks (D'Hooge and DeDeyn, 2001). Among the many brain areas engaged in the acquisition of, or retrieval of, a learned event, the hippocampal-based memory systems have received the most study. For example, the hippocampus has been shown to be critical for spatial-temporal memory, visio-spatial memory, verbal and narrative memory, and episodic and autobiographical memory (Burgess et al., 2000; Vorhees and Williams, 2014). However, there is substantial evidence that fundamental learning and memory functions are not mediated by the hippocampus alone but require a network that includes, in addition to the hippocampus, anterior thalamic nuclei, mammillary bodies cortex, cerebellum and basal ganglia (Aggleton and Brown, 1999; Doya, 2000; Mitchell et al., 2002, Toscano and Guilarte, 2005; Gilbert et al., 2006, 2016). Thus, damage to variety of brain structures can potentially lead to impairment of learning and memory (Sachana et al., 2016).

It was published, that the main learning areas and pathways are similar in rodents and primates, including man (Eichenbaum, 2000; Stanton and Spear, 1990).While the prefrontal cortex and frontostriatal neuronal circuits have been identified as the primary sites of higher-order cognition in vertebrates, invertebrates utilize paired mushroom bodies, shown to contain ~300,000 neurons in honey bees (Menzel, 2012; Puig et al., 2014). From studies in fish, it is clear that a very different brain anatomy may still allow similar brain functions (e.g. Aoki et al. 2013).

However, it is recognised that "the issue of comparability of human and rodent cognitive measures in studies of pharmacology and neurotoxicology has generated a large body of literature, much of which is relevant to the evaluation of DNT data. A few reviews of possible interest are found in D'Mello and Steckler (1996), Sarter (2004), and Sharbaugh et al. (2003)" (NAFTA, 2016). Later, also Pitzer et al. (2018) indicates that further work would be necessary to align human endpoints for learning and memory with the well established rodent endpoints for learning and memory (such as motor activity, acoustic startle response-prepuls inhibition, Morris Water Maze, Cinncinati Water Maze, Radial Water Maze, Conditioned Fear testing).

Moreover, “there are also primate specific features of brain development that increase anatomical, cognitive, and behavioral complexity and may explain why many human neurological and neuropsychiatric diseases are not well modeled in rodents. These features include a larger cortical progenitor pool in the outer subventricular zone not present in rodents and protracted myelination, synapse production, and pruning. Consequently, rhesus monkey and human share a greatly expanded neocortex and specialization of areas (most notably primary visual cortex), compared to mouse, reflecting the more recent common ancestor of human and rhesus monkey (25 million years) than human and mouse (70 million years). Likewise, rhesus monkey and human brain share more similar patterns of gene expression than do mouse and human brain” (Bakken et al. 2016)

2.12.5. How It Is Measured or Detected

Measurement of the startle response is conducted using a variety of methods that all basically record the muscular response to a sudden intense sensory stimulus. A variety of stimuli can be used, including very short noise bursts (msecs), electrical shock, air puff, or light flashes (Davis., 1982). The startle response is a very rapid reaction that is normally measured between 50 and 200 msec after the onset of the stimulus.

Methods used to measure the basic startle response vary between human and most laboratory species. In humans the startle response is routinely measured using the eye-blink EMG response (Ison, 1982), but can also be measured using EMG response in other parts of the body, skin conductance, and cardiac interbeat intervals (Geyer and Swerdlow, 2001).

The most common methods used to record the startle response in rodents, with the most common species used is rats, uses acoustic or tactile stimuli to elicit the response, and either load cell-based or accelerometer based systems to measure the response (NAFTA, 2016 for details).

One of the most important features of the mammalian startle response is that it is highly graded in amplitude so that it is well suited for quantitative analysis in a single animal.

The ontogenicity of specific behaviors, reflexes, and motor functions has been established for laboratory rats and mice, and compared to human developmental patterns (e.g. Fox M., 1965; Altman and Sudarshan., 1975; Wood et al., 2013)

In laboratory animals: in rodents, a variety of tests of learning and memory have been used to probe the integrity of hippocampal function. These include tests of spatial learning like the radial arm maze (RAM), the Barnes maze, passive avoidance and Spontaneous alternation and most commonly, the Morris water maze (MWM). Test of novelty such as novel object recognition, and fear based context learning are also sensitive to hippocampal disruption. Finally, trace fear conditioning which incorporates a temporal component upon traditional amygdala-based fear learning engages the hippocampus. A brief description of these tasks follows.

1) RAM, Barnes, MWM are examples of spatial tasks, animals are required to learn the location of a food reward (RAM); an escape hole to enter a preferred dark tunnel from a brightly lit open field area (Barnes maze), or a hidden platform submerged below the surface of the water in a large tank of water (MWM) (Vorhees and Williams, 2014).
2) Novel Object recognition. This is a simpler task that can be used to probe recognition memory. Two objects are presented to animal in an open field on trial 1, and these are explored. On trial 2, one object is replaced with a novel object and time spent interacting with the novel object is taken evidence of memory retention – I have seen one of these objects before, but not this one (Cohen and Stackman, 2015).
3) Contextual Fear conditioning is a hippocampal based learning task in which animals are placed in a novel environment and allowed to explore for several minutes before delivery of an aversive stimulus, typically a mild foot shock. Upon reintroduction to this same environment in the future (typically 24-48 hours after original training), animals will limit their exploration, the context of this chamber being associated with an aversive event. The degree of suppression of activity after training is taken as

evidence of retention, i.e., memory (Curzon et al., 2009).
4) Trace fear conditioning. Standard fear conditioning paradigms require animals to make an association between a neutral conditioning stimulus (CS, a light or a tone) and an aversive stimulus (US, a footshock). The unconditioned response (CR) that is elicited upon delivery of the footshock US is freezing behavior. With repetition of CS/US delivery, the previously neutral stimulus comes to elicit the freezing response. This type of learning is dependent on the amygdala, a brain region associated with, but distinct from the hippocampus. Introducing a brief delay between presentation of the neutral CS and the aversive US, a trace period, requires the engagement of the amygdala and the hippocampus (Shors et al., 2001).

In humans: A variety of standardized learning and memory tests have been developed for human neuropsychological testing, including children (Rohlman et al., 2008). These include episodic autobiographical memory, perceptual motor tests, short and long term memory tests, working memory tasks, word pair recognition memory; object location recognition memory. Some have been incorporated in general tests of intelligence (IQ) such as the Wechsler Adult Intelligence Scale (WAIS) and the Wechsler. Modifications have been made and norms developed for incorporating of tests of learning and memory in children. Examples of some of these tests include:

1) Rey Osterieth Complex Figure test (RCFT) which probes a variety of functions including as visuospatial abilities, memory, attention, planning, and working memory (Shin et al., 2006).
2) Children's Auditory Verbal Learning Test (CAVLT) is a free recall of presented word lists that yields measures of Immediate Memory Span, Level of Learning, Immediate Recall, Delayed Recall, Recognition Accuracy, and Total Intrusions. (Lezak 1994; Talley, 1986).
3) Continuous Visual Memory Test (CVMT) measures visual learning and memory. It is a free recall of presented pictures/objects rather than words but that yields similar measures of Immediate Memory Span, Level of Learning, Immediate Recall, Delayed Recall, Recognition Accuracy, and Total Intrusions. (Lezak, 1984; 1994).
4) Story Recall from Wechsler Memory Scale (WMS) Logical Memory Test Battery, a standardized neurospychological test designed to measure memory functions (Lezak, 1994; Talley, 1986).
5) Autobiographical memory (AM) is the recollection of specific personal events in a multifaceted higher order cognitive process. It includes episodic memory- remembering of past events specific in time and place, in contrast to semantic autobiographical memory is the recollection of personal facts, traits, and general knowledge. Episodic AM is associated with greater activation of the hippocampus and a later and more gradual developmental trajectory. Absence of episodic memory in early life (infantile amnesia) is thought to reflect immature hippocampal function (Herold et al., 2015; Fivush, 2011).
6) Staged Autobiographical Memory Task. In this version of the AM test, children participate in a staged event involving a tour of the hospital, perform a series of tasks (counting footprints in the hall, identifying objects in wall display, buy lunch, watched a video). It is designed to contain unique event happenings, place, time, visual/sensory/perceptual details. Four to five months later, interviews are conducted using Children's Autobiographical Interview and scored according to standardized scheme (Willoughby et al., 2014).

In Honey Bees: For over 50 years an assay for evaluating olfactory conditioning of the proboscis extension reflex (PER) has been used as a reliable method for evaluating appetitive learning and memory in honey bees (Giurfa and Sandoz, 2012; LaLone et al., 2017). These experiments pair a conditioned stimulus (e.g., an odor) with an unconditioned stimulus (e.g., sucrose) provided immediately afterward, which elicits the proboscis extension (Menzel, 2012). After conditioning, the odor alone will lead to the conditioned PER. This methodology has aided in the elucidation of five types of olfactory memory phases in honey bee, which include early short-term memory, late short-term memory, mid-term memory, early long-term memory, and late long-term memory (Giurfa and Sandoz, 2012). These phases are dependent on the type of conditioned stimulus, the intensity of the unconditioned stimulus, the number of conditioning trials, and the time between trials. Where formation of short-term memory occurs minutes after conditioning and decays within minutes, memory consolidation or stabilization of a memory trace after initial acquisition leads to mid-term memory, which lasts 1 d and is characterized by activity of the cAMP-dependent PKA (Giurfa and Sandoz, 2012). Multiple conditioning trials increase the duration of the memory after learning and coincide with increased Ca2+-calmodulin-dependent PKC

activity (Giurfa and Sandoz, 2012). Early long-term memory, where a conditioned response can be evoked days to weeks after conditioning requires translation of existing mRNA, whereas late long-term memory requires de novo gene transcription and can last for weeks (Giurfa and Sandoz, 2012)."

2.12.6. Biological domain of applicability

2.12.6.1. Taxonomic Applicability

Basic forms of learning behavior have been found in many taxa from worms to humans (Alexander, 1990). More complex cognitive processes such as executive function likely reside only in higher mammalian species such as non-human primates and humans. Recently, larval zebrafish has also been suggested as a model for the study of learning and memory (Roberts et al., 2013).

The startle reflex is highly conserved across a wide variety of taxa including amphibians, fish, insects, bird and mammals (Bullock, 1984; Eaton, 1984; Domenici et al., 2011). Startle reflexes in mammals have been studies in humans, rats, mice, rhesus monkeys and guinea pigs among others mammalian species (Davis., 1982). The startle response offers many advantages as a behavioral measure of central nervous system (CNS) activity: consistency across species, simple neural circuitry, and sensitivity to a variety of experimental manipulations.

2.12.6.2. Life stage applicability

Developmental period

2.12.6.3. Sex Applicability

Mixed

2.12.7. Regulatory Significance of the Adverse Outcome

A prime example of impairments in behaviour, learning and memory, as the adverse outcome for regulatory action is developmental lead exposure and IQ function in children (Bellinger, 2012). Most methods are well established in the published literature and many have been engaged to evaluate the effects of developmental thyroid disruption. The US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OPPTS 870.6300 or OECD 426) both require testing of learning and memory (USEPA, 1998; OECD, 2007) advising to use the following tests; passive avoidance, delayed-matching-to-position for the adult rat and for the infant rat, olfactory conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze, T-maze, and acquisition and retention of schedule-controlled behaviour. These DNT Guidelines have been deemed valid to identify developmental neurotoxicity and adverse neurodevelopmental outcomes (Makris et al., 2009).

Also in the frame of the OECD TG 443 (OECD 2018), learning and memory testing can be applied in the context of developmental neurotoxicity studies. However, many of the learning and memory tasks used in guideline studies may not readily detect subtle impairments in cognitive function associated with modest degrees of developmental thyroid disruption (Gilbert et al., 2012).

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3. Adjacent KERs descriptions

The following KERs were proposed when including only the KE retrieved and assessed in the project using deltamethrin as a stressor. The KERs were therefore built based on (1) the organization of the type of evidence (molecular, cellular, organ and organism responses) and (2) scientific knowledge for inferring the probable change in, or state of, a downstream KE from the known or measured state of an upstream KE (OECD., 2016). The structure of the KERs is following the OECD indications (OECD 2018), by contextualizing the biological plausibility, empirical evidence, essentiality and additional information when available (i.e. analogy and specificity). Empirical support, dose and time concordance, are therefore tailored on evidences retrieved for the type II pyrethroid deltamethrin and this evidence were weighted through the uncertainty analysis and/or an expert knowledge elicitation (EKE) process.

3.1. KER 1: MIE leading to KE1, Binding to VGSC leading to disruption of sodium channel gate kinetics

3.1.1. KER Description

Voltage-gated sodium channels (VGSCs) are critical in generation and conduction of electrical signals in multiple excitable tissues. Natural and synthetic toxins are known to interact with VGSC by altering the gate kinetic of the channel by slowing the activation and deactivation rate of the VGSC and shift to a more hyperpolarized potentials the membrane potential at which the VGSC activate.

The detailed mechanism of voltage sensing and voltage-dependent activation of the voltage sensor of sodium channels through a series of resting and activated states is known at the atomic level.

There is evidence supporting that the binding of pyrethroids to VGSC (Trainer et al 1997; O'Reilly et al., 2006) induces disruption of the sodium channel gate kinetics (Meyer et al., 2008, Soderlund et al., 2002).

3.1.2. Evidence supporting the KER

3.1.2.1. Biological plausibility

It is well known that Ion channels are integral membrane proteins that are critical for the execution of action potential and therefore for neuronal function and activation. Action potentials are the electrical impulses that travel along the axons of neurons and result from the movement of Na+ and potassium (K+) ions across the membrane. Binding of excitatory neurotransmitters to their receptors opens cation-permeable ion channels causing the membrane to depolarize or become more positive. This depolarization activates (opens) VGSCs allowing Na+ to enter the neuron further depolarizing the membrane. This increase in membrane permeability to Na+ is responsible for the rising phase of the action potential, eventually causing the membrane polarity to reverse (overshoot phase). The falling phase of the action potential is caused by the inactivation of the VGSCs and the opening of voltage-gated potassium channels allowing K+ to leave the cell. The efflux of K+ ions results in hyperpolarization (undershoot phase) of the membrane. Ultimately the voltage-gated K+ channels close and the membrane potential returns to its resting state. Type I and II pyrethroids cause stimulus dependent membrane depolarization and conduction block.

It is therefore biological plausible that binding to of a chemical substance to a VGSC leads sodium channels to open at more hyperpolarized potentials and are held open longer (disruption of channel kinetic), allowing more sodium ions to cross and depolarize the neuronal membrane (Shafer et al.2005)

Expression of VGSC are spatial and temporal dependent; however, it is biological plausible that also in developing brain pyrethroids would bound to VGSC isoforms and disrupt the channel gating kinetic (Shafer et al.2005, Soderlund et al. 2002).

3.1.2.2. Empirical evidence

Pyrethroids bind on the sodium channel α-subunit and affect nervous system function by altering their normal gating kinetics. Due to the extreme lipophilicity and the modest potency of pyrethroids radioligand, initial studies attempting to label the binding site were unsuccessful. The subsequent use of more potent radioligands were able to demonstrate high affinity saturable binding to brain sodium channels. However, the high lipophilicity of pyrethroids is still a limitation for the sensitivity of the assay and the identification of a single binding site on any given sodium channel and its mediated action (Soderlund et al., 2002; Trainer et al. 1997).

In hippocampal cells culture from rat postnatal day 2-4 pups, patch clamp preparations of isolated neurons showed that deltamethrin alter the VGSC kinetic and inhibits neuronal activity in glutamatergic networks of hippocampal neurons in a potent and concentration dependent manner (Meyer et al. 2008). Indeed, the actions of DLM are consistent with a decrease amplitude and number of spikes elicited by the current pulse (Meyer et al.2008).In vitro exposure to pyrethroids (the type I permethrin and the type II deltamethrin) has been shown to differently disrupt sodium channel gate kinetics (Meyer et al.2008) on hippocampal cultures from postnatal day 2-4 pups. This in vitro model was considered appropriate to explore effect on the developing brain. Cells were used for electrophysiological recording (patch clamp) 8-12 DIV and hippocampal neurons isolated from early postnatal rodents form spontaneously active networks of interconnected neurons in which both glutamate and GABAergic neurotransmission occurs. Deltamethrin decrease neuronal excitability as measured by the rate of sEPSC activity at the concentration of 0.1 µM. At this concentration decrease in sEPSC interevent interval was rapid, occurring within 1-3 minutes of exposure and persistent, lasting throughout the exposure period (9 minutes). The effect on sEPSC frequency was concentration dependent between 0.01 and 10 µM with an EC50 of 0.037 µM. There was no effect on the sEPSC amplitude at any tested concentration and this was consistent with previous data (Meyer and Shafer 2006), indicating that the effect does not include actions on post-synaptic glutamate receptors (Meyer et al. 2008).

3.1.2.3. Essentiality

Evidence from mutation and knockout models demonstrates that perturbation of VSSC function during development impairs nervous system structure and function. Knockout and mutant mouse models of sodium channel α subunits demonstrate varying degrees of adverse outcomes associated with loss or alteration of specific channel subunits. When mRNA for the Nav1.2 subunit was reduced by approximately 85%, mice exhibited reduced levels of electrical excitability, had high levels of apoptotic neurons in the brainstem and cortex, and died from severe hypoxia within 1–2 days of birth (Planells Cases et al. 2000).

In insect, only VGSC α are codified. Pyrethroid resistant, or knockdown-resistant houseflies are well known. As this mutation does not alter expression or localization of the VGSC, it was suspected to alter the affinity of the channel for pyrethroids. Expression of this mutant channel in Xenopus laevis oocytes resulted in VGSCs that were 10 fold less sensitive to cismethirin as assessed using voltage-clamp experiments (Wakeling et al. 2012).

In humans, a number of mutations have been identified in genes coding for VSSC subunits that result in neuronal hyperexcitability due to subtle changes in channel gating and inactivation (Meisler et al. 2001), these mutations have been linked to various forms of epilepsy (Shafer et al., 2005, Chahine 2018).

Pyrethroids, like these mutations, alter VSSC activation, inactivation, and neuronal excitability. However, the mechanisms and magnitude of mutational versus pyrethroid effects are different as well as the duration of the effect.

3.1.2.3. Dose concordance

For the Type II pyrethroid deltamethrin See Table 6.3_1

Although no evidence is available for the prototype stressor used in this AOP, deltamethrin, on the binding to VGSC, there is indirect evidence measuring the relationship between the MIE and the disruption of the VGSC gate kinetics. At concentration between 0.01 to 1 uM, deltamethrin has been shown to differently disrupt sodium channel gate kinetics (Meyer et al.2008) on hippocampal cultures from postnatal day 2-4 pups. The effect was measured using the restricted patch clamp methodology and the results indicated that the observed change was concentration dependent on the sEPSC without affecting the sEPSC amplitude and therefore excluding a post synaptic excitatory mediated effect.

3.1.2.4. Time concordance

For the Type II pyrethroid deltamethrin See Table 6.3_1

Changes in the VGSC kinetics are evident immediately following exposure in-vitro to deltamethrin and recorded up to 9 minutes (Mayer et al. 2008)

3.1.3. Uncertainties and inconsistencies

The fact that binding of pyrethroids to VGSCs results in altered sodium channel gate kinetics is well accepted and supported by a number of evidences. However, some minor uncertainties can be detected as reported below.

Uncertainties in the overall knowledge remain as the sodium channels' ontogeny is a complex process. Since brain development in both humans and rodents extends from early gestation through lactation is not possible to state with certainty which isoform of the sodium channels' α subunits is preferentially affected by deltamethrin.

For in vitro methodologies, there is still a lack of knowledge on stability of deltamethrin in the medium and the partitioning of this compound with plastic, lipid and protein. Indeed, the high lipophilicity of pyrethroids is still a limitation for the sensitivity of the assays and for the identification of a single binding site on any given sodium channel and its mediated action this may affect the sensitivity of the assays (Ruigt et al.1987). Also, the metabolic competence of the test systems used in various assays is unknown.

Moreover, the study from Meyer et al. (2008) is an indirect measurement of the interaction between the prototype stressor, deltamethrin, and VGSCs. Also, the exact temperature at which patch clamp recording was made is uncertain (in the publication it is stated at room temperature) and it is well-documented that pyrethroids effects on VGSCs are negatively temperature-dependent (reviewed in Narahashi, 2000). Finally, Meyer and colleagues used hippocampal cell culture from rats PND 2-4 which were not characterised and did not contain microglia or oligodendrocyte precursors cells, therefore there are still uncertainties in the knowledge of the interaction between pyrethroids and microglia or oligodendrocytes precursor voltage gated sodium channels.

Some inconsistencies can be observed in experimental studies. They are associated with the electrophysiological technique used to study ionic currents in individual isolated living cells, tissue sections, or patches of cells. The solution used in the bath can be similar to cytoplasm composition or completely different, they can be changed by adding ions or drugs to study the ion channels under different conditions. It is noted that in the study of Meyer et al. 2008 different effects, i.e. burst duration, were observed for permethrin (type I) and deltamethrin (type II) and it was not clear if this represents a

true difference in the mode of action between type I and type II pyrethroids or simply a difference between the two compounds. This could only be determined by the examination of additional chemicals.

See also Appendix B.

3.1.4. References KER1

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3.2. KER2. Long lasting modification of sodium channel gate leads to disruption of action potential generation; membrane depolarization

3.2.1. KER description

Long lasting modification of VGSC increases the channel open time. The direct consequence is a more hyperpolarized potential. These underlie the disruption of neuronal activity with changes in the ions intracellular concentrations and neuronal excitability. Depending on the time the channel is left open, the disruption of the action potential is getting qualitative different and this difference is measurable in electrophysiological recording of the action potential. Limited opening will lead to repetitive firing while

following prolonged opening the membrane potential ultimately becomes depolarized to the point at which generation of action potential is not possible (depolarization dependent block) (Shafer et al. 2005).

3.2.2. Biological domain of applicability

3.2.2.1. Taxonomic applicability

3.2.2.2. Life stage applicability

3.2.2.3. Sex applicability

3.2.3. Evidence supporting the KER

3.2.3.1. Biological plausibility for the KER

The falling phase of the action potential caused by the inactivation of the VGSCs and the opening of voltage-gated potassium channels allowing K+ to leave the cell. The efflux of K+ ions results in hyperpolarization (undershoot phase) of the membrane. Ultimately the voltage-gated K+ channels close and the membrane potential returns to its resting state. It is therefore biological plausible that changing the dynamic of VGSCs leads to a series of complex cellular events resulting in alteration of the firing rate as a final consequence. Type II pyrethroids cause stimulus dependent membrane depolarization and conduction block. Expression of VGSC are spatial and temporal dependent; however, it is biological plausible that also in developing brain pyrethroids would bound to VGSC isoforms and disrupt the channel gating kinetic (Shafer et al.2005, Soderlund et al. 2002).

3.2.3.2. Empirical evidence

Effect on the neuronal electrical activity using the type II pyrethroid deltamethrin is reported in Meyer et al.2008 when using hippocampal cultures from postnatal day 2-4 pups. The electrical changes indicate neuron depolarization and conduction block consequent to disruption of action potential generation with a dose-dependent inhibition of spontaneous glutamate release from hippocampal neurons. Deltamethrin inhibits spontaneous glutamate release from hippocampal neurons as measured by a decrease in sEPSC frequency during bursting release activity (Meyer et al. 2008). The effect is considered presynaptic because the decrease in sEPSC frequency following treatment with deltamethrin was not accompanied by changes in amplitude (Meyer et al. 2008). These data support the fact that deltamethrin decrease neuronal excitation by inhibition of the firing rate (inhibition of the spontaneous spiking activity) and the subsequent release of glutamate from the synapse.

Alterations of calcium dynamics are also reported for pyrethroids (Soderlund et al. 2002, Cao et al 2011). Extracellular calcium, rather than calcium release from the intracellular calcium stores, is the likely source for pyrethroid-induced elevation of calcium in neocortical neurons (Cao et al. 2011). The same paper demonstrates that L-type VGCCs, NMDA receptors, and the Sodium/Calcium exchanger accounted for the majority of pyrethroid-induced calcium entry. TTX completely abolished pyrethroid-induced calcium entry, indicating that these pathways were activated as a result of pyrethroid actions on VGSCs. In the case of L-type VGCCs, activation by deltamethrin is likely to have been secondary to depolarization of the cell membrane as a result of VGSC activation. Although it was not measured, it is likely that the depolarization and calcium entry resulted in glutamate release, which then activated NMDA receptors, resulting in additional Calcium entry. Finally, sodium entry through VGSC may have caused sodium loading of the neurons, which can result in a reversal of sodium/calcium exchange, which accounts for the contribution of this component to pyrethroid-induced calcium entry (Cao et al. 2011).

3.2.3.3. Essentiality

The sodium channel modulator veratridine (VTD) produce the same effect of deltamethrin. In patch recording, this compound rapidly reduced the number of sEPSC without affecting the number of individual burst, but at higher concentration (1uM) completely eliminated all sEPSC activity without affecting mEPSC frequency, similar to treatment to TTX (Meyer et al 2008). Both events - sE(I)PSCs and mE(I)PSCs are similar in the fact that they occur without any artificial stimulation. The difference between sE(I)PSCs and mE(I)PSCs is coming from the fact that in case of the sE(I)PSCs there is a chance of action potential-driven events due to intrinsic properties of presynaptic cell and/or network activity. All the mE(I)PSCs, in turn, are recorded in the presence of tetrodotoxin (TTX) which blocks action potential formation and its propagation, thus mE(I)PSCs are more "spontaneous" events than sE(I)PSCs and can be further used for the quantification of readily releasable pool size. So, it is useful to take both sE(I)PSCs and mE(I)PSCs from the same cell. First, one can record the sE(I)PSCs and then, by introducing TTX into bath solution the mE(I)PSCs. Having both sE(I)PSCs and mE(I)PSCs can help to understand where the changes in synaptic trasmission are coming from. Whether it is from the presynaptic side, or postsynaptic or both (Mayer et al. 2008). Titration with tetrodotoxin (TTX) produces a concentration-dependent reduction in the deltamethrin dependent calcium influx, indicating that the alteration in firing rate is consequent to the disruption in the VGSC (Cao et al. 2011).

3.2.3.4. Dose concordance

For the Type II pyrethroid deltamethrin See Table 6.3_1

Changes in VGSC kinetic and disruption of the action potential are reported in vitro at concentration between 0.01 to 1 uM, in hippocampal or neocortical neurons from postnatal day 2-4 pups (Meyer et al.2008, Cao et al. 2011).

3.2.3.4. Temporal concordance

For the Type II pyrethroid deltamethrin See Table 6.3_1

The two KEs were observed immediately following exposure to deltamethrin when measured in vitro up to 800 seconds recording in Cao et al. 2007 and immediately following exposure to deltamethrin when measured in vitro up to 9 minutes recording in Mayer et al. 2008.

3.3.4. Uncertainties and Inconsistencies

The mechanistic understanding of the generation of membrane potentials, based on Na, K, Cl and Ca ions is broadly accepted and extensive documentation is also available. However, some uncertainties can be detected. The uncertainties and inconsistencies detected in the Meyer et al. 2008 are also applicable for this KER (refer to chapter 3.1.3).

The events investigated by Cao (Cao et al., 2011) e.g. depolarization and calcium entry, glutamate release, activation of NMDA receptors and additional calcium entry, were not directly measured in the study. Moreover, as reported also for VGSCs, the action of pyrethroids on calcium channel is temperature dependent and may have an impact on the deltamethrin-induced calcium influx in neocortical neurons. in the study from Cao et al. 2011 the temperature at which the experiment was carried out is not reported. 9 out of 11 pyrethroids tested were able to produce a concentration-dependent elevation in intracellular calcium concentration in neocortical neurons which occurred secondary to activation of VGSCs. The nine pyrethroids that stimulated calcium influx displayed distinct efficacies. The rank order of efficacy for calcium influx was similar to that for sodium influx (Cao et al., 2009) with the exception of S-bioallethrin, which is the least efficacious compound on calcium influx. Deltamethrin, the prototype stressor for this AOP, is in position 6 (out of 9) in terms of potency.

It should be further noted that other ionic channels may have an impact on the action potential generation and in this regard the knowledge is limited.

Also, in this case, as reported already in paragraph 3.1.3, some inconsistencies can be observed in experimental studies. They can be associated with the electrophysiological technique used to study ionic currents in individual isolated living cells, tissue sections, or patches of cells. The solution used in the bath can be similar to cytoplasm composition or completely different, they can be changed by adding ions or drugs to study the ion channels under different conditions.

See also Appendix B.

3.3.5. References KER2

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3.3. KER3 Disruption of action potential generation leading to depolarization of terminal axon and change in neurotransmitters release.

3.3.1. KER description

Inhibition of activation and increasing channel open times have effect on action the action potential arriving at the axon terminal, alter the synaptic release of Calcium which is regulated by the voltage gate Calcium channels leading to effects on neurotransmitter release and neuronal excitability. VGSC and VGCC are critical to the regulation of neuronal excitability and neurotransmitter release in mammalian and insect neurons. This KER is therefore describing the impact of chemicals affecting the action potential and the impact of neuronal excitability and neurotransmission.

3.3.2. Biological domain of applicability

3.3.2.1. Taxonomic applicability

3.3.2.2. Life stage applicability

3.3.2.3. Sex applicability

3.3.3. Evidence supporting the KER

3.3.3.1. Biological plausibility for the KER

The frequency of all-or-none action potentials arriving at the presynaptic terminal is translated into a graded release of neurotrasmitters. The transmitter chemical rapidly diffuses across the synaptic cleft and then influences the postsynaptic cell. When the neurotransmitter is released from the presynaptic site, it moves through the synaptic cleft and binds to the receptor site on the postsynaptic membrane. This binding changes the permeability of the postsynaptic membrane by opening or closing ion channels and this in turn changes the membrane potential of that cell. At the axon terminal this process is activated by the action potential, opening the voltage gate Calcium channels in the presynaptic membrane. It is the Calcium that diffuse into the presynaptic terminal which triggers the release of neurotrasmitters in the synaptic cleft. It is therefore biologically plausible that disruption of the action potential at the axon terminal will have consequences on neurotransmitter release, neuronal excitability and synaptic transmission.

3.3.3.2. Empirical evidence

In vitro studies with pyrethroids using synaptosomes (isolated presynaptic terminals) showed an increased release of neurotrasmetters which were only partially blocked by TTX. A more sensitive enhancement in the release of neurotrasmitters was observed in preparations of nerve terminal depolarization when treated with pyrethroids. Pyrethroid enhance a Calcium dependent neurotrasmitters release and this effect was also partially blocked by TTX but was fully blocked by a Calcium channel blocker (Soderlund et al. 2002). In vivo studies measuring biochemical changes during the developmental period, although not definitive, suggests that following treatment with pyrethroids there was an increase in the expression of mAChR and alterations in catecholaminergic systems (Shafer et al. 2005).

Hippocampal neurons preparations for patch clamp and microelectrode array recording (MEA) from postnatal day 2-4 pups treated with the pyrethroids deltamethrin and permethrin showed a concentration dependent response on sEPSC, spontaneous spike rate and burst activity which were differently modulated by an inhibitor of GABA receptor and by an inhibitor of Calcium channel, suggesting that the effect on the network was a VGSC dependent mechanism. Similar effect was observed with the specific VGSC modulator veratridine while inhibition of voltage-gated calcium channels had no effect on spontaneous spike rate. The effect on spontaneous glutamate release was most likely presynaptic because although sEPSC frequency decreased, amplitudes were unchanged during and after treatment with both pyrethroids. In addition, in the same experiment, both pyrethroids disrupted the burst characteristics of hippocampal glutamatergic networks in MEA preparation. This would likely indicate that the effect observed originates from disruption of the action potential. The overall pattern of effects indicated a rapid decrease in total activity of excitatory neuronal networks by inhibiting the spontaneous spiking (action potential) and the subsequent release of glutamate from the synapse (Meyer et al. 2008).

In vivo studies with treatment with deltamethrin from PND 2 up to PND 28 PND and effect measured on PND 130 , showed a decrease in norepinephrine (Pitzer et al. 2019). In vivo, treatment of dams until delivery with measurement in pups on 21 PND showed decrease in brain derived neurotrofic factor-BDNF (CA1/hippocampus), decrease of phosphorylation of proteins (pCREB/CREB PTrkB/TrkB) and decrease of GluN1 GluN2A and GluN2B (Zhang et al. 2018).

3.3.3.3. Essentiality

In hippocampal neurons preparations for patch clamp and microelectrode array recording from postnatal day 2-4 pups, both deltamethrin and permethrin increased the spontaneous spike rates. In the presence of inhibitor of GABA receptors, both sEPSCs and and spontaneous spike rates were reduced in a concentration dependent manner. A similar effect was observed with the Similar effect was observed

with the specific VGSC modulator veratridine while inhibition of voltage-gated calcium channels had no effect on spontaneous spike rate (Meyer et al. 2008).

Release of neurotransmitters in synaptosomes from guinea pig cortex was partially blocked by TTX. Deltamethrin induced release on neurtramitters release following nerve terminal depolarization was partially blocked by TTX but completely blocked by calcium channel blocker.

3.3.3.4. Dose concordance

For the Type II pyrethroid deltamethrin See Table 6.3_1. [0.01-1] uM in patch clamp and MEA preparations from neocortical neurons (Meyer et al. 2008).

3.3.3.5. Temporal concordance

For the Type II pyrethroid deltamethrin See Table 6.3_1

The two KEs were observed immediately following exposure to deltamethrin when measured in vitro up to 9 minutes recording in Mayer et al. 2008 in patch clamp preparation and similarly in MEA preparation.

In vivo effect on neurotrasmitters release following deltamethrin administration was observed when measured on PND 130 in Pitzer et al. 2018 with treatment stopped on PND 28 and when measured on PND 21 in Zhang et al. 2018 with treatment performed during gestation.

3.3.4. Uncertainties and inconsistencies

This KER is supported by both in vitro and in vivo data. The biological processes that regulate the generation and propagation of action potential and neuronal transmission are very well known and changes in neurotransmitters release were also observed in vivo following administration of deltamethrin.

Some general uncertainties can be identified for the individual upstream or downstream KEs. For the in vitro methods the uncertainties listed in the previous paragraphs (e.g., lack of knowledge on stability of deltamethrin in the medium and the partitioning of the chemical with plastic, lipid and protein, the metabolic competence of the test systems) are also applicable here.

For the in vivo studies (Pitzer et al. 2019 and Zhang et al, 2018) which investigated the neurotransmitters release there is a lack of knowledge with regards to the real exposure of the mother and the pups. Indeed, few kinetic data is available and the toxicokinetic profile of deltamethrin is not known.

See also Appendix B.

3.3.5. References KER3

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Zhang, C., Xu, Q., Xiao, X., Li, W., Kang, Q., Zhang, X., ... & Li, Y. (2018). Prenatal deltamethrin exposure-induced cognitive impairment in offspring is ameliorated by memantine through NMDAR/BDNF signaling in hippocampus, Frontiers in neuroscience, 12, 615, https://doi.org/10.3389/fnins.2018.00615

3.4. KER 4. Depolarization of terminal axon and changes in neurotransmitter release leading to a decrease of neuronal network function.

3.4.1. KER description

The final outcome of neurodevelopment is the result of a normal endophenotype (brain morphology and functionality) and an healthy exophenotype (behaviour), which are dependent of a series of processes that are critical to nervous system development, including neuronal network formation. When growing in cell culture, neurons form spontaneously active networks of interconnected neurons in which both glutamate and GABAergic neurotransmission occurs. Neuronal networks from rat primary cortical cells culture and neuronal networks derived from human inducible pluripotent stem cells (hiPSC) have been characterized and in addition to excitatory and inhibitory neurons, astrocytes and a small percentage of microglial cells are present (Frank et al. 2017, Masjosthusmann et al. 2020). Depending on the model, immature cells are allowed to develop the neuronal network, modeling in vitro at a higher than cellular level of organization, the effects of toxicant on the neuronal network function. This KER is therefore describing transition from chemically induced alteration of firing rate and changes in neurotrasmitter at neuronal level to an higher level of organization, and this is possible using MEA recording.

3.4.2. Biological domain of applicability

3.4.2.1. Taxonomic applicability

3.4.2.2. Life stage applicability

3.4.2.3. Sex applicability

3.4.3. Evidence supporting the KER

3.4.3.1. Biological plausibility for the KER

Many different fundamental developmental processes must occur to lead to an integrated and functional neuronal network, including neurite outgrowth, maturation of glia, synaptogenesis, excitatory and inhibitory signaling, neurotransmitter recycling, and maintenance of electrochemical gradients (Frank et al 2017; Johnstone et al. 2010, Potter 2001, Radio and Mundy 2008). Control and function of neuronal network is the result of a series of elements like cellular inputs, neurotrasmitters and synaptic changes. The pattern of firing of cellular inputs and the nature of the neuroactive substance released, plus the intrinsic activity of the neuronal cells are critical for the network function (Faingold and Blumenfeld 2013). It is therefore plausible that changes in firing rate and changes in neurotransmitters would decrease network activity in networks containing both excitatory and inhibitory inputs.

3.4.3.2. Empirical evidence

Hippocampal neurons isolated from early postnatal rodents, form spontaneously active networks of interconneted GABAergic and glutamatergic neurons and their spontaneous activity at network level was recorded using microelectrode array recording (MEA) after exposure to the pyrethroids deltamethrin and permethrin. Both pyrethroids inhibited spontaneous spike rate in a concentration dependent manner. In this experiment burst analysis was conducted on data collected at the IC50

concentration. Burst duration and the percentage of spikes occuring in a burst were decreased. In the same experiment, but in patch clamp preparation, both pyrethroids inhibited spontaneous glutamate release from hippocampal neurons as measured by a decrease in sEPSC frequency during bursting release activity. Because in the patch clamp preparation sEPSC frequency decreased but amplitudes were unchanged during and after treatment with both pyrethroids, this would likely indicate that the effect observed originates from disruption of the action potential (Meyer et al. 2008).

Evaluation of pyrethroid effects on network formation in rat cortical neurons grown on microlelectrode arrays (rNNF), showed that 11 out 12 pyrethroids tested were active on at least one parameter in the MEA recording. Mutual information (a parameter indicative of neuronal network syncrony) appeared to be a sensitive endpoint for pyrethroids (Shafer et al. 2019; Frank et al. 2017). In a human cell based model of neuronal network formation (hNNF), deltamethrin showed a concentration response inhibition of 6 parameters, including mutual information. Most of the affected parameters in the hNNF preparation were also affected in the rNNF in a similar concentration range (Masjosthusmann et al 2020).

Table C.3. Summary table for effects of pyrethroids observed on changes in network activity

Reference	Cell-type	Exposure	Parallel viability or cytotox measured?	Chemicals tested
Alloisio et al. 2015 (926)	primary Wistar rat foetal day 18 cortical neurons	acute, 35 minutes on mature network. i.e. after 21-35 DIV	No	11 pesticides: insecticides (carbaryl, chlorpyrifos, deltamethrin, fipronil, Imidacloprid), herbicides (glyphosate, quinmerac, paraquat); fungicides: (dimoxystrobin, Orysastrobin), plant grown regulator (mepiquat. T) *Insecticides affected, except for Imidacloprid* *Herbicides not affected, except for Quinmerak* *Fungicides affected* *Growth regulator not affected*
Krishnan et al. 2016 (2842)	primary Wistar rat foetal day 18 cortical neurons	acute, 30 minutes on mature network, i.e. after 24 to 35 DIV	No	6 Pyrethroids: beta-cyfluthrin, bifenthrin, cypermethrin, deltamethrin, lambda-cyhalothrin, permethrin *rank order of IC50 of MBR was beta-cyfluthrin < lambda-cyhalothrin < deltamethrin < cypermethrin < bifenthrin < permethrin*

Vassallo et al. 2017 (2959)	A) primary cultures from embryonic (E16-18) rat cortex (Labs 1 and 3) B) primary cortical cultures from newborn (0–24 h) rats (Lab 2) C) cryopreserved embryonic (E14-15) mouse cortex (Lab 4)	acute, ? minutes on mature network, i.e. 3-4 weeks in vitro	no, but refers to earlier studies	6 chemicals: In vivo neurotoxic chemicals: Deltamethrin, Domoic Acid, Chlorpyrifos oxon In vivo non-neurotoxic chemicals: Dimethyl Phatalate, Glyphosate, Acetaminophen *Deltamethrin and other neurotox ref. chemicals positive in similar magnitude, negative reference chemicals are negative*
Frank et al. 2017 (30344475)	primary Long-Evans rat 0-24h postnatal cortical cells	developmental, exposure immediately after cell plating, re-administered at DIV 5 and 9; the 5 measurements were at DIV 2, 5, 7, 9, 12	yes at 12 DIV (CTB and LDH)	87 chemicals: 60 with in vivo DNT evidence (Mundy et al. 2015) + 4 tested positively in this assay earlier (bisindolylmaleimide I, loperamide, mevastatin, and sodium orthovandate) Acetaminophen as assay negative control. 21 wo in vivo DNT evidence (unknowns), at least 4 of them generally considered as safe (1,2-propylene glycol, amoxicillin, sodium benzoate, sorbitol) *Deltamethrin ranked between position 20 to 30.*
IUF/UKN report 2020	hiPSC-based GABAergic/ glutamatergic neurons and primary human astrocytes known proportion of cell types at start of experiment	developmental, exposure from 7 to 35 DIV, weakly measurement, with wash-out before each of the 5 measurements	yes	Deltamethrin & Flufenacet *Deltamethrin affected several endpoints, Flufenacet not.*

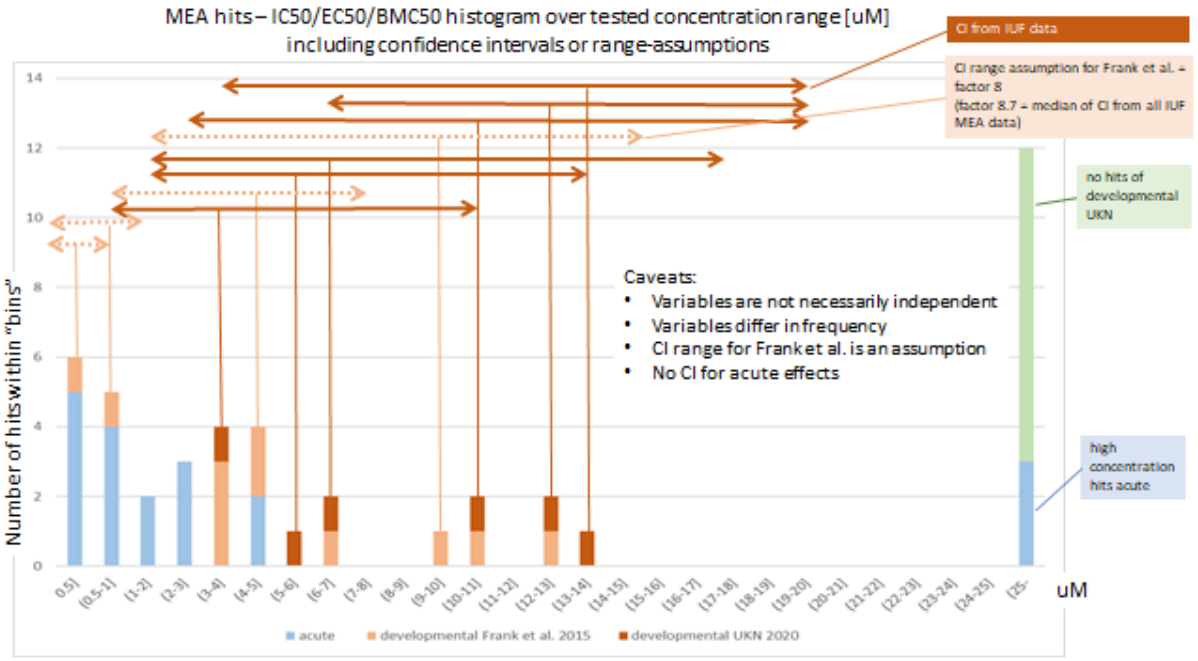
3.4.3.3. Essentiality

In Meyer et al. 2008, the spontaneous activity recorded on MEA chips, resulted in a rapid and complete cessation of spiking and bursting activity following exposure to TTX, indicating that the events were

dependent on VGSC activity. In both MEA and patch preparation, the sodium channel agonist veratridine showed a rapid and reversible decrease in spike rate of hippocampal neurons (Meyer et al., 2008).

3.4.3.4. Dose concordance

For deltamethrin see Table 6.3_1. For deltamethrin effects on the MEA was observed in a range of concentration from 0.45 to 11.2 µM, depending on the test system and test method applyied (Alloisio et al. 2015; Krishnan et al. 2016; Vassallo et al. 2017; Frank et al. 2017; Shafer et al. 2019; Masjosthusmann et al., 2020).



3.4.3.5. Time concordance

For deltamethrin see table 2. Acute exposure to deltamethrin of rNNF preparations on day 21 to 35 in vitro (DIV) showed effects from few minutes to 35 minutes (Alloisio et al. 2015; Krishnan et al. 2016; Vassallo et al. 2017). Chronic exposure to deltametrin on rNNF from the first week up to 28 DIV or in hNNF from day 12 to 35 DIV (Frank et al. 2017; Shafer et al. 2019; Masjosthusmann et al., 2020).

3.4.4. Uncertainties and inconsistencies

It is well recognized that the maintenance of electrochemical gradients and neurotransmitter release are fundamental processes that must occur to lead to functional network.

For this KER some uncertainties but no inconsistencies are identified. First of all, there is a lack of direct evidence assessing that the upstream KEs is associated with a corresponding in the occurrence of KE4.

Moreover, deltamethrin effects on the MEA were observed in a range of concentration from 0.45 to 11.2 µM, which depends on the test system and test method applied. The choice of this range was based on an Expert Knowledge Elicitation (EKE) approach (see Appendix B) and considers different uncertainties that can have affected the results e.g. the variability of the different tests and systems, the shape of the different dose-response and the lack of a pragmatic approach to guarantee sufficient separation among concentrations.

In addition to this, the impact of the protective role of microglia remains an uncertainty to be further investigated.

See also Appendix B.

3.4.5. References for KER4

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3.5. KER5. Decrease of neuronal network during development causes altered behavioral function (Non-adjiacent KER)

3.5.1. KER Description

Within the AOP conceptual framework, disruption of ion channel function and changes in cellular excitability may serve as MIE and KE, leading to neurotoxicity following compound exposure. These effects were empirically supported by a series of assays conducted with deltamethrin and pyrethroid

substances, most of them conducted in vitro; therefore, this evidence support mechanistically and empirically the biological plausibility that exposure to deltamethrin and pyrethroids can potentially lead to neurotoxicity. Neurotoxicity of pyrethroids, including deltamethrin has been well characterized (Soderlund et al., 2002). Although age dependent differences in sensitivity to pyrethroids are known (Shafer et al. 2005), available studies of pyrethroids linking target tissue events and AOs are lacking or only partially covering the sequence of events when considering the potential role of perturbation of VGSCs and developmental neurotoxicity. Considering the methodological and scientific uncertainties in defining causation for changes observed in animal testing including sensory motor reflex and cognitive processes (Tsuji and Crofton, 2012), the link between decrease of neuronal network formation and the AOs will be mainly based on the empirical evidence scrutinized for deltamethrin and the biological plausibility. Dose concordance will additional benefit of the available information provided to support the physiologically based pharmacokinetic modeling for deltamethrin; see note to the dose and time concordance table (US EPA 2017).

3.5.2. Biological domain of applicability

3.5.2.1. Taxonomic applicability

3.5.2.2. Life stage applicability

3.5.2.3. Sex applicability

3.5.3. Evidence supporting the KER

3.5.3.1. Biological plausibility for the KER5

There is consensus that chemical mediated alterations on cellular events that are critical to normal development, can induce neurodevelopment disorders (Bal-Price et al. 2018, Mundy et al. 2015, Masjosthusmann et al. 2020). These cellular events include neural stem cell proliferation and differentiation, migration, neurite outgrowth, synaptogenesis, network formation, myelination and apoptosis (Harrill et al. 2018, Masjosthusmann et al. 2020). Many different fundamental neurodevelopment processes must occur to lead to functional networks *in vitro*, including neurite outgrowth, maturation of glia, synaptogenesis, excitatory and inhibitory signaling, neurotransmitter recycling, and maintenance of electrochemical gradients (Frank et al., 2017). Pyrethroids, and deltamethrin can alter the neuronal electrochemical homeostasis and disrupt neuronal network formation in *in vitro* models relevant to assess chemical contribution to developmental neurotoxicity.

Shafer et al. 2005, reviewed the outcome of a series of available and peer reviewed in vivo studies conducted with type I and type II pyrethroids, including deltamethrin. Because of the methods variability and limitations in the data set and because there is an unresolved conceptual gap on lack of knowledge to conclude on relationship between the observed biochemical and behavioral changes observed and ion channels disruption, they conclude that there are still several research needs in the area of developmental neurotoxicity and pyrethroids.

These studies were however not included in the analysis of evidence performed by EFSA and the AOs proposed in this AOP is based on the outcome from studies with the exposure of pregnant dam (Zhang et al. 2018) or in post-natal period (Pitzer et al. 2018). The proposed AOs are therefore the result of an uncertainty analysis followed by an expert knowledge elicitation and the biological plausibility is based on the scientific consensus that disruption of neuronal network formation during the process of brain development can lead neurodevelopment disorders.

3.5.3.2. Empirical evidence

It appears practically impossible to measure decreased neuronal network activity in parallel to altered behavioural function within the same test system. Moreover, neuronal network activity is measured in human cell system and behaviour is measured in the rat.

However, several biochemical and behavioral changes are described for type I and type II pyrethroids including deltamethrin (Shafer et al. 2005). Moreover, clear evidence for a decreased neuronal network activity is available for DLM and other pyrethroids (see section 4.4.3.2) on the one hand, and evidence is also available for DLM and other pyrethroids on rat behavioural endpoints, on the other hand:

Deltamethrin induced changes in sensory motor reflex only in males in Pitzer et al. 2019 and impairment of learning functions in two different learning and memory behavioral tests (CWM; MWM) when orally administered by gavage to pups until PND 28 (Pitzer et al. 2019, CWM) and when administered to dams during pregnancy (Zhang et al. 2018, MWM).

Table C.4. Summary table of evidence for Type II pyrethroid DLM on altered behavioural function in rats

Reference	Testing	Endpoints affected within NOAEL-LOAEL range
Pitzer et al. 2019: Sprague-Dawley rat pups exposure by gavage from PND 3 to 20 at 0.25, 0.5, 1 mg/kg bw day	acoustic and tactile startle response, peak amplitude and pre-pulse inhibition	↑ Peak amplitude in males only (Traditionally both male and female rats have been used in measures of startle reflex magnitude and plasticity. However, the effects of rat gender on this variable has not been studied systematically (Geyer and Swrdlow. 1998). NOAEL/LOAEL = 0.25/0.5 mg/kg bw d No effect on pre-puls inhibition
TG 426 OECD (2007) Wistar rat dam exposure by diet from GD6 to LD21 at 1.8, 7, 16 mg/kg bw d	acoustic startle response, peak amplitude, latency to peak and habituation for both	No statistically significant and biologically relevant effect
Pitzer et al. 2019: Sprague-Dawley rat pups exposure by gavage from PND 3 to 20 at 0.25, 0.5, 1 mg/kg bw day	Cincinnati Water Maze Test	↑ acquisition latency and related increase in number of errors to find the exit in a labyrinth in males pups gavage NOAEL/LOAEL= 0.5 / 1 mg/kg bw d
	Water Morris Maze	no statistically significant and biologically relevant effect
	Conditioned Freezing Test	
Zhang et al. 2018 Sprague-Dawley rat dam exposure by gavage from GD0 to delivery at 0.54, 1.35, 2.7, 9 mg/kg bw day	Water Morris Maze	↑ escape latency and swimming distance to find the hidden platform (effect in acquisition phase) ↓ time spent in searching the removed platform in the correct target quadrant (effect in the retention phase) NOAEL/LOAEL= 2.7 / 9 mg/kg bw d
TG 426 OECD (2007) Wistar rat dam exposure by diet from GD6 to LD21 at 1.8, 7, 16 mg/kg	letter M Water Maze	no statistically significant and biologically relevant effect between dams dietary levels of 0.25 and 1 mg/kg bw d

bw d		

Further studies with DLM testing locomotor activity in zebrafish using different protocols (Dach et al., 2019, Awoyemi et al., 2019, Kung et al., 2015) were in first instance considered to contain a low risk of bias. However, after a detailed review, it was concluded that in these studies the probability that locomotor activity was affected is lower than 66%. Therefore, these study results for not further considered in the subsequent ad hoc AOP generation.

For other pyrethroids rat in vivo behavioural data are also available, and these are concordant with the KE4 or KE5 data for about 50% of the pyrethroids with available data for both of these AOP levels. This may, at least partly be due to uncertainties in the rat data (see section 4.5. and 6.3).

Various human observational studies, prospective birth cohort studies, are available in literature linking measurement of specific and non-specific pyrethroids metabolites in urine of mothers during pregnancy or at the first year of age and developmental neurotoxicity impairments in children such as behavioural effects, impaired psychomotor development, cognitive impairment, ADHD and communication disorders (for a literature review of the evidence for deltamethrin see EFSA scientific opinion human contextualization).

However, causal association is difficult to demonstrate with these observational studies and there is a big uncertainty in these studies' since direct exposure to deltamethrin was not assessed, instead a surrogate was used consisting of measuring specific and non-specific metabolites (cis-DBCA and 3-PBA, respectively), when measuring 3-PBA, whereas not for cis-DBCA, may suggest that the effect observed may be related to pyrethroids other than deltamethrin specifically.

3.5.3.3. Essentiality

3.5.3.4. Dose concordance

3.5.3.5. Time concordance

3.5.4. Uncertainties and inconsistencies

It is well recognized that the knowledge about the variability of the current regulatory rodent in vivo DNT testing and assessment results is uncertain. This is due to the high number of potentially relevant DNT endpoints and the flexibility for their selection and way of measurement, recognized potential interferences from the experimental set-ups, paucity of method specific positive and historical control data and the assessment of many complex data, which these methods provide (summarized in Paparella et. 2020).

Moreover, uncertainties are also recognized for the use of regulatory in vivo DNT testing and assessment for human hazard and risk extrapolation. These may be due to the difficulty to differentiate primary DNT effects from those that are secondary to more general toxicity, some interspecies differences in brain anatomy development and function and differences in kinetics and biotransformation of chemicals as well as the impossibility to test with animals many orthogonal endpoints and include higher cerebral and nervous system tasks (summarized in Paparella et al.,2020). Last but not least, DNT may have multifactorial causes with chemical exposure just being one of these and others include epigenetic and genetic background, socio-economic status, diet, life style, stress and co-exposure including environmental contaminants and drugs or maternal infection and viruses, for instance (De Felice et al. 2015). Such human variability is an additional huge challenge for the extrapolation to exposure values resulting in a defined proportion of population under risk for defined effects (sizes).

These aspects of uncertainty may explain that KER5 as well as the relationship between AOs at rodent organism level and AOs at human population level effects may be largely based on biological plausibility.

Uncertainties specific for the rodent in vivo DNT findings for DLM are summarized in the section for AO (see paragraph 6.2) .

3.5.5. References KER5

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4. Non-Adjacent KER descriptions

4.1. KER6. Disruption of sodium channel gate kinetics leading to decreased oligodendrocytes differentiation

4.1.1. Biological Domain of Applicability

4.1.1.1. Taxonomic Applicability

Vertebrates and Invertebrates.

4.1.1.2. Life Stage Applicability

All life stages. This AOP specifically refers to the developmental period.

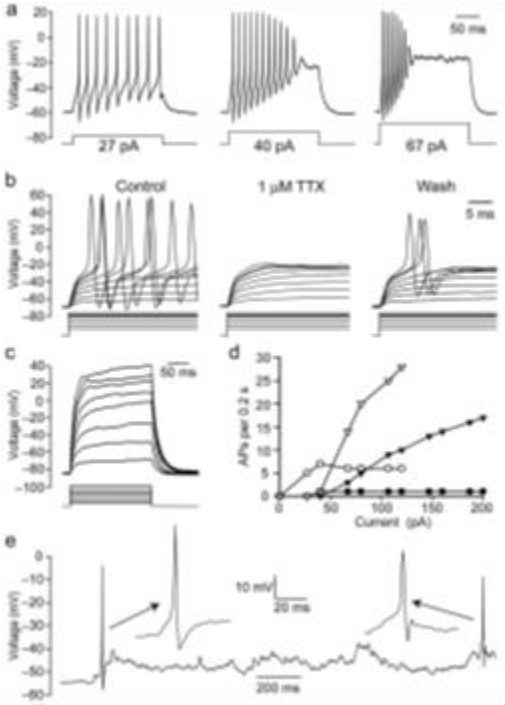
4.1.1.3. Sex Applicability

Mixed.

4.1.2. KER Description

Voltage-gated sodium channels are found to be present on oligodendrocytes progenitor cells (OPCs) derived both from rodents' preparations (Sontheimer et al. 1989, Barres et al. 1990, Williamson et al. 1997, Paez et al. 2009) and human pluripotent stem cells (Livesey et al. 2016, Marton et al. 2019). These studies on OPCs in cultures identified voltage-activated Na+ currents that possess features associated with neurons, including rapid kinetics of activation and inactivation and sensitivity to TTX. In particular it was reported by Káradóttir and coworkers, that 46 % of OPCs in the cerebellum of PND 7 rats, exhibited a transient inward current on depolarization beyond -50mV, which was reversibly blocked by TTX, and was inactivated by maintained depolarization to -30 mV, indicating that it is mediated by voltage-gated Na+ channels. The peak amplitude of the Na+ current was 1 nA (1.09 ± 0.13 nA, n=76) on depolarization from -70 mV to -10 mV (Figure C. 13).

Figure C. 13. OPCs with Na+current generate action potential
(Source: Káradóttir et al. 2008).



The expression of Na+ channels is downregulated as OPCs mature into differentiated OLs. As reported by Sonthemeier (

Figure C. 14), sodium currents are present in most A2B5-positive cells, observed in a fraction of 04 positive cells and absent in 01 positive cells [1]. This progressive reduction of functional sodium channels in the oligodendrocyte's lineage could be suggestive of a role of these channels in the process observed at early stage of development. Recent work demonstrated the involvement of the VGSC $Na_v1.2$ in axon myelination (Berret et al. 2017).

[1] In oligodendrocytes development, the sequential expression of developmental markers, identified by a panel of cell specific antibodies (e.g. A2B5; 04 and 01) divide the lineage into distinct phenotypic stages (Baumann e al. 2001)

Figure C. 14. Left: expression of proton-activated ion channels in neurons and oligodendrocytes in contrast to Shwann cell and Astrocytes. Right: expression of proton-activated ion channels in glial precursor cells and oligodendrocytes; precursors can be distinguished from the more mature oligodendrocytes by the ability to generate voltage-activated Na+ and K+ currents

(source: Sontheimer et al. 1989)

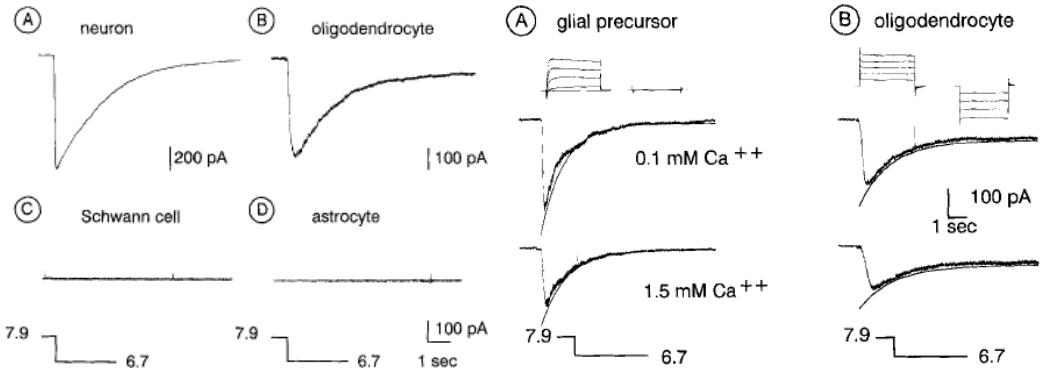
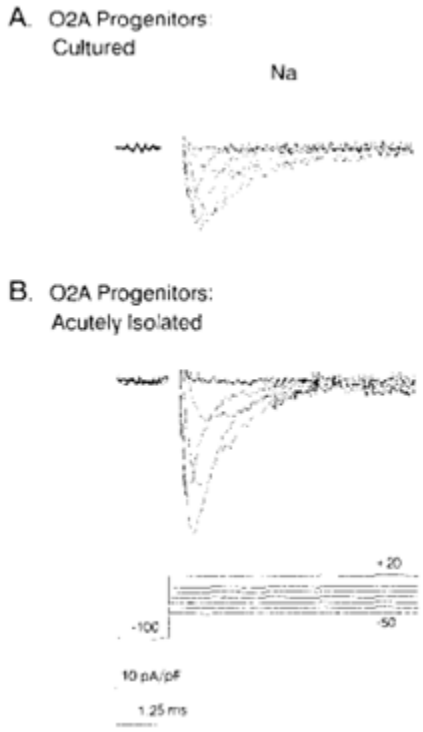


Figure C. 15. Voltage sensitive currents in O-2A Progenitors

(modified from Barres et al. 1990).



4.1.3. Biological domain of applicability

4.1.3.1. Taxonomic applicability

4.1.3.2. Life stage applicability

4.1.3.3. Sex applicability

4.1.4. Evidence Supporting this KER

4.1.4.1. Biological Plausibility

The association between these two KEs is supported by a number of studies. Alteration of voltage gated sodium channels kinetics is expected to encompass different steps including changes in intracellular ions concentration and changes in the action potential. Besides sodium channels, cells of the oligodendrocyte lineage express and are capable of responding to a diverse array of ligand-receptor pairs, including neurotransmitters and nuclear receptors such as γ-aminobutyric acid, glutamate, adenosine triphosphate, serotonin, acetylcholine, nitric oxide, opioids, prostaglandins, prolactin, and cannabinoids These signals might interact with each other and commonly contribute to toxicity (reviewed in Marinelli et al. 2016).

Berret and colleagues in 2017, demonstrated that Nav1.2 mediated excitability is required for Pre-myelinated Oligodendrocytes (pre-OLs) to form and extend their processes, which facilitate proper contacts with axons for myelination. The study has been conducted on brain slices (PND 5-62) from aN auditory region of rat brainstem, called medial nucleus of the trapezoid body (MNTB), a highly myelinated and synapse-rich area. To understand the role of Nav 1.2 in excitable pre-OLs, they induced a **knockdown** of Nav1.2 using an adenovirus expressing hairpin RNS (shRNA) against rat Nav1.2 and demonstrated that sodium channels are necessary for a proper elaboration of pre-OLs structure and interaction with axons and that their loss has an impact on myelination (Berret et al., 2017).

Volpe and colleagues in 2011, while studying the brain injuries affecting premature infants, reported that pre-OLs are highly vulnerable to a series of maturation-depending factors such as: microglial activation, excitotoxicity, and free radical attack. Excitotoxicity vulnerability of the pre-OL cells has been studied in both experimental animal models and human brain to understand the pathogenesis of **Paraventricular Leukomalacia (PVL)** the main white matter injury observed in premature infant brains. In a sheep model of PVL elevated extracellular glutamate have been documented and the extend of glutamate increase has been correlated directly to the ultimate extend of white mater injury. The source of glutamate in cerebral white matter are the glutamate transporters. Indeed, under conditions of ischemia and a failure of the ATP-dependent Na+/K+ pump and the consequent loss of the Na+ gradient across the plasma membrane, the high affinity Na+-dependent glutamate transporters fail and operate in reverse. The elevation of glutamate in the extracellular space lead to receptor mediated toxicity in pre-OLs, eventually lead to cell injury (reviewed by Volpe et al., 2011).

A number of *In vitro* and *in vivo* models of **ischemia** support the evidence that an activation of AMPA/KA receptors in pre-OLs lead to cell death. Rats on post-natal day 7 subjected to unilateral carotid ligation and hypoxemia eventually incurred in pre-OLs death and subsequent failure of myelination, as in human PVL. In the same study it was reported that both pre-OL injury and hypomyelination could be prevented by systemic administration of an AMPA/KA antagonist, called NBQX (Follett et al., 2000) suggesting the important role of an altered electrophysiology. Subsequently, also topiramate, an AMPA blocker, has been shown to markedly attenuate pre-OL injury in a neonatal rat model (PND7) of selective hypoxic-ischemic white matter injury (Follett et al., 2004). Similarly, the NMDA receptor blocker, memantine, also

has been protective to pre-OLs in the same model of selective white matter injury (Manning, 2004). An additional study conducted on mice cerebellar slices on postnatal day 7 (PND7), demonstrated that OPCs expressing VGSCs are more susceptible to ischaemia conditions. In this study, PND 7 cerebellar slices were exposed to ischemia condition for 1 hour in the presence of propidium iodide to label dead cells, and then fixed the slices and labelled them with antibodies to NG2 and to Na+ channels. Consistent with this, 1 h of ischemia killed 35% of Na+ channel–expressing cells, but only 2% of cells lacking Na+ channels, and this selective vulnerability was mediated by glutamate receptors. This suggests that the class of OPCs expressing Na+ channels will be preferentially damaged in conditions leading to cerebral palsy or in ischemia caused by stroke or spinal cord injury in the adult and implies that future therapeutic strategies should focus on these cells (Káradóttir et al., 2009).

Altogether these studies support the association between disruption of voltage sodium gated channels kinetics and decreased of oligodendrocytes differentiation.

4.1.4.2. Empirical Evidence

It was recently demonstrated by Masjosthsumann et al. 2020 that pyrethroids, such as deltamethrin, may lead to an impairment of oligodendrocyte differentiation at low concentrations (0.5 uM with a CI [0.25-0.76] uM) decreasing the pool of functional myelinating cells.

As it is scientifically accepted that pyrethroids operate through the blockage of voltage gated sodium channel, the following alteration of electrophysiological membrane properties could be considered as the leading reason of a reduction of mature oligodendrocytes observed in the work of Masjosthsumann and co-workers (Masjosthusmann et al., 2020).

4.1.4.3. Essentiality

4.1.4.4. Dose Concordance:

Table C.5. Dose concordance

Reference	Result
Masjosthusmann et al., 2020 NPC 5 (primary human NPCs from fetus): 3D assay, 120h differentiation (by counting O4 positive cells versus all cells)	Clear dose-response with mean BMC30 = 0.5 - CI 95: [0.25-0.76] uM

The KE of oligodendrocyte differentiation was considered to be affected within this specific assay with a probability of ≥ 66% based on the result of an Experts-Knowledge-Elicitation assessment.

4.1.4.5. Temporal concordance

Table C.6. Temporal concordance

Reference	Result
Masjosthusmann et al., 2020 NPC 5 (primary human NPCs from fetus): 3D assay, 120h differentiation (by counting O4 positive cells versus all cells)	Clear dose-response with mean BMC30 = 0.5 - CI 95: [0.25-0.76] uM

4.1.4.6. Incidence Concordance

Not applicable

4.1.5. Uncertainties and Inconsistencies

Currently only one assay and report are available addressing this endpoint (Masjosthsumann et al. 2020; NPC5). With this assay a wide variety of chemical stuctures was tested including heavy metals, organophospahtes, organochlorines, phenylpyrazole, alkylamine derivative, glucocorticoid, pyridine derivate, neonicotinoid, polybrominated diphenyl ethers, bisphenol, carbamate, piperidine. In the work of Masjosthsumann et al. 2020; 49 out of 117 chemicals tested were positive for this endpoint, if chemicals with unspecific effects were excluded (including borderline and specific effects, based on comparison of BMC30 oligodendrocyte differentiation with BMC30 viability within the table A3 from Annex to the report). Also Flufenacet provides a hit in this assay, but Cypermethrin is negative.

However, the potency among the 47 hits ranged between 0.2 – 18 uM and Deltamethrin is on position 6 in terms of potency, Flufenacet on position 47. If chemicals are ranked for the ratio BMC30 oligo. diff/BMC30 viability the total range is 1 – 154 and Deltamethrin is on position 3, Flufenacet remains on position 47. Beyond hit rate, the relative potency of chemicals may be relevant to consider the specificity of this assay.

Moreover, due to the fact that cells of the oligodendrocyte lineage express and are capable of responding to a diverse array of ligand-receptor pairs, including neurotransmitters and nuclear receptors such as γ-aminobutyric acid, glutamate, adenosine triphosphate, serotonin, acetylcholine, nitric oxide, opioids, prostaglandins, prolactin, and cannabinoids (reviewed in Marinelli et al. 2016) and are specifically vulnerable to oxidative stress and excitotoxicity (Volpe et al. 2011), there is a scientific rationale for these findings. However, mechanistic establishment of individual compound effects on oligodendrocyte lineage cells needs to be established.

4.1.6. References KER6

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4.2. KER7. Decrease oligodendrocyte differentiation leading to impairment behavioral function.

4.2.1. Biological Domain of Applicability

4.2.1.1 Taxonomic Applicability

Vertebrates and Invertebrates.

4.2.1.2. Life Stage Applicability

All life stages. This AOP specifically refers to the developmental period.

4.2.1.3. Sex Applicability

Mixed.

4.2.2. KER Description

Oligodendrocytes (OLs) are cells that myelinate axons providing saltatory conduction of action potentials which establish subcortical white matter of the brain. It has been shown that myelination is a dynamic and plastic process with an excess of oligodendrocytes precursor cells being generated and then abolished if not integrated into neural circuits. Decrease of oligodendrocytes or a lack of these cells as well as an interference with OLs differentiation and myelin formation during brain development can impair brain performance in child due to reduce level of myelin (hypomyelination), resulting in various adverse outcome, including different behaviour alterations (e.g. cognitive, attentional, behavioural, and social deficits).

4.2.3. Evidence Supporting this KER

4.2.3.1. Biological Plausibility

It has been observed that of the 65000 very low birth infants born each year in the United States almost 25-50% exhibit cognitive/attentional/behavioural/socialization defects that impair the quality of life (reviewed by Volpe et al. 2011).

Brain injury in the premature infants is a problem of major importance. The dominant lesion found is a form of cerebral white matter injury termed periventricular leukomalacia (PVL) (reviewed by Volpe et al. 2011; Perlman, 1998). Characterized by complex pathological processes, including the death of pro-oligodendrocytes (Rezaie & Dean, 2002).

Several studies underline that injuries to pre-OLs are responsible for hypomyelination indicating that a premature loss of these precursors lead to a reduction of mature functional oligodendrocytes.

Back and co-workers observed that late OL progenitors (positive 04+ cells) are selectivly vulnerable to hypoxia-ischemia and this may underlie the cellular basis for PVL (Back et al. 2001). In addition to this, in a study conducted by Follett and colleagues, rats on post-natal day 7 subjected to unilateral carotid ligation and hypoxemia eventually incurred in pre-OLs death and subsequent failure of myelination, as in human PVL (Follett et al., 2000).

It was further demonstrated that in the premature infant brain affected by PVL, focal necrosis with loss of all cellular elements and specific acute loss of early differentiating oligodendrocytes occurred (Figure C. 16). Overall, PVL can be cystic or noncystic but in both cases the diffuse injury to pre-OLs occurs and is followed by a deficit in mature, myelin producing OLs and as consequence cerebral hypomyelination and cognitive defects take place (reviewed by Volpe et al. 2011).

Figure C. 16. Periventricular leukomalacia. Coronal section of cerebrum (H+E stain) in a premature infant
(source Volpe et al. 2011).

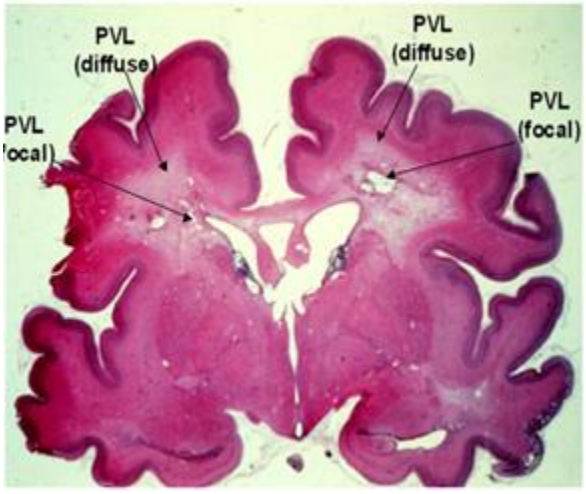
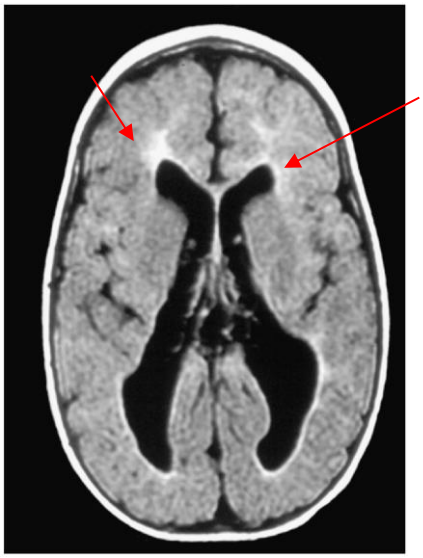


Figure C. 23. Axial MRI (FLAIR) of cerebrum in a 20-month old infant with PVL.
(adapted from Volpe et al. 2011).



4.2.3.2. Empirical Evidence

not applicable

4.2.3.3. Essentiality

4.2.3.4. Dose concordance

Dose and temporal concordance is considered moderate low, since dose concordance between effect levels for in vitro level effects and in vivo behavioural effects could be supported with available kinetic data. Moreover, within the rat studies for deltamethrin there is evidence that doses that are affecting developmental stages in the rat are not affecting adult rats.

4.2.3.5. Time concordance

Temporal concordance is shown indirectly, since the effect concentration from in vitro methods resembling developmental stages are concordant with in vivo concentrations affecting the development stages in the rat rather than adult stage

4.2.4. Uncertainties and inconsistencies

It is commonly accepted that a decrease in myelination can lead to a reduction of the speed of axon propagation, eventually reducing the startle reflex. However, there is a lack of knowledge with regard to the relationship between the measured behavioural impairment and the underlying brain structure for many neurological functions. This is the reason why the KER is considered non-adjacent. Further knowledge is needed to investigate relationship between decreased oligodendrocytes differentiation (KEupstream) and behavioural impairment (AO).

Moreover, it is important to underline that the oligodendrocytes differentiation assay (used as evidence to support the upstream KE) was conducted on human test system therefore a species-specificities cannot be excluded and remain an uncertainty to be addressed.

See also Appendix B.

4.2.5. References KER7

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4.3. KER8. Disruption of sodium channel gate kinetics leading to increase of intracellular sodium in microglia cells.

4.3.1. Biological Domain of Applicability

4.3.1.1. Taxonomic Applicability

Vertebrates and Invertebrates.

4.3.1.2. Life Stage Applicability

All life stages. This AOP specifically refers to the developmental period.

4.3.1.2. Sex Applicability

Mixed.

4.3.2. KER Description

Disruption of VGSCs kinetics in microglia cells may lead to an alteration of intracellular sodium contents in these cells.

4.3.3. Evidence Supporting this KER

4.3.3.1. Biological Plausibility

It has been demonstrated that the blockade or modification of the VGSC in microglia, affects multiple functions of these immune cells. For instance, neuronal cell death induced after a cell injury by the activation of microglia, can be mitigated by sodium channel blocker (e.g. antiepileptic drugs, TTX) via inhibiting inflammatory responses such as phagocytosis, cytokines release and microglia migration (Aloisi et al. 2001; Black et al. 2009; Black and Waxmann, 2012; Jung et al. 2013). Similar evidence has been reported in an experimental autoimmune encephalomyelitis (EAE) model mice, an inflammatory/demyelinating model of multiple sclerosis (Black and Waxmann, 2012).

Based on the above-mentioned evidence, it is biologically plausible to assume that the prolonged activation of VGSC in primary microglia cells leads to a rapid influx and accumulation of Na+ which eventually results in microglia activation.

4.3.3.2. Empirical Evidence

One of the mechanism responsible for microglia activation could be explained through the results obtained in a recent study by Hossain and colleagues (2017). Based on the presence of sodium channels on microglia cells and the knowledge commonly accepted that pyrethroids pesticides delay the inactivation of VGSC, the group measured Na+ influx and Na+ intracellular accumulation in BV2 (immortalized mouse microglia cells) and primary microglia cells (PND 1-2 pups mice) after the exposure to permethrin and deltamethrin insecticides.

At concentration of 1 uM both deltamethrin and permethrin caused a rapid increase of sodium influx by about 43 % in BV2 cells and 28 % in primary cells. [(Na+)i] was measured at 24 and 48 hours after permethrin or deltamethrin exposure. Both pyrethroids caused a dose-dependent and time dependent accumulation of sodium in BV2 and primary microglia cells (after 24 hours at 1 uM 125% of intracellular sodium compared to control; Figure C. 18).

Figure C. 18. Sodium influx changes after deltamethrin exposures (source Hossain et al. 2017).

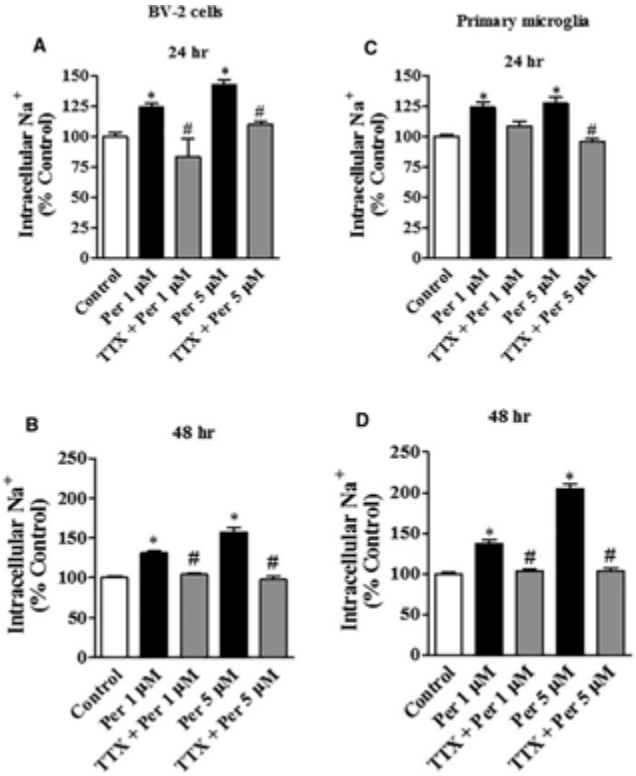
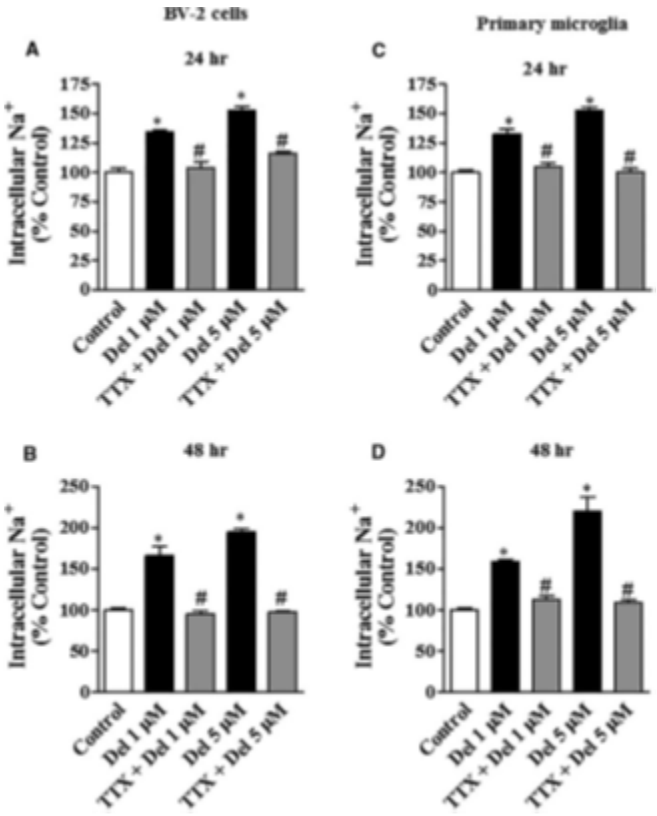


Figure C. 19. Changes in sodium intracellular accumulation in microglia following the exposure to deltamethrin
(source: Hossain et al. 2017)

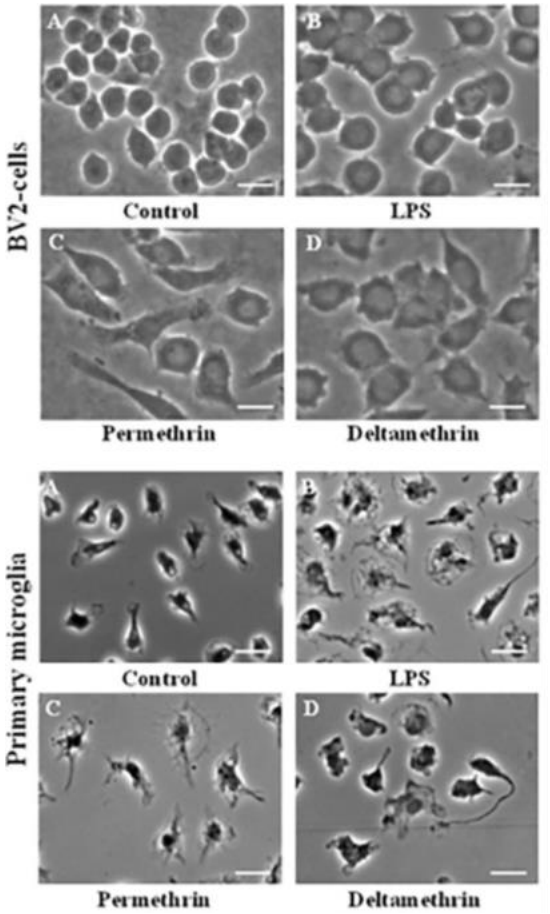


In addition to this it has been demonstrated that the excessive accumulation of sodium in cells resulted in microglia activation and release of the pro-inflammatory cytokines TNF-α, playing a pivotal role in initiating the inflammatory process (Hossain et al. 2017).

Microglia activation after pyrethroids exposure has been examined through an analysis of microglia morphology. These cells become much larger and with a thick cytoplasmic projection (Figure C. 19).

Figure C. 20. Morphology changes observe in primary microglia cells and BV2 after pyrethroids exposures.

LPS has been used as positive control (source Hossain et al. 2017).



Overall, primary microglia cells isolated from 1- 2- day-old pups are more sensible to pyrethroids effects rather than immortalized microglia cells, BV2. This may be the results of differences in VGSC isoforms expression, as Na_v 1.6 is most sensitive to pyrethroids modification, is more expressed in primary microglia cells. In addition, primary microglia cells are isolated from neonatal mice, and developing brain in mammals is more sensitive than adult to pyrethroids.

4.3.3.3. Essentiality

In the study from Hossain et al. 2017 it has been demonstrated that the blockage of VGSC with TTX in microglial cells resulted in a reduction of intracellular Na+ accumulation and decreased production of TNF-α. This demonstrated that the prolonged activation of VGSC, induced by pyrethroids, lead to the accumulation of sodium in microglia and initiate the inflammatory process.

4.3.3.4. Dose Concordance

Table C.7. Dose concordance

Reference	Result
Hossain et al. 2017 Primary microglia isolated form 1- to 2 day-old C57BL/6 pups and immortalized microglia cells from mice (BV2): Measurement of Na+ influx every 30 s for 30 minute with fluorescence and measurement of intracellular Na+ [(Na+)i] 24 and 48 h after permethrin or deltamethrin exposure	Increase Na+ influx and dose-time dependent accumulation of Na+ after exposure at 1 uM

4.3.3.5. Temporal concordance

Table C.8. Temporal concordance.

Reference	Result
Hossain et al. 2017 Primary microglia isolated form 1- to 2 day-old C57BL/6 pups and immortalized microglia cells from mice (BV2): Measurement of Na+ influx every 30 s for 30 minute with fluorescence and measurement of intracellular Na+ [(Na+)i] 24 and 48 h after permethrin or deltamethrin exposure	Increase Na+ influx and dose-time dependent accumulation of Na+ after exposure at 1 uM

4.3.3.6. Incidence Concordance

not applicable

4.3.4. Uncertainty and inconsistencies

No inconsistencies can be identified for this KER, however some uncertainties are present.

It is biological plausible that VGSC present in microglia affects their role as immune cells. There is also evidence for deltamethrin causing a rapid influx of sodium and accumulation of intracellular sodium in the microglia cells. However, there is a lack of direct evidence assessing that the KE downstream is caused by the KE upstream. Further data investigating in parallel both KE upstream and KE downstream should be generated (see also Appendix B).

4.3.5. References KER8

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4.4. KER9. Increase of intracellular sodium leading to altered neuronal network function

4.4.1. Biological Domain of Applicability

4.4.1.1. Taxonomic Applicability

Vertebrates and Invertebrates.

4.4.1.2. Life Stage Applicability

All life stages. This AOP specifically refers to the developmental period.

4.4.1.3. Sex Applicability

Mixed.

4.4.2. KER Description

The culmination of neurodevelopment is the formation of functional neuronal networks. It has been demonstrated that alteration of sodium kinetics in microglia may lead to an over-activation of these cells, that eventually lead to neuronal cell-death and impairment of neuronal network functions.

4.4.3. Evidence Supporting this KER

4.4.3.1. Biological Plausibility

VGSCs are targets of pyrethroid actions in insect and mammals and they are critical in the regulation of a number of processes. From a biological point of view, it is plausible that modification of these channels functions leads to activation of inflammatory processes though microglia activation and eventually to neuronal cell death.

Many different fundamental neurodevelopmental processes must occur to lead to formation of functional network in vitro, including neurite outgrowth, maturation of glia, synaptogenesis, excitatory and inhibitory signaling, neurotransmitter receptors expression, neurotransmitter recycling, and maintenance of electrochemical gradients (from Frank et al 2017; Johnstone et al. 2010, Potter 2001, Radio and Mundy 2008). Therefore, a modification of electrophysiological equilibrium in microglia cells may lead to disruption and modification of neuronal cell networks.

4.4.3.2. Empirical Evidence

MEA studies have been conducted with the Type II pyrethroid deltamethrin on rodents' cells (Frank et al. 2017). Deltamethrin was positive in several MEA endpoints in a screening of 86 compounds during neural network formation (Frank et al. 2017). The cultures contained astrocytes, neurons and a minor population of microglia characterized by immunocytochemistry. This screening experiment was conducted to assess in 86 compounds of which 60 of them have known in vivo DNT effects.

4.4.3.3. Essentiality

4.4.3.4. Dose Concordance

For deltamethrin effects on the MEA was observed in a range of concentration from 0.5 to 12.5 µM (Frank et al. 2017).

4.4.3.5. Temporal concordance

Chronic exposure to deltametrin on rNNF from the first week up to 28 DIV (Frank et al. 2017).

4.4.3.6. Incidence Concordance

Not applicable.

4.4.4. Uncertainty and inconsistencies

For this KER it is recognized that dysregulation of microglia signalling may affect the synaptic and circuit function, however, the knowledge beyond the process is very limited.

With regards to the study from Frank et al. 2017, uncertainties are also present, the cell culture used in the study contains only a limited number of microglia. Therefore, a study directly investigating the effect of microglia on alteration of neuronal functions in vitro (through MEA) is missing.

The above-mentioned uncertainties are the reason why this KER is considered non-adjacent.

See also Appendix B.

4.4.5. References KER9

Frank, C. L., Brown, J. P., Wallace, K., Mundy, W. R., & Shafer, T. J. (2017). From the cover: developmental neurotoxicants disrupt activity in cortical networks on microelectrode arrays: results of screening 86 compounds during neural network formation. Toxicological Sciences, 160(1), 121-135. https://doi.org/10.1093/toxsci/kfx169

Johnstone, A. F., Gross, G. W., Weiss, D. G., Schroeder, O. H. U., Gramowski, A., & Shafer, T. J. (2010). Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century. Neurotoxicology, 31(4), 331-350.

Masjosthusmann, S, Blum, J, Bartmann, K, Dolde, X, Holzer, A-K, Stürzl, L-C, Hagen, Keßel E, Förster, N, Dönmez, A, Klose, J, Pahl, M, Waldmann, T, Bendt, F, Kisitu, J, Suciu, I, Hübenthal, U, Mosig, A, Leist, M, Fritsche, E, (2020). Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity, *EFSA Supporting Publications 2020:* 17(10), 1938E, 152 pp, doi: 10.2903/sp.efsa.2020.EN-1938

Potter, S. M. (2001). Distributed processing in cultured neuronal networks. In Progress in brain research (Vol. 130, pp. 49-62). Elsevier.

Radio NM, Mundy WR (2008). Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. Neurotoxicology. 29(3):361-76. doi: 10.1016/j.neuro.2008.02.011.

4.5. KER10. Binding to Ryanodine receptors leads to a disruption of Ca channel kinetics

4.5.1. Biological Domain of Applicability

4.5.1.1. Taxonomic Applicability

Vertebrates and Invertebrates.

4.5.1.2. Life Stage Applicability

All life stages. This AOP specifically refers to the developmental period.

4.5.1.3. Sex Applicability

Mixed.

4.5.2. Evidence supporting this KER

4.5.2.1. Biological plausibility

Ryanodine receptors (RyRs), together withinositol-1,4,5-trisphosphate receptors influence Ca2+ release from sarcoplasmic reticulum endoplasmic reticulum stores, and thus mediate a myriad of Ca2+-regulated cellular processes (Lanner etal.2010; Pessah et al., 2010).

4.5.2.2. Empirical evidence

Using as a model mouse cortical primary cultured neurons from post-natal day 0 or 1 (both male and female) from wild type C57BL/6JWT (Cao et al.2011; Zheng et al., 2019) acute exposure to nanomolar deltamethrin at 6, 8, 10 and 12 DIV caused suppression of the amplitude and frequency of the Synchronous Ca2 Oscillations in a concentration-dependent manner, and the degree was dependent on the stage of development of the neuronal network with a maximum of complete suppression at 6 DIV (neuronswithIC50 values of 139.6 nM and 37.6 nM, respectively; Zheng et al., 2019).

4.5.2.3. Essentiality

In the same in vitro study when using cortical primary cultured neurons from post-natal day 0 or 1 from RyR1T4826I=T4826I knock-in mice, the SCO frequency in neurons expressing $RyR1^{T4826I=T4826I}$ was comparable with that measured in wild type but the SCO amplitude in $RyR1^{T4826I=T4826I}$ neurons was significantly smaller. The inhibition of SCO amplitude was greater in $RyR1^{T4826I=T4826I}$ cortical neurons compared with wild type neurons and these neurons showed to be more responsive to deltamethrin for this event.

Furthermore, deltamethrin influenced ryanodine receptor function ((Morisseau et al.2009) at 30 nM altered gating kinetics of RyR1 channels, increasing mean open time, decreasing mean closed time, and thereby enhancing overall open probability

4.5.2.4. Dose concordance

4.5.2.5. Temporal concordance

4.5.2.6. Incidence concordance

4.5.3. Uncertainties and inconsistencies

Overall, the role of ryanodine receptor in the regulation of intracellular calcium homeostasis is well known. Uncertainties remain in the knowledge of the additional factors that may influence calcium homeostasis, other than chemicals binding to ryanodine receptors.

Some inconsistencies can be observed in experimental studies. In an initial screening it was found that > 3 uM of deltamethrin are required to elicit changes in ryanodine receptors whereas in the study from Zheng et al. 2019, it is demonstrated that level as low as 10 nanomolar are required. This inconsistencies in the result may be explained by the high lipophilicity of the prototype stressor deltamethrin, that may lead to an underestimation of its potency.

See also Appendix B.

4.5.4. References for KER10

Cao, Z., Shafer, T. J., & Murray, T. F. (2011). Mechanisms of pyrethroid insecticide-induced stimulation of calcium influx in neocortical neurons, Journal of Pharmacology and Experimental Therapeutics, 336 (1), 197-205. American Society for Pharmacology and Experimental Therapeutics. doi: https://doi.org/10.1124/jpet.110.171850.

Lanner, J. T., Georgiou, D. K., Joshi, A. D., & Hamilton, S. L. (2010). Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harbor perspectives in biology, 2(11), a003996.

Morisseau, C., Merzlikin, O., Lin, A., He, G., Feng, W., Padilla, I., ... & Hammock, B. D. (2009). Toxicology in the fast lane: application of high-throughput bioassays to detect modulation of key enzymes and receptors. Environmental Health Perspectives, 117(12), 1867-1872.

Pessah, I. N., Cherednichenko, G., & Lein, P. J. (2010). Minding the calcium store: Ryanodine receptor activation as a convergent mechanism of PCB toxicity. Pharmacology & therapeutics, 125(2), 260-285

Zheng, J., Yu, Y., Feng, W., Li, J., Liu, J., Zhang, C., ... & Cao, Z. (2019). Influence of nanomolar deltamethrin on the hallmarks of primary cultured cortical neuronal network and the role of ryanodine receptors, Environmental health perspectives, 127(6), 067003, https://doi.org/10.1289/EHP4583

4.6. KER11. Disruption of intracellular Calcium channel kinetics leads to disruption of action potential generation; membrane depolarization

4.6.1. Biological domain of applicability

4.6.1.1. Taxonomic Applicability

Vertebrates and Invertebrates.

4.6.1.2. Life Stage Applicability

All life stages. This AOP specifically refers to the developmental period.

4.6.1.3. Sex Applicability

Mixed.

4.6.2. KER description

Synchronous Calcium Oscillation (SCO) depends on electrical spike activity and these two processes are critical for neuronal development (Spitzer et al. 1995). Changes in the amplitude of SCO can occur in the absence of changes in the baseline intracellular Calcium concentration through a chemical mediated influence on regulation of intracellular Calcium stores.

4.6.2.1. Biological plausibility for the KER

In vitro, spike activity is an early event while synchrony and SCO occur later and gradually increase and stabilize, contributing to the long-distance network connectivity. RyRs and IP3P regulate the influence of calcium release from the sarcoplasmic/endoplasmic reticulum and therefore influencing several Calcium mediated processes. Alteration of this complex process could therefore alter the intracellular Calcium dynamic and the electrical spike activity, two interrelated and essential biological processes that affect neuronal network refinement (Rash et al. 2016).

4.6.2.2. Empirical evidence

In cortical neuronal networks developed in vitro, SCO frequency and ESA increased in concert with morphometric complexity. The temporal increase in morphological complexity coincides with enhanced functional connectivity across the neuronal network that likely mimic many aspects of the developing brain (Feller 1999; Stafford et al.2009). Nanomolar concentrations of deltamethrin was shown to be capable of altering both morphometric and functional parameters. Cortical neurons exposed sub chronically to DM (<1 uM) for 6 day in vitro displayed a concentration dependent decreases in SCO frequency. Similarly, cortical neurons receiving deltamethrin (10 and 30 nM) exposure for 4 days after plating displayed concentration-dependent inhibition of electrical spike frequency and burst frequency (Zheng et al 2019).

4.6.2.3. Essentiality

Primary cultured cortical neuronal network from the mouse pups carrying gain-of-function mutation exhibited significantly lower SCO amplitude. Deltamethrin altered RyR1 gating kinetics at levels as low as 10 nM, providing the evidence that RyR1 was a direct sensitive target of DM (Zheng et al 2019).

4.6.2.4. Dose and temporal concordance

Neuronal networks responded to DM (30–70 nM) as early as 5 DIV, reducing SCO amplitude and depressing ESA and burst frequencies 60–70%. DM (10–300 nM) in a concentration dependent manner (Zheng et al. 2019)

4.6.2.5. Incidence concordance

4.6.3. Uncertainty and inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties.

The first uncertainty stems from the paucity of data for the other events that may influence calcium homeostasis in the cells and, even if there is evidence that the blockage of ryanodine receptor lead alteration of intracellular calcium release from ER, this is not enough to fully understand the process. In addition, the role of calcium and its intracellular homeostasis in a complex system are not well studied and should be further investigated.

See also Appendix B.

4.6.4. References for KER11

Feller, M. B. (1999). Spontaneous correlated activity in developing neural circuits. Neuron, 22(4), 653-656.

Rash, B. G., Ackman, J. B., & Rakic, P. (2016). Bidirectional radial Ca2+ activity regulates neurogenesis and migration during early cortical column formation. Science advances, 2(2), e1501733.

Spitzer, N. C., Olson, E., & Gu, X. (1995). Spontaneous calcium transients regulate neuronal plasticity in developing neurons. Journal of neurobiology, 26(3), 316-324.

Stafford, B. K., Sher, A., Litke, A. M., & Feldheim, D. A. (2009). Spatial-temporal patterns of retinal waves underlying activity-dependent refinement of retinofugal projections. Neuron, 64(2), 200-212.

Zheng, J., Yu, Y., Feng, W., Li, J., Liu, J., Zhang, C., ... & Cao, Z. (2019). Influence of nanomolar deltamethrin on the hallmarks of primary cultured cortical neuronal network and the role of ryanodine receptors, Environmental health perspectives, 127(6), 067003, https://doi.org/10.1289/EHP4583

5. Overall Assessment of the putative AOP

5.1. Introduction

This putative network AOP was developed for use in an IATA for the potential developmental neurotoxicity of deltamethrin. Thus, it was derived using a stressor-based approach, systematically collecting from scientific literature all available rlevant data for the DNT hazard identification and characterization of deltamethrin. In addition, data from the DNT IVB was included in a second step in the IATA framework. The available evidence was mapped into the AOP and included concentrations and doses with the related ranges or probabilistic distributions resulted from the uncertainty analysis. MIEs, KEs and AOs were added to the network, only if there was empirical evidence supporting a causal association with the stressor deltamethrin. The putative pathways in the network were evaluated using a quantitative probabilistic approach (I.e. the Bayesian Network model) summarising biological plausibility, essentiality, empirical support, and quantitative understanding for each KE relationship. This approach is summarized below. More details are provided in the **Appendix B**.

5.2. Summary of the putative AOP network

The KEs included in the putative AOP network were first identified mapping evidence from a systematic review and the underlying known biology.

The next step involved assessing the probability that a causal association between exposure to deltamethrin and the activation/occurrence of the KE would occur in the various populations (i.e. humans, rodents, neuronal cells in development). To support this judgement an uncertainty analysis has been performed assessing sets of pre-defined uncertainty domains tailored to each line of evidence (see Appendix B). KEs for which the probability of a causal association was judged by consensus to be 66% or above were included in an initial list that was then subject to further scrutiny. The threshold of 66% was considered sufficiently conservative since it means that it is judged at least twice as probable that the deltamethrin is activating the KE, rather than not activating the KE.

Among the MIEs/KEs/AOs initially judged as causally associated with deltamethrin, all were selected for the putative AOP network except for in vitro evidence for decreased neuronal crest cell migration since the specific effect was only observed at high concentrations. Based on available evidence and expert judgement, the KEs were connected leading to a putative AOP entailing 11 KERs Figure C.2).

To characterize the certainty within this putative AOP network and the KER, a Bayesian Network approach was developed based on available data and expert knowledge for deltamethrin. Experts were engaged to elicit conditional probabilities for couples and triplets of connected KEs in the network based on evidence for biological plausibility, essentiality and dose/temporal concordance for all KERs. Description of the results of the elicitation process for the KEs status combinations, looking at the downstream KE occurring given the activation of the upstream KE(s), is provided in Figure C. 21 and

Table 10. More details on the BN approach and all the results of the elicitation process to derive the conditional probability tables can be retrieved in Appendix B and Table C.10. Summary table for KERs: WoE based on Biological plausibility, Essentiality, Dose and Temporal Concordance..

The BN approach also allows exploration of partial pathways, or AOP-strings, within the network. An AOP string is defined herein as a set of connected KERs that are linear and may or may not include the entire pathway from MIE to AO. For example, the putative AOP string including the adjacent KERs from MIE1 to KE4, (measurable with in vitro methods), describe the well-established knowledge about pyrethroids binding to VGSC (MIE1), and subsequent disruption of sodium channel gate kinetics (KE1), action potential generation (KE2), axon terminal depolarization and changes in neurotransmitter release (KE3), and altered neuronal network function (KE4).

The partial strings leading to the AO (KER 5, 7, 9) are all considered non-adjacent, due primarily to knowledge gaps. These gaps are in some cases not due to biological uncertainty between the KEs, but instead because the systematic review resulted in no data for any intermediate KEs for DLT. For example, there are KEs missing between KE5 and the AO even though decreases in oligodendrocyte differentiation are known to alter the neuron/oligo ratio, myelin formation, and likely network function, but there were not systematically reviewed deltamethrin studies for this endpoint. The same argument is valid for KER9 (increase of intracellular sodium in microglia cells leading to altered neuronal network function). In other cases, there are possible intermediate KEs for which biological knowledge is lacking. For example, the uncertainty in the relationship between KE4 and the AO is based on both a lack of empirical data on any intermediate KEs and the biological uncertainty of what KEs exist between behavioural-based outcomes and the underlying anatomy and physiology in the brain. One exception is that many spatial based behaviours are known to require proper functioning of the hippocampus and related structures.

Experimental data is available individually for the most downstream KEs and the AO. And, while disturbed neuronal network function (KE4) and adverse effects on oligodendrocyte differentiation (KE5) are well documented using in vitro methods, the relationship between these KEs and the AO of altered behavioural function is more uncertain.

Data and knowledge for other non-adjacent KERs are available for deltamethrin, resulting in additional putative AOP strings. When put together, these putative AOPs form an AOP network. These additional AOP strings describe potential additional MIEs and KEs for deltamethrin. Binding to ryanodine receptors may lead to disruption of intracellular calcium channel kinetics (KER10), which in turn may lead to the disruption of action potential generation and membrane depolarization (KER11). Furthermore, disruption of sodium channel gate kinetics may also lead to decreased oligodendrocyte differentiation (KER6) and consequent hypomyelination and ultimately impaired behavioural function (AO) (KER7). Disruption of sodium channel gate kinetics may also lead to increase of intracellular sodium in microglia cells (KER8), which may affect neuronal network function (KER9), which may further impair behavioural function.

It is appreciated that the altered network function (KE4) is likely to be an intermediate KE within KER7. Moreover, it is well known that the microglia function (KE6) may also be affected by calcium channels. However, these and other considerations for intermediate and additional KEs are not included and assessed as such within this putative AOP network, since the intention was to select the KEs based on experimental data available specifically for deltamethrin (for more details on the underlying uncertainties see section 6.2).

There is also uncertainty in the outcomes found in the in vivo studies. Some have reported effects (e.g., Pitzer et al.2019, Zhang et al. 2018) and others no effects on similar adverse outcomes (e.g., startle and learning/memory endpoints). These discrepancies are likely due to differences in experimental variables between the studies, including differences in exposure routes, and/or exposure ages. The latter two variables will affect chemical kinetics and thus concentrations during different periods of brain development, and therefore influence the in vivo outcome. The comparative tissue concentrations can

be calculated for the dose-concordance for KE4 and KE5 in *in vitro* studies, with the AO in the Pitzer et al (2019) in vivo study, that employed postnatal gavage application to pups. It seems that also within the standard TG426 study (feeding of dams from GD6 till weaning), which did not indicate any DNT findings, a similar but lower c_{max} of deltamethrin could have been reached, but the respective kinetic data are more uncertain. In a third in vivo study (Zhang et al. 2018) that used gavage exposure to dams only during gestation changes AO (e,g, learning in a water Morris maze) was observed. In this study embryo/foetus exposure was probably highest compared to the other studies, but pup brain concentrations were probably very low (for more details see section 6.2). In addition, other experimental design differences, such as the endpoint selection, measurement, and assessment, notably also the different specific testing methods used for learning and memory, the rat strains and number of animals used as well as biological variability may contribute to the observed differences. Furthermore, the in vivo rat AO measurements is in principle accepted by regulators as a useful basis to measure to estimate human relevant adversity effects and effect levels;however some regulators also stressed that their sensitivity and relevance for human real-world effects is necessarily limited and at least as uncertain as other standard in vivo endpoints (Paparella et al. 2020;see table in 5.5. KER5&7). Human epidemiological data are available, but they inherit other uncertainties.

In summary the AO measurements are uncertain, but there is consensus that it is biologically plausible that in vitro measured chemical mediated alterations on cellular events that are critical to normal development, may indicate a chemicals potential to induce human neurodevelopment disorders (see section 4.5.3.1.).

Overall, the development of this AOP network identified two major uncertainties. The first uncertainty is in the network as it is missing potentially important KEs. This is due to the decision to include only data and KEs that have been studied for the stressor deltamethrin. The second is due to a knowledge gap that drives uncertainty in the last two KERs, which is the lack of empirical data that provide correlative and/or causal relationships between disturbed neuronal network function (KE4) and/or hypomyelination (as a result of KE5) and altered behavioural function (AO). These uncertainties can be lowered by additional KEs and KERs from the inclusion of stress-agnositic biological knowledge. The knowledge gap for linking KE4 and the AO may be more difficult to address and has been identified in several other AOPs with adverse neurological outcomes (Bal-Price et al., 2015; Sachana et al., 2016; Tschudi-Monnet and Fitzgerald, 2018; Crofton et al., 2019). and some suggestions for this were discussed in related work (Paparella et al. 2020,).

From the quantitative Weight of Evidence analysis, it is concluded that the mechanistic knowledge underlying the putative AOP informed and supported the experimental observations for deltamethrin to a moderate degree. Moreover, the mechanistic AOP knowledge base supporting that deltamethrin affect voltage gated sodium channels, which ultimately leads to altered network function (KE4), is strong. This information would therefore be relevant if KE4 needed to be recognised as immediately useful for regulatory toxicology decisions (see section 5.4. Biological domain of applicability of the AOP. and 5.6. Quantitative WoE considerations – the Bayesian Network approach.).

5.3. Potential relevance of the putative AOP for the chemical class of pyrethroids

Knowledge and data for other pyrethroids were considered to inform the reliability and relevance of the assay results for deltamethrin. The in vitro data for effects of other pyrethroids on the MIE and early KEs is supported by decades of research and was not systematically reviewed herein. Data from later KEs was available from the same reports scrutinized for deltamethrin (e.g. Masjosthsumann et al., 2020; Frank et al., 2017). Inclusion of all available in vivo AO data for the broad chemical class of pyrethroids was beyond the scope of this project and will require a systemic review. However, an initial consistency comparison was made with all available OECD 426 studies; data on the AO from the OECD 426 studies has been therefore retrieved from EFSA, JMPR and US EPA reports (see Table C.9). Any additional

work will also need to focus on other critical experimental variables, including kinetics modelling of embryo/foetus and pup brain exposure.

Although, these considerations are making the comparative effort unlikely, there is evidence that consistency exists for the mechanistic data (MiE1, MiE2, KE1 KE2, KE3, KE4, KE5); indeed, the mechanistic characteristics are similar across the pyrethroids chemical class. Further assessment of the AO is recommended using an evidence-based approach in order to consider all the uncertainties. This additional work on the full chemical class has the potential to support also the current expert-based probability assignment to the most downstream KERs 5 and 7.

Table C.9. Consistency table

Chemical Name	DNT IVB	MIE1	MIE2	KE1	KE2	KE3	KE4	uM	KE5	uM	KE7	KE6		AO - ASR	AO - L&M	MIGRATION	References for KEs and AO
Allethrin	z	1		1	1		1	0.38					4				**KE4**: Shafer et al. 2019
Bifenthrin	nt	1	1	1	1	1	1	0.1					5	0	0		**KE 3**: Syed et al. 2016 **KE 4:** Shafer et al. 2019;
Bioalletrhin S	z	1		1	1		1	0.28					4				**KE3**: Pauluhn & Schmuck, 2003 **KE3:** Eriksson & Fredriksson, 1991; **KE3:** Ahlbom et al, 1994 **KE 4**: Shafer et al. 2019;
Cyfluthrin, beta	x	1	1	1	1		1	0.27	1	2.6			6	0	0		**KE 5:** Masjosthusmann et al. 2020; **AO:** OECD 426 from CalEPA (2015); **AO:** EPA (2007) TXR #: 0052372
Cyfluthrin	z	1		1	1		1						4				**KE4:** Shafer et al. 2019
Cyhalothrin, ?-													0	0	1		**AO:** OECD 426 from EPA (2007) TXR 0053099
Cyhalothrin, ?-													0	0	0		**AO:** OECD 426 from JMPR (2007)
Cyhalothrin, ?-	x	1		1	1		1		1	0.9			5	0	1		**KE5**: Masjosthusmann et al. 2020; **AO:** OECD 426 from EFSA RAR 08 vol3 B6
Cypermethrin	x	1		1	1		1	0.567	0				4	0	0		**KE4:** Frank et al. 2017
Cypermethrin, alpha	z	1	1	1	1		1						5	0	0		**AO:** OECD 426 from EFSA (2018) doi: 10.2903/j.efsa.2018.5403
Cypermethrin, beta	x	1		1	1				1	4.4			4	1	0		**KE5**: Masjosthusmann et al. 2020; **AO:** OECD 426 from EFSA Peer review EFSA Journal 2014;12(6):3717
zeta-Cypermethrin	nt												0	0	0		**AO:** OECD 426 from EPA (2006) TXR # 0053857
Deltamethrin	x	1	1	1	1		1	1.05	1	0.5	1	1	8	1	1	1	**KE5**: Masjosthusmann et al. 2020 **AO:** EFSA Systematic Review
Deltamethrin													0	0	0		**AO:** OECD 426 from EPA (2007) TXR #:

																0054481
Etofenprox	x	1		1	1		1	0.35	1	15.2			5	1	0	**KE4**: Shafer et al. 2019; **KE5**: Masjosthusmann et al. 2020 **AO:** OECD 426 from JMPR 2011
Fenpropathrin	nt						1						0	1	0	**AO:** OECD 426 from JMPR (2012)
Fenvalerate Es	nt	1		1	1		1	0.1					4			**KE4:** Shafer et al. 2019
Fenvalarate	nt	1	1	1	1								4			
Flumethrin	nt												0	0	0	**AO:** OECD 426 from EPA (2011) TXR#: 0055615
Permethrin	nt	1		1	1	1	1	0.87				1	5			**KE3:** Nasuti et al. 2007; **KE4:** Frank et al. 2017; **KE6:** Hossain et al. 2017
Phenothrin	nt												0			
Prallethrin	nt	1		1	1		1	0.05					4			**KE4:** Shafer et al. 2019
Pyrethrins	nt												0			
Resmethrine	nt	1		1	1		1	5					4			**KE4:** Shafer et al. 2019
Tau-fluvalinate	x	1		1	1				1	1.7			4			**KE5**: Masjosthusmann et al. 2020
Tefluthrin	nt	1		1	1		1	0.05					4			**KE4:** Shafer et al. 2019
Tetramethrin	nt	1		1	1		0						3			**KE4:** Shafer et al. 2019
Transfluthrin	nt												0	0	0	**AO:** OECD 426 from EC (2013), **AO:** EPA (undated) TXR#: 0057699

x = all assays/ z=some IVB/nt= not test

5.4. Biological domain of applicability of the AOP

5.4.1. Taxonomic Applicability

VGSC are present in multiple taxa. However, the AO only apply to mammals.

5.4.2. Life Stage Applicability

This AOP specifically refers to the developmental period.

5.4.3. Sex Applicability

Mixed.

5.5. Evidence supporting all KERs

The biological plausibility, essentiality and dose/temporal concordance are established for the more upstream adjacent KERs (full arrows) and MIE and early KEs are a dogma for pyrethroids. There is more uncertainty for the non-adjacent KERs (dashed arrows) and more downstream KERs (e.g., KER5, KER7). For the latter, intermediate KEs are needed (see Section 5.1). Currently they are based on biological plausibility and empirical data correlations, but they lack experimental data for essentiality and dose/temporal concordance within the same test system.

Table C.10. Summary table for KERs: WoE based on Biological plausibility, Essentiality, Dose and Temporal Concordance.

KER: KE downstream being activated given the statuses/status combination of upstream KE(s), i.e.: KEupstream=1 / KEdownstream=1 or 0 NB Complementary probability for the KE downstream not being activated is derived by difference (sum must be always 1)		BP	E*	C**	Prob	Brief explanation
n.r.	MIE1 (Binding to VGSC) activation given Deltamethrin exposure					
	MIE1=1/ exposure DMT=1	H	H	n.r.	0.99	**Biological plausibility** and **essentiality** are considered **high**, since deltamethrin is a pyrethroid and its interaction with VGSC is a dogma (very extensive and consistent literature).
n.r.	MIE2 (Binding to Ryanodine Receptor) activation given Deltamethrin exposure					
	MIE2=1/ exposure DMT=1	M	M	n.r.	0.7	**Biological plausibility** and **essentiality** are considered **moderate**, since MEI2 is experimentally documented for deltamethrin, but only from one study within one laboratory. Relative to MIE1 this is significantly less evidence.
KER1	KE1 (disruption of sodium channel gate kinetics) activation given the status of MIE1 (Binding to VGSC)					
	KE1=1/ MIE1=1	H	H	H	0.99	**Biological plausibility** is considered **high**, based on extensive documentation and broad acceptance of the mechanistic understanding of the function of VGSCs. **Essentiality** is considered **high** based on data from mutation and knock out mouse, housefly and xenopus models as well as human data for mutations in VGSC subunits linked to various forms of epilepsy. **Dose and temporal concordance** are considered as **high,** though only indirect measures are available for changes in VGSC kinetics after exposure to deltamethrin, and these are also dose dependent. From the range (0.85-1] **0.99,** the highest value was considered the most plausible **quantitiatve WoE estimate.**
	KE1=1/ MIE=0	L	H	nr.	0.01	see above, the **biological plausibility** that MIE1 not leads to KE1 is considered very **low**
	KE2 (disruption of action potential generation and membrane depolarization) activation given the combined status of KE1 (Disruption of sodium channel gate kinetics) AND KE7					

KER2 and KER11	(disruption of intracellular calcium channel kinetics)					
	KE2=1/ (KE1=1 and KE7=1)	H	M	M	0.8	**Biological plausibility** **KER2:** Biological plausibility is considered high, based on extensive documentation and broad acceptance for the mechanistic understanding of the generation of membrane potentials, based on Na, K, Cl and Ca ions. **KER11:** There is uncertainty, to what extent changes of intracellular Ca concentrations due to release from the endoplasmatic reticulum (by Ryanoide receptor binding of pyrethroids) may affect the action potential generation. **KER2 & KER 11:** Biological plausibility is considered **high**. **Essentiality** **KER2:** Essentially is considered high. Neuronal activity was measured in primary rat hippocampal neuronal cultures using patch-clamp. In the presence of GABA receptor antagonists (BIC and SCH50911), deltamethrin and permethrin had similar effects on spontaneous excitatory post-synaptic currents (sEPSCs) like the voltage gated sodium channel modulator VTD, but different effects on sEPSCs compared to the voltage gated calcium channel antagonist MVIIC. This indicates that VGSCs are likely to be the main target for these pyrethroids (Meyer et al., 2008). . **KER11:** Essentiality is considered moderate. Primary cultured cortical neuronal network from the mouse pups carrying Ryanodine Receptor gain-of-function mutation exhibited significantly lower Synchronous Calcium Oscillation (SCO) amplitude. Moreover, Deltamethrin altered RyR1 gating kinetics at levels as low as 10 nM. Deltamethrin affected also SCO frequency and electrical spike frequency in primary mouse cortical neurons and it is known that SCO and electrical spike frequency in neurons are strongly interrelated and relevant for neuronal development (Feller 1999,). Thus, there is indirect evidence that disruption of intracellular calcium channel kinetics leads to disruption of action potential generation and membrane depolarization. However, no experiment exists with block of just the intracellular Ca release and its impact on Na influx and disruption of action potential. **KER2 & KER11:** Data are available that indicate that Deltamethrin activates both KERs in the same cellular system (Zheng et al., 2019). However, the evidence is indirect and there is uncertainty to what extent KER11 contributes to KE2 activation and evidence for KER11 is not as abundantly demonstrated as KER2. Therefore, essentiality is considered **moderate** **Dose and temporal concordance** **KER2:** Dose and temporal concordance is shown, see table 6.3_1 **KER11:** Dose and temporal concordance is shown between the reduction of the SCO amplitude and depressing ESA and burst frequencies (30-70 nM) within primary cortical mouse cells (Zheng et al. 2019) **KER2 & KER11:** Dose and temporal concordance is shown in the same cellular system (Zheng et al., 2019). However, the evidence for KER11 is not as abundantly demonstrated as for KER2. Therefore, dose and

						temporal concordance is considered as **moderate** in the context of this triplet. **Quantitative WoE for KER2 and KER7** From the range (0.75-0.85] **0.8,** was considered the most plausible, since biological plausibility is considered high and also the KER combinations are substantiated by experimental data, though not as abundant as for the KER2 in isolation
	KE2=1/ (KE1=1 and KE7=0)	H	M	M	0.8	**KER2 & KER 11:** see above, **biological plausibility** is considered **high** that KER2 is activated also without activation of KER11.
	KE2=1/ (KE1=0 and KE7=1)	M	L	L	0.5	**KER2 & KER 11**, see above, **biological plausibility** is considered **moderate** that KER2 is activated also without activation of KER11, due to uncertainty in the KER11.
	KE2=1/ (KE1=0 and KE7=0)	L	M	n.r.	0.01	**KER2 & KER 11,** see above, **biological plausibility** is considered very **low** that KE2 is activated without neither KE1 nor KE7 being activated, due to very high biological plausibility for KER2.
KER3	KE3 (depolarization of terminal axon and change in neurotransmitters release) activation given the status of KE2 (Disruption of action potential generation)					
	KE3=1/ KE2=1	H	H	H	0.95	**Biological plausibility** as well as essentiality is considered **high**, based on extensive documentation and broad acceptance of the mechanistic understanding of how action potentials at the presynaptic membrane open voltage gated calcium channels which translates to neurotransmitter release. **Essentiality** is considered **high**, e.g. deltamethrin and permethrin showed an effect on spontaneous excitatory post-synaptic currents (sEPSCs) and spontaneous spike rates in rat hippocampal neurons, which was modulated by inhibitors of GABA receptors (Meyer et al. 2008). Neurotransmitter release was affected in vivo following administration of DLM during the post natal period (Pitzer et al. 2019) or when administer to dams during pregnancy (Zhang et al. 2018). Effect of pyrethroids on biochemistry of neurotransmitter is also reported for others type I and II pyrethroids (Meyer et al., 2008 and Soderlund 2002 and 2012). **Dose and temporal concordance** is considered **high**, based on deltamethrin concentrations being concordant in available in vitro studies for both KEs (see table 6.3_1). From the range (0.85-1] **0.99**, the highest value was considered the most plausible **quantitative WoE estimate.**
	KE3=1/ KE2=0	L	H	n.r.	0.05	**Biological plausibility** is considered **low** that KE3 is activated without KE2 being active, due to the high biological understanding of this fundamental biological process.
	KE4 (decrease of neuronal network function) activation given the combined status of KE3 (Depolarization of terminal axon & changes in neurotransmitter release) AND KE6 (					

KER4 and KER9	increase of intracellular sodium in microglia cells)					
	KE4=1/ (KE3=1 and KE6=1)	H	L	L	0.66	**Biological plausibility** **KER4:** Biological plausibility is considered high based on extensive documentation and broad acceptance of the mechanistic understanding of cellular developments and functions leading to neuronal network function. **KER9:** The impact of microglia activation on this biological process is biologically plausible, though it is not similarly well documented **KER4 & KER9:** The biological plausibility is considered **high**. **Essentiality** **KER4**: Essentiality is considered moderate. Available MEA experiments demonstrate that VGSC blockers or agonists block or decrease network activity (KER4). This does not represent direct, but indirect evidence for essentiality, since it demonstrates the link of more upstream KEs (MIE1, KE1) with KE4. **KER9:** Essentiality is considered low. No experimental evidence is available, how modulation of sodium kinetics or activation of microglia affects network function (no reference in this AOP?). **KER4 & KER9:** Essentiality is considered **low.** No experimental evidence is available, how neurotransmitter release and sodium kinetics in microglia interact to decrease neuronal network function. **Dose and temporal concordance** **KER4**: Dose and temporal concordance is high. Available in vitro experiments indicate dose and temporal concordance for KE3 and 4 (see table 6.3_1). **KER9:** Dose and temporal concordance is low. It is demonstrated (see pyrethroid concordance table 6.3_2). Microglia is a component of the human and the rat cellular system for neuronal network activity measurements. This means some indirect evidence is available. However, sodium kinetics activation in microglia and decreased network function were not measured in the same test system. **KER4 & KER9:** The concordance is considered **low**. No experiment is available demonstrating concurrent activity of both KERs. **Quantitative WoE for KER4 and KER9** From the range (0.-0.66] **0.66**, was considered the most plausible since the biological plausibility is high and the dose and temporal concordance is high for KER4 and indirect evidence is available for KER9, and essentiality for KER4 is moderate.
	KE4=1/	H	L	L	0.66	See above, **biological plausibility** is **high** that KER4 is activated without KER9 being activated, since

	(KE3=1 and KE6=0)					biological understanding is high for KER4 and less so for KER0.
	KE4=1/ (KE3=0 and KE6=1)	L	L	L	0.2	See above, **biological plausibility** is **low** that KER9 is activated without KER4 being activated, since there is biological plausibility for KER9, though with much less experimental evidence. Furthermore, altered network function (KE4) may require changes in neurotransmitter release (KE3) in a direct or at least indirect way (KE6). Probability is higher than for the 0-0-1 combination (see below), since some evidence for KE6 and KER9 is available (which lead to the inclusion of KE6 into the AOP).
	KE4=1/ (KE3=0 and KE6=0)	L	L	n.r.	0.05	See above, **biological plausibility** is **low due** to high biological understanding of this fundamental process.
KER5 and KER7	AO (altered behavioural function) occurrence given the combined status of KE4 (decrease of neuronal network function) AND KE5 (inhibition of oligodendrocyte differentiation)					
	AO=1/ (KE4=1 and KE5=1)	M	L	M	0.66	**Biological plausibility** **KER5 as well as KER7:** Biological plausibility is considered **moderate.** On the one hand there are knowledge gaps for possible other intermediate KEs and potential modulating influences and for the relationship between measured rat behaviour and underlying brain structures and for the complex regional and temporal ontogeny of VGSCs. On the other hand, there is consensus that chemical mediated alterations on cellular events that are critical to normal neurodevelopment, can result in adverse neurological development (Bal-Price et al. 2018, Mundy et al. 2015, Masjosthusmann et al. 2020). Many different fundamental neurodevelopment processes must occur to lead to functional networks *in vitro*, including neurite outgrowth, maturation of glia, synaptogenesis, excitatory and inhibitory signalling, neurotransmitter recycling, and maintenance of electrochemical gradients (Frank et al,2017). In terms of experimental support of this hypothesis, for deltamethrin clear effects were observed on neuronal network activity (prob. >>0.66) and oligodendrocyte differentiation (prob. >0.66).However, there is more uncertainty for the AO of altered behavioural function for deltamethrin (prob. >0.66), since inconsistent results are available from different in vivo rat studies. This inconsistency may be due to the variability between in-vivo study protocols resulting e.g. in different brain concentrations, but it may also be due the differences in the endpoints -selection, -measurement, –assessment, selection of rat strains, number of animals used, biological variability and the resulting uncertainty in data variability. Furthermore, the in vivo rat AO measurements are in principle accepted by regulators as a useful basis to estimate human relevant adverse effects and effect levels, but some regulators also stressed, that their sensitivity and relevance for human real world effects is at least as uncertain as other standard in vivo endpoints but differencesfor kinetics and biotransformation during development, difficulties to directly compare human and rodent cognitive measures, the practical limitations for a fully comprehensive set of behavioural measures and primate specific features of brain development may add to this. Therefore, behavioural endpoints in rat studies may provide “just“indicators for a general DNT potential in humans. It was claimed that conceptually the same may be true for KE4 and KE5 data that may be used as indicators for doses probably not causing some type of DNT or other toxicities, rather than as predictors for any specific in vivo findings (Paparella et al. 2020;) . Human population level data are available for the deltamethrin´s metabolites, which theoretically may be used to support the AO, but they contain other uncertainties. It is noted that KE4 was measured in rat and human cellular systems and the results support that -at the cellular level- interspecies differences were not apparent. KE5 was measured in a human cellular system only and species specificities cannot be excluded for this endpoint (Dach et al. 2017,). Thus, the uncertainty in KER5 and KER7

					may be more due to the inherent limitations and uncertainties in the in vivo AO measurement than in the uncertainty of the biological relevance of the KEs (and different experts may have different views on this).
					Essentiality **KER5:** Essentiality is considered low based on no data identified/appraised specifically supporting essentiality. **KER7:** Essentiality is considered low based on no data identified/appraised specifically supporting essentiality. **KER5 & KER7:** Essentiality is considered **low** based on no data identified/appraised specifically supporting essentiality**.**
					Dose and temporal concordance **KER5 as well as KER7:** Dose and temporal concordance is considered moderate. Neurotransmitter release was affected in vivo following administration of deltamethrin during the post natal period (Pitzer et al. 2019) or when administered to dams during pregnancy (Zhang et al. 2018). Dose concordance between effect levels for in vitro level effects (KE4 and 5) and in vivo behavioural effects (AO) could be supported with available kinetic data, though the in vivo AO data are not consistent (see above, line for biological plausibility). Moreover, within the rat studies for deltamethrin there is evidence that doses that are affecting developmental stages in the rat are not affecting adult rats. Temporal concordance is shown indirectly, since the effects (concentration) from in vitro methods resembling developmental stages are concordant with in vivo effects (concentration) at the development stages in the rat rather than adult stage. Yet, evidence for dose and temporal concordance for KER5 and KER7 were not identified/appraised. **KER5 & KER7:** Dose and temporal concordance for KER5 and KER7 in combination were not identified/appraised. However, based on the evidence for the individual KERs summarized above, dose and temporal concordance is considered **moderate**.
					Quantitative WoE for KER5 and KER7 From the range (0.00-0.66] **0.66** was considered the most plausible based on the assessment of biological plausibility.
AO=1/ (KE4=1 and KE5=0)	M	L	M	0.66	see above, **biological plausibility** is considered **moderate** that KER5 is activated but KER7 is not activated. In principle it is expected that KE5 would activate the AO via KE4 but the neuronal network assay doesen't contains oligodendrocytes. However, the effect on oligodendrocytes within the neuronal network activity was not specifically measured for deltamethrin. Thus, there is uncertainty, if the in vitro assay captures this pathway (and

						therefore KER7 connects directly to the AO instead of KE4 for the purpose of this putative AOP). However, the biological plausibility for KER7 as a non-adjacent KER is given (see above).
	AO=1/ (KE4=0 and KE5=1)	M	L	M	0.55	see above, **biological plausibility** is considered **moderate** that KER7 is activated but KER5 is not activated. However, this probability is lower than the probability for the inverse situation as explained in the line above.
	AO=1/ (KE4=0 and KE5=0)	L	L	n.r.	0.20	see above, **biological plausibility** is considered **low** that the AO occurs without KE4 and KE5 being activated. Given the biological relevance attributed to the KEs4 and 5 by including them into the AOP, this probability needs to be lower than for a situation where any of the two KEs are active.
KER6	KE5 (decreased oligodendrocyte differentiation) activation given the status of KE1 (disruption of sodium channel gate kinetics)					
	KE5=1/ KE1=1	M	L	L	0.66	**Biological plausibility** is considered **moderate**. There is evidence for functional VGSC in oligodendrocytes and it was shown that deltamethrin and other pyrethroids affects oligodendrocyte differentiation (Masjosthusmann et al., 2020). However, the effect of deltamethin on the VGSC in oligodendrocytes was not yet demonstrated. **Essentiality** is considered **low**, since there are no experimental data demonstrating VGSC induced modulation of oligodendrocyte differentiation. **Dose and temporal concordance** is considered **low**, since there are no experimental studies, which measured both KEs. From the range (0-0.66] **0.66**, the highest value was considered the most plausible **quantitiatve WoE estimate** considering the data provided by the testing battery (Masjosthusmann et al., 2020) with several pyrethroids.
	KE5=1/ KE1=0	L	L	n.r.	0.50	see above, **biological plausibility** is considered **low** that KE5 occurs without KE1 being activated, since there is a paucity of data supporting KER6. However, it should be less likely than the situation that KE5 occurs with KE1 being activated (see the line above).
KER8	KE6 (altered sodium kinetics in microglia) activation given the status of KE1 (disruption of sodium channel gate kinetics)					
	KE6=1/ KE1=1	M	L	L	0.66	**Biological plausibility** is considered **moderate**. There is evidence for functional VGSC in microglia affecting their role as immune cells in vitro and in a mouse model. There is also evidence for DLM and permethrin causing a rapid Na influx and increased accumulation of intracellular sodium in the microglia cells in a dose- and time-dependent manner (Hossain et al. 207). However, the total available evidence is less compared to KER1 or KER3.

						Essentiality is considered as **low**. Inhibition of VGSC with TTX significantly reduced the intracellular Na accumulation after 24 and 48h of permethrin or deltamethrin treatment leading also the assessment of essentiality as moderate. However, the total available experimental evidence for deltamethrin and other pyrethroids is less compared to KER1 or KER3. From the range (0-0.66] **0.66**, the highest value, was considered the most plausible **quantitiatve WoE estimate** since the biological plausibility was considered as moderate and essentiality was considered low, but borderline to moderate.
	KE6=1/ KE1=0	L	L	n.r.	0.50	see above, **biological plausibility** is **low** that KE6 occurs without KE1 being activated, since there is a paucity of data supporting KER8. However, it should be less likely than the situation that KE6 occurs with KE1 being activated (see the line above).
KER10	KE7 (disruption of intracellular Ca channel gate kinetics) activation given the status of MIE2 (Binding of Ryanodine receptor)					
	KE7=1/ MIE2=1	M	M	M	0.66	**Biological plausibility** is considered **moderate**. Ryanodine receptors (RyRs) are Ca2+ channels that regulate Ca2+ release from the sarco/endoplasmic reticulum (Pessah et al.2010). All three sub-types of RyRs were reported to be expressed in the central nervous system (Feng et al.2017; Galeotti et al.2008; Giannini et al.1995). However, only one paper is available for the support of the biological plausibility of the KER following exposure to DLM (Zheng et al. 2019). **Essentiality** is considered **moderate**. Primary cultured neocortical neuronal network, carrying RyR1T4826I=T4826I gain-of-function mutation, exhibited significantly lower synchronous Calcium oscillation (SCO) amplitude and much longer axons. This was consistent with the demonstration that RyRs were responsible for producing temporally and spatially defined Calcium signals (Zheng et al 2019). However, the total available experimental evidence for deltamethrin and other pyrethroids is less compared to KER1 or KER3. **Dose and temporal concordance** is considered **moderate.** Measurements of SCOs using Ca2+ imaging, MEA in primary murine cortical neurons from wild-type and ryanodinereceptor1 (RyR1T4826I=T4826I) gain of function mutations are available. (Zheng et al., 2019). However, the total available experimental evidence for deltamethrin and other pyrethroids is less compared to KER1 or KER3. From the range (0-0.66] **0.66**, was considered the most plausible **quantitiatve WoE estimate** since, though just from one publication, clear data are available with deltamethrin for assessing direct biological plausibility, essentiality and empirical evidence.
	KE7=1/ MIE2=0	M	M	n.r.	0.66	see above, **biological plausibility** is **moderate** that KE7 occurs without MIE2 being activated. It is known, that the binding of deltamethrin to the Ryanodine receptor affects calcium channel gate kinetics and the intracellular calcium homeostasis; however, additional factors can influence the intracellular calcium channel gate kinetics.

Dose and temporal concordance

The following section includes a summary of the dose and temporal concordance along the adjacent KEs and a qualitative uncertainty analysis. For a better understanding and interpretation of the correspondence between the external administered doses in studies used to describe the AO and the expected concentration at the target site, a summary of the exposure scheduling is reported in Fig. 21.

Table C.11. Dose- and temporal- concordance summary table for the AOP string MIE1 KE1 KE2 KE3 KE4 AO.

	MIE	KE1	KE2	KE3	KE4	AO
	Binding to VGSC	Disruption of sodium channel gate kinetics;	Disruption of action potential	Disruption of axon terminal depolarization; changes in neurotransmitter release (in vivo KE)	Altered neuronal network function	Impairment behavioral function (sensory motor reflex and learning)
Time	Simultaneously observed when measured *in vitro* up to 9 minutes recording (1)	Simultaneously neobserved when measured *in vitro* up to 9 minutes recording (1)	Simultaneously observed when measured *in vitro* up to 9 minutes recording (1,2)	*In vivo* up to 28 PND treatment and measured on PND 130 (3) *In vivo*, treatment of dams until delivery and measured in pups on 21 PND (4) *In vitro*, following exposure of DLM in patch clamp and MEA preparations(immediately after exposure until the end of recording) (1)	Acute exposure after 21 to 35 days *in vitro* (DIV) from few minutes to 35 minutes (5,6,7). Chronic exposure lasting up to 28 DIV on 12 to 35 DIV (8,9).	Pups exposure from PND 3 to PND 20 (3); Dams exposure during pregnancy from GD0 until delivery (4).
Concentration/ dose	**[0.01-1] µM** (KE1UA)	**[0.01-1] µM** (KE1UA)	**[0.01-1] µM** (1,2)	**0.25 -9 mg/kg in vivo**. (3) (4). (4): **In vitro [0.01-1] µM** (1)	**0.04-5 µM in vitro** (0.04 µM, corresponds to 19.3 ng of deltamethrin for gram of brain, 5 µM corresponds to 2,4 µg of deltamethrin for gram of brain	**(0.25-7.25] mg/kg bw/d in vivo** (3,4) oral gavage doses of 0.25 -1 mg/kg/day in pups at PND 15 by gavage (single dose) correspond to a brain concentration of 10.7 to 42.8 ng/g of brain assuming linearity.

(1) Mayer et al 2008
(2) Cao et al. 2011
(3) Pitzer et al. 2019
(4) Zhang et al. 2018
(5) Alloisio et al. 2015
(6) Krishnan et al. 2016
(7) Vassallo et al. 2017- 4 studies
(8) Frank et al. 2017
(9) Masjosthusmann et al., 2020

A key element of the dose-concordance analysis is the availability of a model able to describe the relationship between external dose and exposure at the target. For deltamethrin, life-stage rat and human PBPK models that predict the disposition of deltamethrin after oral exposure are available (Song et al., 2019). Although the model does not include the gestational and lactation periods, two life-stage models were developed to investigate and validate an IVIVE approach based on *in vitro* metabolism data. These models predict the time course of plasma and brain deltamethrin concentration in rats at different ages following single dose exposures. Available in vivo data were used to estimate the amount of deltamethrin in plasma and brain across a range of doses, volumes of administration and dissolving vehicle relevant for the comparative analysis., The dose administered in vivo in the studies was used to derive the empirical support for the AOs. Similarity in the administered volume and vehicle are relevant as it is known that they can significantly affect both the kinetics of many chemicals, including deltamethrin (Kim et al. 2007; Mortuza et al. 2018). Vehicle type and volume are also known to impact the potency of pyrethroids on behavioural AOs (e.g., Crofton et al., 1995; Wolansky et al.,2007).

For the current work to assess dose-concordance, the model of Song et al was implemented and the mathematical representation and computer implementation were accurate and carefully documented, inlcuding sensitivity and uncertainty analyses. However, a quantitative assessment of the uncertainties concerning the data used herein in the dose concordance analysis for this AOP was not conducted and they are qualitatively summarized at the end of this chapter.

Based on the deltamethrin kinetic data reported in Mortuza et al. (2018), and the known correlation between pyrethroid neurotoxicity and Cmax (Scollon et al., 2011; Starr et al., 2012), the Cmax in plasma and brain were used as a measure of internal and target tissue exposure. Single oral doses of 0.1-0.25 and 0.5 mg/kg deltamethrin dissolved in 5 mL/kg in Corn Oil were administered by gavage to rats on PND 15 and 90. Plasma and brain were collected up to 48 h following dose administration and were analysed upon serial sacrifice for total (free and bound) DLM by gas chromatography-negative chemical ionization-mass spectrometry. Plasma unbound fraction was evaluated by using C14-labeled DLM (Mortuza et al. 2018).

1ng DLM/g of brain equal 2.05 nM of DLM (considering a brain density of 1046 kg/m^3 and a molecular weight for deltamethrin of 505,21 g/mol).

Doses of 0.25 -1 mg/kg/day in pups at PND 15 by gavage (single dose) correspond to a brain concentration of 10.7 to 42.8 ng/g of brain assuming linearity (Mortuza et al. 2018).

The lowest concentration with 66% probability of affecting the KE 4 (decrease in neuronal network function) is 0.04 um, corresponding to 19.3 ng of deltamethrin for gram of brain.

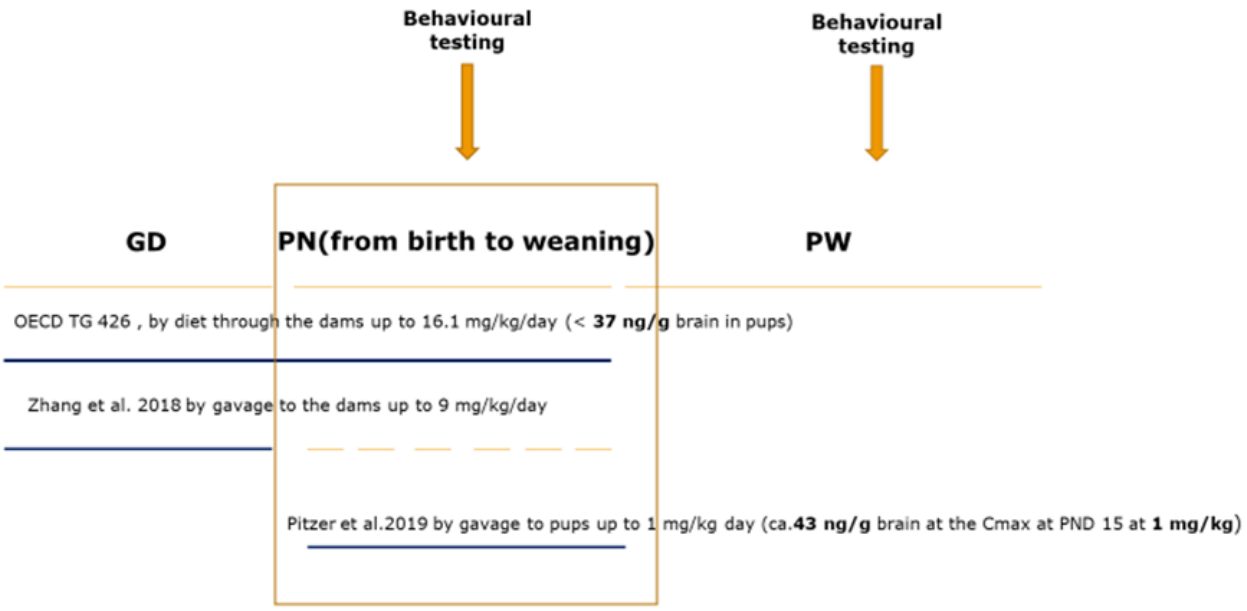
For the study conducted following the OECD TG 426 for deltamethrin, a preliminary dose-finding study is available where brain concentration was measured at a toxic dose of 20 mg/kg/day administered in the diet to the dams. A concentration of 39 ng/g of brain was measured and reported in Fig.1 and this dose lead to increased pup loss and cannibalization by the dams. Therefore the final dose in the main study was of 16 mg/kg/day administered in the diet, which using linear extrapolation would correspond to about a concentration of 31 ng deltamethrin/g brain, I.e. about 30% less than in the pups gavage study (Pitzer et al. 2018). Since the dose-finding study was not systematically reviewed, these data should be considered with caution.

Table C.12. Qualitative uncertainty analysis for dose-concordance.

Uncertainty	Comment
The in-vitro concentration used to assess the dose concordance corresponds to the nominal concentration as reported in the assay protocol.	There is uncertainty in the dose concordance estimation since for all the in vitro assays included to measure the MIE and the KEs the partitioning of the chemical with the plastic, proteins or lipidic fractions was not calculated. Similarly, the intracellular concentration was not estimated. In addition, in the in-vitro chronic models there was no estimation of potential cumulative effects at cellular levels. The nominal concentration is likely overestimating the active concentration in the in-vitro cell system.
The different routes of exposure applied across the different in-vivo studies is an uncertainty.	Mixing the test item with food in multiple dose studies in rat can result in internal and target tissue exposure uncertain. During dietary administration rats eat over several hours, resulting in a long period of feeding associated with a low Cmax when compared to direct dosing by gavage. Deltamethrin is rapidly adsorbed, distributed and cleared from the body and neurotoxicity is expected to be correlated with peak concentration. Therefore, the direct dose administration of deltamethrin by gavage is expected to result in a greater internal and target tissue exposure when compared to rodent dietary administration. There is also uncertainty, how to interpret DNT effects observed at 42.8 ng/g brain concentration with gavage application, while at about 39 ng/g brain concentration with feed exposure pup loss and cannibalization was observed. However, the latter data were not scrutinized to the same level. When considering potential correlation (or not) to human observational data, additional uncertainties are noted, such as: 1) Human exposure situations and resulting kinetics

	(V_{max}) are more likely to correspond to repeated feeding exposure than daily gavage. 2) Major brain sprout occurs prenatal in humans, but post-natal in rats. This means that the kinetics and brain concentrations in humans relate to placental exposure and maternal biotransformation and in rats they relate to the pup's gastrointestinal exposure (via gavage or milk) and pups biotransformation. In the absence of robust kinetic interspecies extrapolation models, these uncertainties add to the rat to human extrapolation uncertainties.
Window of exposure across studies is an uncertainty for the assessment of the dose concordance.	The in vivo studies used for the assessment of the dose concordance were conducted with different exposure schemes making extrapolation of the external dose to internal and target tissue exposure only suitable for Pitzer at al. 2019 where vehicle, volume of administration and the range of administered external doses were similar or the same
The kinetic study measuring the internal and target tissue exposure was conducted by single dose administration at PND 15, and the lack of prenatal kinetics is an uncertainty	The concentration of deltamethrin at the steady state in the brain was not experimentally assessed. This uncertainty can potentially underestimate the exposure of deltamethrin in the brain of pups.

Figure C. 21. Schmatic summary of the treatment scheduling for the studies used to describe the AO.



5.6. Quantitative WoE considerations – the Bayesian Network approach

5.6.1. Introduction to the use of Bayesian Network for quantitative WoE characterization within AOP

Lack of approaches to quantitatively model AOPs and AOP networks including the associated uncertainty have been recently identified as an obstacle to successfully implement the AOP framework in the regulatory context (LaLone et al. 2017). The Bayesian Network (BN) framework represents a promising approach in this respect. The incorporation of statistical or probabilistic relationships into an AOP creates a probabilistic quantitative AOP (pqAOP). As with any quantitated AOP (qAOP), a BN can serve as a computational tool for translating or extrapolating from mechanistic measurements of upstream KEs to a predicted severity/status of the AO (Fenton & Neil 2012). More sophisticated models could have been developed using quantitative dose-response and response-response to model the KE relationship (Muller et al 2015, Perkins et al., 2019), this option would have required more ample data to be implemented. That data was not available.

Bayesian Networks are graphical models that allow to represent the probabilistic structure of multivariate data using a graphical display (Scutari & Denis 2015). They entail: 1) a set of random variables, the MIEs/KEs/AO in the AOP framework with the associated joint probability distribution (named *global probability distribution*); 2) a graphical representation, namely a Directed Acyclic Graph (DAG), describing the dependencies/independencies within the set of variables. In a DAG, the random variables in the set *X* are represented as nodes (MIEs, KEs and AOs in the context of an AOP) and the links between variables (KE relationships in an AOP) as directed arcs. BN-AOP can have different applications. It can be used to perform prognostic inferences i.e. to prospectively predict the probability of the occurrence and/or the severity of an adverse outcome based on different scenarios (i.e. different combinations of the possible status of the upstream variables/events in the networks). BN-AOP can also be used to derive diagnostic inference, running the model from the Adverse Outcome backward to identify KEs that are the main determinants of the AO. Furthermore, the model can be run from any intermediate MIE or KE backwards and forwards. The BN approach has recently started to be applied successfully in the context of AOP (Moe et al 2020, Jeong et al. 2019, Jaworska et al 2013, Pirone et al 2014). Advantages of its implementation in this context include fitting naturally the concept of the AOP network, allowing the integration of data coming from different lines of evidence such as -invitro high throughput assays and in-vivo toxicological data and measuring the impact of introducing a new information in the system. Finally, being a probabilistic model, it allows accounting and propagating uncertainty also in complex assessment systems.

Three types of probabilities are associated with the Bayesian Network structure and can be used to infer conclusions on the KEs and the triggering stressor:

Conditional probability distributions: the conditional probability is the probability of each of the possible statuses of a downstream event given each possible status (or combination of statuses) of the connected upstream event(s) (i.e. the conditioning event). For each combination of the conditioning upstream KE(s) and downstream KE status, the conditional probabilities can be higher or lower than the marginal probabilities of the conditioned KE depending on the strength of the KER (i.e. the stronger the association the more different the conditional and marginal distribution will be).

Marginal probabilities: the marginal probability distribution describes the probabilities associated to each possible state of a KE/variable (e.g. activated/not activated, occurrence/not occurrence) irrespective of the state of all the others. This probability distribution can be used to infer what is the most probable status of a KE/AO (e.g. altered behavioural function) assuming exposure to a stressor (e.g. deltamethrin) and therefore to classify the stressor as able to trigger or not the KE/AO. The marginal probability (as

the conditional and joint probabilities) can be updated once new 'evidence' (e.g. Disruption of action potential generation activated with certainty) becomes available.

Joint probability: the joint probability distribution of a set/network of KEs describes the probability of each of the possible combinations of the status of the KEs in the network. A natural choice for a joint distribution representing a set of binary variables (i.e. variables assuming only two possible values such as active/not active) is a multinomial distribution. Since the number of combinations dramatically increases when the number of KEs raises, so does the number of distributional parameters. As illustrative examples for a network with 10 KEs each of which entails only two statuses (active/not active), the number of combinations is 1023. Consequently, the probability attached to each combination is rarely extremely high unless the evidence is supporting it with high certainty.

Four single AOP-BN strings could be derived from the AOP network. The related AO-BNs and the associated conditional probability tables are provided in Appendix B.

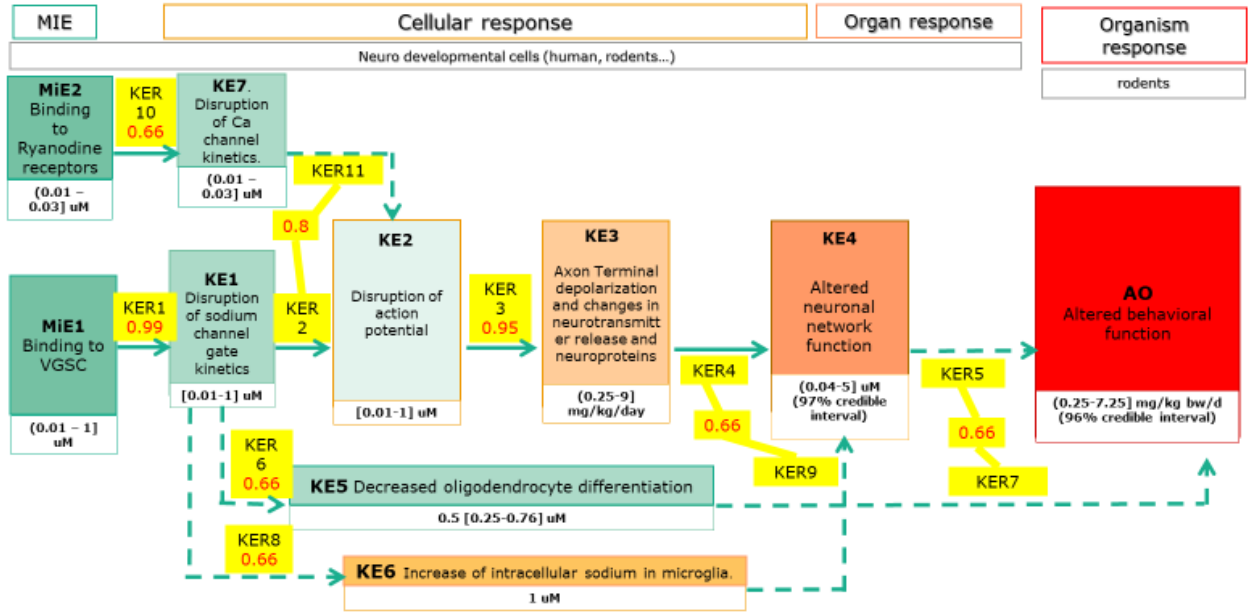
5.6.2. Conditional probability distributions

An expert judgement approach was adopted to estimate the parameters of the Conditional probability distributions. This choice was made necessary by the lack of large data sets that provided empirical data for large portions and/or the whole pathway.

For each pair of MIE/KE/AO in the BN the following question was addressed: 'what is the probability that the KEx is activated given the upstream (parent) KE is activated/not activated'. For each triplet the question to answer was: 'What is the probability that the KEx is activated given all possible combinations of the state of the upstream (parents) KEs (i.e. active/active, active/non active, non-active/active, non-active/non active). The judgement was based on the assessment of the three criteria: the biological plausibility, essentiality and empirical evidence indicated in the AOP handbook (OECD 2018) as the three critical Bradford Hill considerations for AOPs elements to characterise the KEs, KER and their uncertainty in view of demonstrating causality. The assessment of the criteria was first translated into probability ranges according to pre-defined rules and then into single probabilities agreed by the experts based on a collegial discussion. The details of the procedures are described in Appendix B, the rationale justifying the assessment of the criteria and the related conditional probability tables are given in Table C.1.

Figure C. 22 only shows the conditional probabilities (in yellow highlight) associated to the case when a downstream KE is affected (e.g. KE2) under the condition that the individual or two parallel upstream KEs are affected (e.g. KE1 and KE7). Multiplying these probabilities provides the probability for all the KEs and the AO being activated concurrently, i.e. 6.5% (see section 6.3). The rationale for assigning the conditional probability values and the complete conditional probability tables are provided in the Appendix B and in Table C.1. Table C.1 therefore describe of the results of the elicitation process for the KEs status combinations looking at the downstream KE occurring given the activation of the upstream KE(s).

Figure C. 22. Postulated AOP network; conditional probability that a downstream KE is activated given the connected upstream KE(s) is(are) activated.



It is immediately clear from the conditional probability tables, that the evidence for KER5 and KER7, leading to the AO "altered behavioural function" in rodents, contains relatively high uncertainty within the network (see Figure C. 22). The probability of the AO being activated under the condition that the two upstream KEs (KE4 and KE5) occur is estimated as 0.66. This is in the same range as for the other non-adjacent KERs, but clearly lower than the conditioned probabilities for KERs within the string describing the best-established KEs for deltamethrin and pyrethroids, i.e. binding to voltage gated sodium channels ultimately leading to inhibition of neuronal network function (MIE1-KEs1-2-3-4).

5.6.3. Marginal probability for the AO to occur

Integrating the expert-derived conditional probabilities over all KERs by using the Bayesian Network approach provides marginal probabilities for the AO to be activated/not activated. In this specific stressor based AOP, those probabilities are derived under the assumption that exposure to deltamethrin occurred. Based on the hypothesised structure of the AOP, the marginal probability for the AO identifies the 'occurrence' as more probable than not irrespective of the status of the other MIEs/KEs. For the putative AOP network elaborated here, this marginal probability is 0.55.

Table C.13 displays the marginal probabilities for all the MIEs/KEs/AO in the network. It is worth noting that all the KEs have a probability to be activated greater than 0.5 leading to the conclusion that their activation is more probable than not when exposure to deltamethrin occurs.

The marginal probabilities also illustrate how the uncertainty propagates across the pathway. In fact KEs closer to the root of the network (MIEs as triggered by exposure to the stressor – deltamethrin) generally have, with few exceptions, a high probability to be activated whereas for KEs related to higher complexity

(i.e. organ and organism response) this probability tends to approach the maximum uncertainty (i.e. 0.5).

Table C.13. Marginal probabilities for all the MIEs/KEs/AO in the network

MIE/KE/AO		Probability	
		to be activated	to be not activated
MIE1	Binding to VGSC	0.99	0.01
MIE2	Binding to Ryanodine receptors	0.7	0.3
KE1	Disruption of sodium channel gate kinetics	0.98	0.02
KE2	Disruption of action potential generation; membrane depolarization	0.79	0.21
KE3	Disruption of axon terminal depolarization; changes in neurotransmitter release	0.76	0.24
KE4	altered neuronal network function	0.54	0.46
KE5	decreased oligodendrocyte differentiation	0.66	0.34
KE6	increase of intracellular sodium in microglia cells	0.66	0.34
KE7	Disruption of intracellular Ca channel kinetics	0.66	0.34
AO	Impairment behavioural function	0.55	0.45

5.6.4. Joint Probability: Quantitative estimate for mechanistic knowledge within the putative AOP network

The probabilities for the individual KEs to occur under the condition that the upstream KEs were activated, were used to estimate the joint probability that all events (MIEs, KEs, AO) in the network are activated/occur. This probability is 6.5% (see Appendix B). This was achieved by resorting to the Bayesian theorem and property of the nodes in a BN to be conditionally independent from all non-descendent nodes given the parents (conditioning nodes).

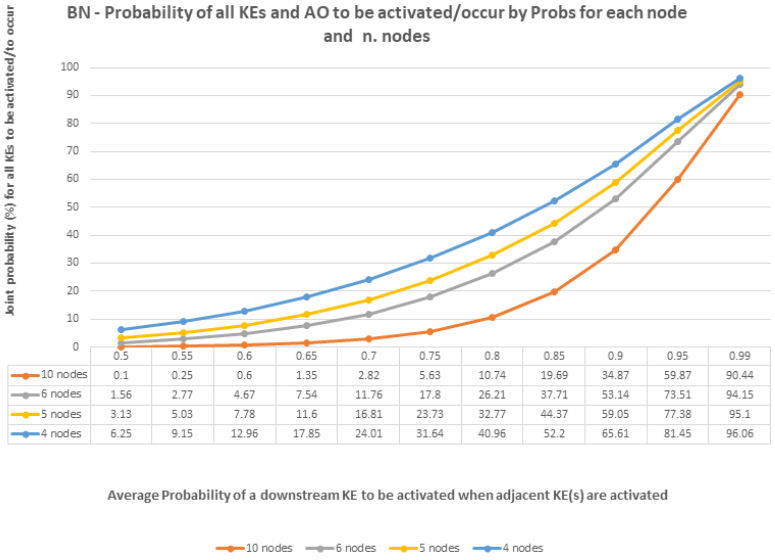
While it is appreciated that a network cannot be stronger than its weakest link, a characterization for the uncertainty averaged over the network may provide insight into the total AOP knowledge gathered. It is noted that the joint probability for all KEs and the AO to be activated within a network depends also on the number of nodes within the network. Therefore, an interpretation of this probability of 6.5% is neither meaningful in absolute terms, nor by comparison with absolute probabilities for other AOPs or AOP-networks (see Figure C. 23). Rather, it is meaningful to consider the probabilities for each KE being activated, averaged over all KEs in the network. The 6.5% probability for all KEs and the AO in a 10-nodes network being activated corresponds to a situation, where the average probability for the individual KEs being activated under condition that upstream KEs are activated is about 0.76. This is a moderate probability, considering that it represents a situation between random, where KEs would have an average probability of 0.5 and a situation of very high certainty, where KEs would have an average probability of 0.95. In comparison, the probability for all KEs and the AO being activated is 32.4% for the AOP string including only the best documented KERs for deltamethrin (MIE1-KEs1-2-3-4- AO). This corresponds to a situation where KEs would have an average probability of 0.83, which is somewhat higher compared to the average probability of 0.76 for the KEs in the complete network (see table 6.5_1).

Moreover, as mentioned, within this experimentally best established AOP string (MIE1-KEs1-2-3-4- AO), the KER5, connecting the last KE4 with the AO, is contributing most of the uncertainty. As discussed in section 4.5.4, the uncertainty in this KER is also due to uncertainties in the in vivo rodents AO measurements. Considering that from basic biological knowledge it is likely that effects on neuronal network function will affect behaviour, it appears useful to analyse this AOP string (MIE1-KEs1-2-3-4-AO) also without the in vivo rodent AO. This results in a relatively highest average probability of 0.87 for the KEs being activated within this reduced KER string (last column in Table C.14). Therefore, the mechanistic AOP knowledge base supporting that deltamethrin and other pyrethroids affect voltage

gated sodium channels which ultimately leads to altered network function (KE5) is strong. This information would be relevant if KE4 was recognised as immediately useful for regulatory toxicology.

In summary, the mechanistic knowledge underlying the putative AOP helps explain and support the experimental observations for deltamethrin (all KEs and AO activated) to a moderate degree (0.76 probability averaged over all KEs) and the non-adjacent KERs, including the most upstream KERs leading to the AO, are the elements limiting the total strength (0.66 probability for AO to occur with upstream KEs activated). Nevertheless, though it is always desirable to reduce uncertainty and increase knowledge, the mechanistic information collected within the putative AOP network, may already now help to support the overall conclusion on the adverse outcome.

Figure C. 23. Probability for all KEs and AO to be activated by number of nodes in the network



	0.5	0.55	0.6	0.65	0.7	0.75	0.8	0.85	0.9	0.95	0.99
10 nodes	0.1	0.25	0.6	1.35	2.82	5.63	10.74	19.69	34.87	59.87	90.44
6 nodes	1.56	2.77	4.67	7.54	11.76	17.8	26.21	37.71	53.14	73.51	94.15
5 nodes	3.13	5.03	7.78	11.6	16.81	23.73	32.77	44.37	59.05	77.38	95.1
4 nodes	6.25	9.15	12.96	17.85	24.01	31.64	40.96	52.2	65.61	81.45	96.06

Table C.14. Probabilities for all KEs and the AO being activated, number of nodes and per-node average conditional probably of the downstream KEs to occur given the activation of the connected upstream KE(s) for the AOP network and the linear strings

	AOP_net	AOP1: MEI1-KEs1-2-3-4-AO	AOP2: MEI2-KEs7-2-3-4-AO	AOP3: MIE1-KEs1-5-AO	AOP4: MIE1-KEs1-6-4-AO	MIE1-KEs1-2-3-4
Achieved Prob (%)	6.53	32.45	15.18	39.41	23.5	49.16
Average prob per node (%)	0.76	0.83	0.73	0.79	0.75	0.87
n. nodes	10	6	6	4	5	5

5.6.5. Influence of the upstream KEs on marginal probability for the AO to occur

The BN approach allows also to perform scenario analyses assessing the impact of hard evidence such as an individual MIE/KE occurring/not occurring with certainty (probability of occurring equals to 1 or probability of not occurring equal to 1 irrespective of the upstream KEs status). In this way it can be assessed, which MIEs/KEs-have the highest influence on the marginal AO probability to occur. MIEs/KEs can be ranked for their impact and this ranking could be used for recommending the best most useful test within an IATA. For the putative AOP network outlined here, the strongest impact is

from the most downstream KEs, i.e. KE4 (altered neuronal network function), followed by KE5 (decreased oligodendrocyte differentiation; see Table C.15.

Table C.15. Impact of uncertainty in MIEs/KERs on certainty in AO to occur within the putative AOP

Marginal Probability of AO to occur = 0.55					
MIEs/KEs		MIEs/KEs not active with certainty i.e. Prob (KEx=0)=1	MIEs/KEs active with certainty i.e. Prob		
		probability for AO to occur	probability for AO to occur	Difference in the Prob for AO to occur	Rank for influence
MIE1	Binding to VGSC	probability for AO to occur	0.55	0.08	6
MIE2	Binding to Ryanodine receptors	0.55	0.55	0	8
KE1	Disruption of sodium channel gate kinetics	0.46	0.56	0.10	5
KE2	Disruption of action potential generation; membrane depolarization	0.47	0.58	0.11	4
KE3	Disruption of axon terminal depolarization; changes in neurotransmitter release	0.46	0.58	0.12	3
KE4	altered neuronal network function	0.43	0.66	0.23	1
KE5	decreased oligodendrocyte differentiation	0.45	0.61	0.16	2
KE6	increase of intracellular sodium in microglia cells	0.55	0.56	0.01	7
KE7	Disruption of intracellular Ca channel kinetics	0.55	0.55	0	8

5.7. References

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