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About the OECD

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Foreword

This publication constitutes the proceedings of the OECD Workshop on Critical Innovations in pesticides safety testing and chemical risk assessment for developmental neurotoxicity (DNT). This event was organised under the auspices of OECD's Expert Group on DNT In vitro Battery (IVB) and held on 28-30 October 2024.

These workshop proceedings were reviewed by the Expert Group on DNT-IVB and the WPHA in March-April 2025 and received approval by the WPHA at the June 2025 meeting. It is published under the responsibility of the OECD Chemicals and Biotechnology Committee.

The OECD Workshop on Critical Innovations in Pesticide Safety Testing and Chemical Risk Assessment for developmental neurotoxicity was sponsored by the OECD Co-operative Research Programme: Sustainable Agricultural and Food Systems, whose financial support made it possible for some of the invited speakers to participate in the Conference in person.

Disclaimer: *The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries, nor does mention of trade names or products represent endorsement for use. The workshop discussion will be published separately in a scientific journal.*

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Executive summary

This publication constitutes the proceedings of the OECD Workshop on Critical Innovations in pesticides safety testing and chemical risk assessment for developmental neurotoxicity (DNT). This event was organised under the auspices of OECD's Expert Group on DNT In vitro Battery (IVB) and held on 28-30 October 2024. Seventy-nine participants attended the conference, in addition to seven participants from the OECD Secretariat. Participants represented government agencies from OECD member countries, intergovernmental organisations, industry, contract research organisations, academia, and non-governmental organisations. Participants were from a wide range of scientific backgrounds and provided expertise in developmental neurotoxicology, in vitro toxicology and development of new approach methodologies (NAMs), physiological based kinetic modelling (PBK), and regulation of pesticides and chemicals.

The OECD Expert Group on DNT IVB, was established by the Working Party of National Coordinators of the Test Guidelines Programme (WNT) in 2017 to facilitate the drafting and review of the Initial Recommendations on evaluation of data from the DNT in vitro testing battery (DNT-IVB) [ENV/CBC/MONO(2023)13]. The application and interpretation of the DNT-IVB were also elaborated in five IATA Case Studies endorsed by the WPHA in 2021. The learnings from the Case Studies, along with addressing critical steps identified in the 2023 Initial Recommendations, are part of a project added to the WPHA workplan in 2024. The intention of the WPHA project is to develop an IATA framework for combining and interpreting the DNT-IVB data and include aspects necessary for assessing DNT potential of chemicals. To this end, the Workshop on Critical Innovations included presentations on state-of-the-art science to address critical steps in building a DNT-IVB IATA Framework.

The workshop objectives were to drive toward a more efficient regulatory process by implementing new approach methodologies (NAMs) for developmental neurotoxicity (DNT) risk assessment of chemicals and pesticides in particular. The increased investment and innovative capacity of the chemicals industry to provide safe and sustainable chemicals are important to offer new solutions to the market. However, chemicals with hazardous properties can cause harm to human health and the environment. The existing chemicals and pesticides policies are evolving to respond more rapidly and effectively to the challenges posed by hazardous chemicals. This workshop provided a forum for exchanging experiences and increasing policymakers' understanding and support for the development and application of appropriate NAMs for faster and cost-effective chemical risk assessment methodologies to assess DNT.

The proceedings present a collection of papers from the speakers, providing an overview of the issues associated with DNT from the perspective of research, industry, and regulatory experts. The workshop focused on promoting dialogue on DNT In vitro Battery (IVB) and initiating a process to make recommendations for improvements by exchanging information on governments' or organizations' experiences and challenges in this area. The document also includes discussions on the development of a tiered exposure physiologically based kinetic (PBK) modelling framework and the identification of knowledge gained from CROs' or researchers' experience with additional assays to cover neurodevelopmental processes not represented in the DNT-IVB.

The break-out groups' discussions addressed the use of DNT-IVB data as complementary information and part of the Weight of Evidence (WoE) in an Integrated Approaches to Testing and Assessment (IATA). Standardisation of these data through an IATA framework template was emphasised to ensure consistency and reliability in their regulatory application. It further identified gaps and areas to be addressed in a draft tiered testing strategy to ensure a comprehensive and effective approach to DNT testing. The discussions also focused on assessing and identifying the steps needed to reach standardised testing methods, through the development of a defined approach.

1 Introduction

The proceedings present a collection of papers from the speakers published in the context of the OECD Workshop on Critical Innovations in pesticides safety testing and chemical risk assessment for developmental neurotoxicity (DNT). It aims to provide an overview of the issues associated with this topic from the perspective of research, industry, and regulatory experts and provide input to the potential future development of recommendations for ongoing and possible additional OECD work.

The workshop focused on promoting a dialogue on DNT In vitro Battery (IVB) and initiating a process to make recommendations for improvements by exchanging information on governments' or organisations' experiences and challenges in this area.

Developmental neurotoxicity refers to "any adverse effects on the normal complex developmental processes of the nervous system structure or function". DNT is one of those hazards which happen early in the development of human brain and manifest in the early years or later in life through either loss of cognitive function or sensory and motor deficits, leading to increased risk for neurological disorders later in life. Screening of chemicals for potential developmental neurotoxicity involves multiple targets and complex biological processes. The current regulatory testing paradigm is based on triggered OECD guideline in-vivo studies following prenatal and/or postnatal exposure. However, due to its complexity, cost, and resource limitations, the need for development of NAMs (New Approach Methodologies), has been emphasised by regulatory agencies and stakeholders.

The increased investment to protect the environment and human health, in particular that of vulnerable groups such as pregnant and nursing women, the unborn, infants and children, aims at innovative safety testing and chemical risk assessment to improve the quality, efficiency, and speed of chemical hazard and risk assessment but also to reduce dependency on animal testing in line with the 3Rs principle (Replacement, Refinement, and Reduction).

Participants

People attending the Workshop included:

- members of the OECD Expert Group on DNT-IVB;
- invited experts from key stakeholder groups such as the pesticide and chemical industry (BIAC);
- invited experts from research institutes (academia), and
- regulators, risk assessors and evaluators from governmental or intergovernmental bodies.

Purpose and Scope of the Workshop

This Workshop was an excellent opportunity to exchange information on experiences that OECD countries and relevant stakeholders have in the area of Integrated Approaches to Testing and Assessment (IATA) when applying DNT-IVB data. The main aim was to:

- Discuss what was learned so far and identify the focus of additional efforts to accelerate the uptake of DNT-IVB in the chemical risk assessment allowing faster action and optimisation of resources.
- Prioritise future efforts by identifying common strengths and limitations of the approaches used among the various case studies.
- Take a step forward from those efforts and discuss specific solutions to specific issues that were raised from the regulatory case studies; to identify specific questions or specific items that need to be addressed in a short-medium timeline for regulatory implementation.
- Develop a tiered exposure physiologically based kinetic (PBK) modelling framework that will allow the use of the DNT-IVB in vitro bioactivities to derive a Point of Departure (PoD) for human health risk assessment.
- Identify any knowledge gained from CROs' or researchers' experience with additional assays to cover neurodevelopmental processes not represented in the DNT-IVB and non-mammalian animal models.

Structure of the Workshop

The Workshop programme is provided in Annex A. Invited speakers included:

- International experts in this field;
- Government representatives; and
- Representatives from industry and research institutes.

Presentations were grouped into the following four themes and three sessions:

- Leverage on available experience from applying DNT-In vitro Battery (IVB) data
  - Lessons learned from existing IATA case studies using DNT-IVB data
  - Exposure assessment and Quantitative In vitro to in vivo extrapolation (QIVIVE)
- Regulatory implementation of the DNT-IVB
- Tiered testing strategy for application of DNT-IVB
  - Tiered testing, Additional Assays and Non-Mammalian Animal Models– What do they have to offer to the DNT-IVB?
- Develop Action Plans to Implement Recommendations

There was a short discussion after each set of presentations and a more general discussion at the end of the workshop. Break-out groups were formed to discuss:

- Use the DNT-IVB data as complementary information and part of the WoE in an IATA. What are the next steps for standardisation?
- Moving forward to an agreed-tiered testing strategy. What is still missing?
- Defined approach/es development for DNT testing. Are we there yet?

2 Lessons learned from existing IATA case studies using DNT-IVB data

US Division of Translational Toxicology (DTT): IATA case study for DNT to prioritise a class of chemicals

Helena Hogberg, NICEATM, US Division of Translational Toxicology (DTT), NIEHS, United States

Organophosphorus flame retardants (OPFRs) are abundant and persistent in the environment but have limited toxicity information. Their similarity in structure to organophosphate pesticides presents great concern for developmental neurotoxicity (DNT). However, current in vivo testing is not suitable to provide DNT information on the amount of OPFRs that lack data. An integrated approach to testing and assessment (IATA) to prioritise compounds for further testing was developed using the DNT in vitro battery (IVB) and behavioural data from zebrafish embryos. Eight OPFRs were evaluated, including aromatic OPFRs and halogenated FRs. Two representative brominated flame retardants (BFRs) with known DNT potential were selected for toxicity benchmarking.

Human biomonitoring and exposure data were identified and physiologically-based toxicokinetic models were applied to relate in vitro toxicity data to human exposure based on maximum plasma concentration. In the previous OECD published IATA, most critical uncertainties included lack of data across assays in the battery, limited mechanistic and exposure data, various confidence in the assays and data analysis and interpretation. The IATA has since then been updated with additional mechanistic and exposure data from the DNT IVB, the Integrated Chemical Environment (ICE) and the literature. Moreover, various prioritisation ranking methods have been proposed. Data from the DNT battery indicate that the aromatic OPFRs have activity at similar concentrations as the BFRs and should therefore be evaluated further. However, the DNT IVB assays provide limited information on the mechanism of these compounds. By integrating information from ICE and the literature, endocrine disruption was identified as a potential mechanism. Emphasising the necessity for additional

endpoints, this IATA case study suggests that combining the OECD DNT IVB with other NAMs could improve confidence in DNT assessment. New exposure data indicates that human exposure to some OPFRs could lead to a plasma concentration similar to those eliciting in vitro activities, indicating potential concern for human health.

Introduction

Flame retardants (FRs) are a group of chemicals that are added to various consumer products to reduce the spread of fire, including electronics, furniture, car seats, textiles, baby products and plastics (Dishaw et al., 2014; EPA 2005; Jarema et al., 2015). Historically in the US, brominated flame retardants (BFRs) were most commonly used but due to human health concern they have been phased out (Birnbaum and Staskal, 2004; Feo et al., 2013; Watanabe and Sakai, 2003). Instead, they have been replaced by organophosphorus FRs (OPFRs) causing increased human exposure to these novel chemicals (Blum et al., 2019; Stapleton et al., 2014). OPFRs are abundant and persistent but have limited toxicity information. However, their similarity in structure to organophosphate pesticides presents concern for developmental neurotoxicity (DNT) (Burke et al., 2017; Grandjean and Landrigan, 2014; Mie and Ruden, 2023). OPFRs as a class, consist of about 20-50 chemicals including commercial and isomeric mixtures, making in vivo testing impractical in terms of time and cost, to provide DNT information on all chemicals that lack data (NAS, 2019).

Purpose

An integrated approach to testing and assessment (IATA) case study was developed to prioritise OPFRs of highest concern for further testing using the DNT in vitro battery (IVB) and behavioural data from zebrafish embryos and planarian. Eight OPFRs were evaluated, including five aromatic OPFRs (triphenyl phosphate (TPHP), isopropylated phenyl phosphate (IPP), 2-ethylhexyl diphenyl phosphate (EHDP), tricresyl phosphate (TMPP), isodecyl diphenyl phosphate (IDDP), tert-butylphenyl diphenyl phosphate (BPDP)) and two halogenated OPFRs ((Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), tris(2-chloroethyl) phosphate (TCEP)). Two representative brominated flame retardants (BFRs) (2,2'4,4'-tetrabromodiphenyl ether (BDE-47) and 3,3',5,5'-tetrabromobisphenol A (TBBPA)) with known DNT potential were selected for toxicity benchmarking. The initial IATA case study was published by the OECD and described the most critical uncertainties including lack of data across assays in the DNT IVB, limited mechanistic information, limited exposure data, various confidence in the assays, data analysis and interpretation (OECD, 2022). To decrease these uncertainties the IATA case study was updated with additional mechanistic and exposure data (Kreutz et al., 2024).

Description of Workflow

For this workshop the speakers were instructed to specifically consider which parts of the case study can be standardised and incorporated into an IATA framework template and supporting guidance. Sections to include were description of workflow, data gathering, information sources, integrations, and interpretations.

The workflow of this IATA case study started with a problem formulation to “Select high priority chemicals within a class of OPFRs for further DNT hazard evaluation” (Fig 1).

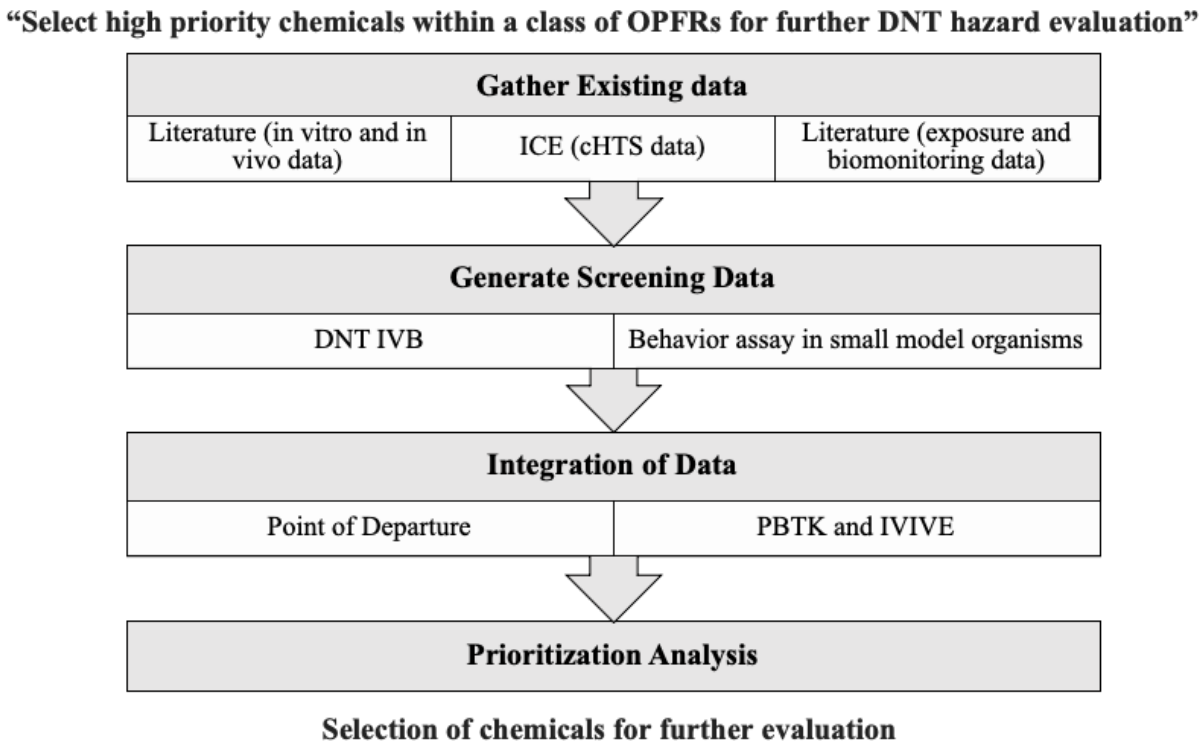

Fig 1. Workflow of the IATA case study for DNT to prioritise a class of chemicals containing various modules. 1) Gather existing in vitro and in vivo data from the literature, curated high throughput screening (cHTS) data from the Integrated Chemical Environment (ICE) and exposure and biomonitoring data from the literature. 2) Generate screening data using the DNT in vitro battery (IVB) and behaviour assays in small model organisms. 3) Integrating data using point of departure analysis and physiologically based toxicokinetic (PBTK) modeling for in vitro to in vivo extrapolation (IVIVE). 4) Prioritisation analysis to select high priority chemicals for further DNT evaluation.

Gather Existing Data

In the first step existing information on the OPFRs was gathered from the Integrated Chemical Environment (ICE) (https://ice.ntp.niehs.nih.gov) and the literature. ICE was developed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to support the development, evaluation and application of New Approach Methodologies (NAMs). ICE contains curated high-throughput screening (cHTS) data sets from the US federal Tox21 collaboration and EPA's ToxCast program that is annotated using control vocabulary terminology from the Open Biological and Biomedical Ontology (OBO) Foundry (http://obofoundry.org) (Daniel et al., 2022). The data extraction followed a systematic approach where all available testing data for the selected FRs were downloaded. Concentration-response curves for active calls were manually quality checked, curve fits that were not reliable, e.g., high variability or no data point above threshold were excluded. For the remaining curves activity concentration at cut-off (ACC) were collected as the data output. The approach taken here was to include all available endpoints, but it can be flexible by only selecting a specific endpoint of interest, e.g., DNT. The advantage with inclusion of all endpoints is the potential to identify mechanisms and other sensitive targets such as cardiotoxicity or endocrine disruption and compare the potency to the DNT endpoint. Such information could guide experimental design, and tailor follow up studies of selected high priority chemicals.

Additional in vivo and in vitro data were collected from the literature. A comprehensive narrative review (Patisaul et al., 2021) served as a source to identify publications on OPFRs. Lowest observed effect concentrations (LOEC) for each study and endpoint were collected as the data output. In addition, regulatory in vivo points of departure (POD) were gathered but were only available for three of the chemicals. In this case study, a full systematic review and bias analysis were not performed but could be considered. The advantage with such approach would be a lower uncertainty but due to the large number of chemicals it would be more time consuming and likely not favorable for prioritisation. There is a higher uncertainty using literature data without bias analysis because raw data are rarely available, data analyses are not standardised between studies and the interpretation is dependent on the author.

Generate Screening Data

As there was limited DNT information available for the selected OPFRs, screening information from the DNT IVB (OECD, 2023) and behaviour assays in small model organisms was generated. The division of translational toxicology (DTT) has its own pipeline for data analysis that provides benchmark concentration (BMC) (https://cran.r-project.org/package=Rcurvep) as the POD that is similar to the US EPA's ToxCast Analysis Pipeline (tcpl). Some uncertainties remained in the updated IATA case study, including, i) data generation was not performed in all the DNT IVB assays, and ii) one chemical had limited testing data from the battery. Some additional assays were included instead, such as neurite outgrowth assays in cortical and peripheral neurons differentiated from induced pluripotent stem cells (iPSC) (Li et al., 2021) and behavioural assays in zebrafish embryo (Quevedo et al., 2019) and planarian (Hagstrom et al., 2019).

Human Exposure Prediction

Human biomonitoring and exposure data from breast milk, house dust, hand wipes, urine and plasma were collected from the literature. Biomonitoring data was converted to oral doses using standardised parameters. The generic physiologically based toxicokinetic (PBTK) model provided by the US EPA's high throughput toxicokinetic (httk) R package (v.2.2.2) was then applied to relate in vitro toxicity data to human exposure based on maximum plasma concentration. For standardisation purposes, the R-script can be shared but is also accessible through a user-friendly interface provided in ICE.

Integration of Data

Data from the various data sources were integrated and revealed that the replacement OPFRs are quite active in the DNT battery and appear to have comparable activity as the phased-out BFRs. Moreover, the generated data showed that for most of the chemicals, the zebrafish embryo behaviour assay was the most sensitive, followed by the assay for oligodendrocyte differentiation. The majority of the most sensitive endpoints derived from ICE and the literature were annotated to endocrine disruption and effects on astrocytes and microglia populations, processes that are currently not measured in the battery. Follow up studies should therefore consider inclusion of such endpoints. Comparing in vitro POD with estimated plasma concentrations from human exposure and biomonitoring data showed an overlap for some of these chemicals which indicate potential concern for human health. The integration of data from multiple sources increased the confidence and proposed a mechanism of these chemicals to induce DNT.

Prioritisation Analyses

The final step in this IATA case study was to select high priority compounds for further DNT studies. Various prioritisation analyses were considered, including Pareto (Lotov and Miettinen, 2008), ToxPi (Marvel et al., 2018), most sensitive (lowest BMC) endpoint (Kreutz et al., 2024), most selective sensitive endpoint and ratio between estimated exposure and in vitro POD (Kreutz et al., 2024). Top prioritised chemicals differed between these analyses, and each of them has its own uncertainties that need to be

further evaluated. DTTs decision was to select TPHP, for further DNT assessment using tailored in vivo studies (Witchey et al., 2023). This was based on the most sensitive endpoint and overlap of in vitro POD and estimated human plasma from human biomonitoring data. Furthermore, DTT selected IPP because it has a different chemical structure compared to TPHP (presence of side-chain) and may provide information on how this impacts the toxicity.

The updated version of this IATA case study (Kreutz et al., 2024) decreased some of the uncertainties identified in the first version (OECD, 2022), mainly by incorporating additional mechanistic and exposure data. Each module of this IATA case study has been directed towards standardisation but also suggests where flexibility can be considered. Various approaches for how to prioritise chemicals were presented but more guidance on the best context of use still needs to be addressed.

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Case studies from large European (Horizon 2020) projects to refine testing strategies

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In the context of several projects (e.g. EU-ToxRisk and Riskhunt3R) overarching strategies for NAM-based risk assessment have been developed. They are being integrated into the Alternative Safety Profiling Algorithm (ASPA), which is an IATA with a very broad scope, and a defined structure. The latter means that not only building blocks have been defined, but that defined connections between these blocks are being established and further refined. The ultimate goal is to generate a structure that resembles a defined approach (DA). Various case studies have been set up to probe the functionality of the ASPA and to use the learning from the studies to further improve the ASPA. Two exemplary studies deal with the DNT hazard assessment of agrochemicals. Exemplary compounds are picoxystrobin (from the group of strobilurin fungicides) and thiacloprid (from the group of neonicotinoid insecticides). A number of steps along the ASPA will be demonstrated, using picoxystrobin as example. Strobilurins serve as example for challenges posed by the uneven distribution of toxicants in a test system and for the types of approaches required to quantify key event relationships in an AOP.

Summary

The development and initial use of the developmental neurotoxicity (DNT) in vitro test battery (DNT-IVB) has been a milestone in the transition from traditional animal-based testing for DNT towards next generation risk assessment (NGRA) approaches. As all new approach methodologies (NAM) of the DNT-IVB have a high readiness level, the data output is considered to be robust. However, the transition from test data to toxicological knowledge and further to the use in risk assessment needs to be defined. Here, a two-step process is presented. In a first step, the activity calls generated during a screen need to be followed up to define potential toxicants (confirmation testing). In a next step, information from all DNT-IVB NAM needs to be integrated and combined with other information sources. In the course of such an integrated approach to testing and assessment (IATA) process, also toxicokinetics need to be considered for risk assessment. An algorithmically structured, multi-tier approach to such an IATA was presented. This alternative safety-profiling algorithm (ASPA) has been established and is being refined by EU-funded projects of the ASPIS cluster, with a leading role being taken by Riskhut3R. It was outlined in the presentation and then exemplified, using the strobilurin fungicide picoxystrobin as example. Based on toxicity to neural crest cell function (UKN2 assay for migration), on a mechanistic rationale (mitochondrial respiratory chain inhibition) and on a careful toxicokinetics extrapolation, it was suggested that there is an at least 80-fold offset between the highest expected internal exposure and the lowest concentrations triggering adversity. Such data may be used in a final ASPA module for risk assessment.

Introduction

Developmental neurotoxicity (DNT) is one of the more complex domains of toxicology, compared with e.g. the assessment of acute topical toxicity to skin and eyes. The animal-based guideline studies, e.g. according to OECD TG426, are amongst the most complex and resource requiring of all test methods in toxicology. The reason for this is that developmental neurotoxicants may act through many targets, affect multiple pathways and cell types and lead to a large panel of adverse outcomes on the level of nervous system structural organisation or function (Smirnova et al., 2014, 2024; Celardo et al., 2025). It has, therefore been considered extremely difficult to assess chemicals for DNT hazards, based on non-animal novel approach methodologies (NAM).

DNT is often assessed in humans by complex neuro-functional and cognition tests. To allow the testing by NAMs, the concepts of toxicity endophenotypes (TEP) and key neurodevelopmental processes (KNDP) have been important (Bal-Price et al., 2015, 2018; Kadereit et al., 2012). Briefly, it is assumed that all apical endpoints assessed during DNT testing are due to structural or functional disturbances of neural connectivity (TEP) and that this may be caused by the disturbance of one or more KNDPs (e.g. migration, neurite growth, differentiation, myelination, synapse formation or neural network formation). The latter processes and their disturbance by chemicals can all be modelled and assessed by NAMs (Celardo et al., 2025).

Based on this, it has been hypothesised that a battery of NAMs, assessing KNDPs, can be assembled (Bal-Price et al., 2015, 2018). With a sufficiently broad coverage of KNDPs, such a battery may identify a potential DNT hazard of test chemicals (Smirnova et al., 2014, 2024). Indeed, initial versions of a DNT in vitro battery (DNT-IVB) have been assembled and tested with reference chemicals (Aschner et al., 2017) and a large number of additional compounds to explore the response dynamics (Blum et al., 2023; Carstens et al., 2022). The accuracy is > 80%. To increase confidence in the results generated by NAMs of the DNT-IVB, readiness criteria have been developed and evaluated. Important questions for the future are how the battery hits are further processed, how test positives relate to AOPs (Leist et al., 2017; Hartung et al., 2024) and how this knowledge is integrated and interpreted in the context of integrated approaches to testing and assessment (IATA) (Blum et al., 2023; Cöllen et al., 2025).

Connecting NAM-based screening and risk assessment

The very first NAMs that were validated and formed the basis of OECD test guidelines (e.g. for phototoxicity or eye irritation) were assumed to make predictions on apical toxicity outcomes that may be used for risk assessment or classification of chemicals according to GHS. Since then, it has become clear, that individual NAMs can hardly provide such information, especially in the more complex toxicological domains (developmental and reproductive toxicity, systemic target organ toxicity, carcinogenicity) (Leist et al., 2014). A more refined concept for the interpretation of NAM data has been developed (Fig. 1). According to this concept, it can be assumed that many NAMs are a tool to sort test chemicals into hits and non-hits (according to the data interpretation procedure (DIP) of the NAM). Each of the DNT-IVB NAM has such a DIP, as defined in the respective ToxTemp forms (Cöllen et al., 2024; Krebs et al., 2019, Blum et al., 2025). For assays, used in a screening mode, several follow-up steps are necessary (e.g. confirmation testing in various assay setups, control for potential technical artefacts, etc.) to show that a hit (activity call) should be considered a candidate toxicant (Smirnova et al., 2024). The follow-up and confirmation of hits should be considered a general principle of toxicology, be it traditional or next-generation risk assessment (NGRA)-based (Leist et al., 2024; Magel et al., 2024).

At this stage, data on the test chemical would, in most cases, be integrated with other types of information (information on toxicokinetics and external exposure as well as data from other assays) for a risk assessment or classification of the chemical. This process of collecting, integrating and interpreting data from different information domains takes place within an IATA process (Fig. 1). To some degree, these considerations also apply to non-hits. Altogether, this means that hits from the DNT-IVB need to be fed

into a processing procedure in order to come to regulatory conclusions. A special form of structured IATA, i.e. the Alternative Safety Profiling Algorithm (ASPA) may offer some advantages for this procedure and will be exemplified below. The ASPA is currently under development in the EU Riskhunt3R project, and this is accompanied by the graphical user interface NAMASTOX (https://www.risk-hunt3r.eu/aspa/), which is scheduled for public presentation and release in 2025. More detailed background information may be found using following links: (https://www.youtube.com/watch?v=8z63MGg71HM; https://www.youtube.com/watch?v=fqk17Y3wz9o; https://www.risk-hunt3r.eu/wp-content/uploads/RH3R_newsletter_Issue4_FV.pdf. or in Celardo et al., 2025).

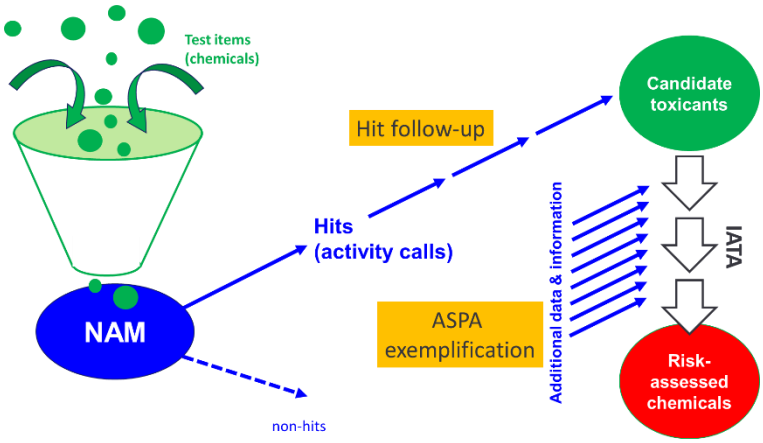

Fig. 1: Overview of the processes required to move from screening in NAM to risk assessment

Construction principles of an algorithmic IATA, named ASPA

During the past 15 years, many teams and organisations suggested key elements that need to be considered for an integrated risk assessment (e.g. Leist et al., 2014). The OECD IATA case studies program provides a lot of examples. The ASPA uses the same canonical building blocks as suggested in many other publications (Fig. 2). The problem formulation is considered to be the entry point and it feeds in e.g. the definition of the test chemical and the regulatory questions to be answered. The problem formulation is important for exact definitions of more downstream decision points in the IATA/ASPA procedure. In a next step, already available information would be collected. If not sufficient for risk assessment, three major modules of data generation are activated. The exposure module defines the external exposure, depending on use scenarios, and then links to the toxicokinetics module (ADME) to determine the internal exposure. Important for this is the setup and parametrization of suitable PBK models. A further function of the ADME module would be to provide information on potentially toxic metabolites and specific distribution phenomena (e.g. via specialised transporters). The hazard module would e.g. contain the DNT-IVB for screening. Ideally, relevant mode of action (MoA) and AOPs would be identified (Tal et al., 2024; Leist et al., 2017). PoD would be used for IVIVE for prediction of adverse doses. This information (and the linked uncertainties) would then need to be integrated and interpreted to allow risk assessment.

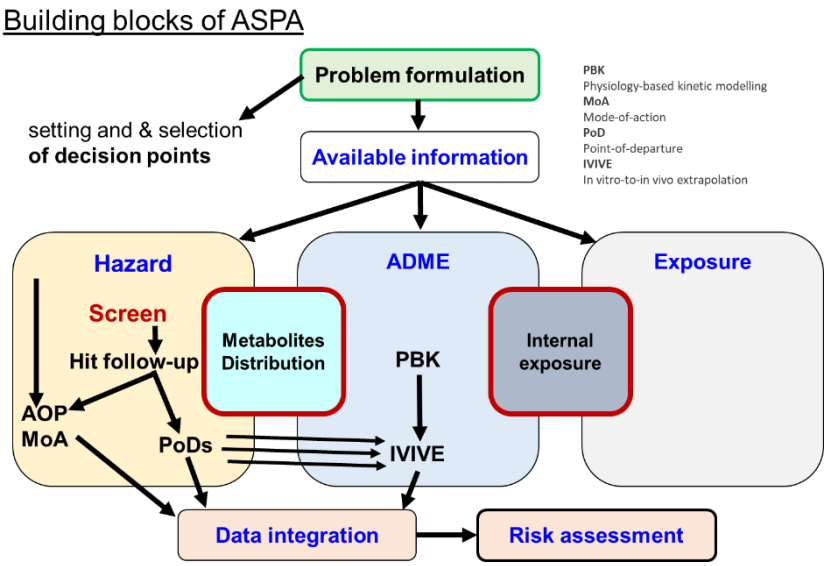

Fig. 2: Overview of the main building blocks of ASPA and of a simplified workflow.

The conventional IATA procedure contains all essential elements and is very flexible. However, the steps taken, the sequence of the steps, the methods used, the decision points and resultant decisions, and the way how information is integrated and used for risk assessment are not defined. Thus, it is likely that groups of experts may arrive at different conclusions, even when faced with similar problem formulations and similar sets of data. ASPA attempts a solution to this problem by suggesting an algorithmic procedure for data generation and interpretation, but also by providing a rationale for the setup of this structure and the selection of methods (Fig. 3). Accompanying the ASPA, a software dashboard is being developed that leads through this procedure, and tracks/documents each step. The vision is that such data are stored for many compounds so that a historical reference base is generated that helps making expert decisions more consistent (Fig. 3).

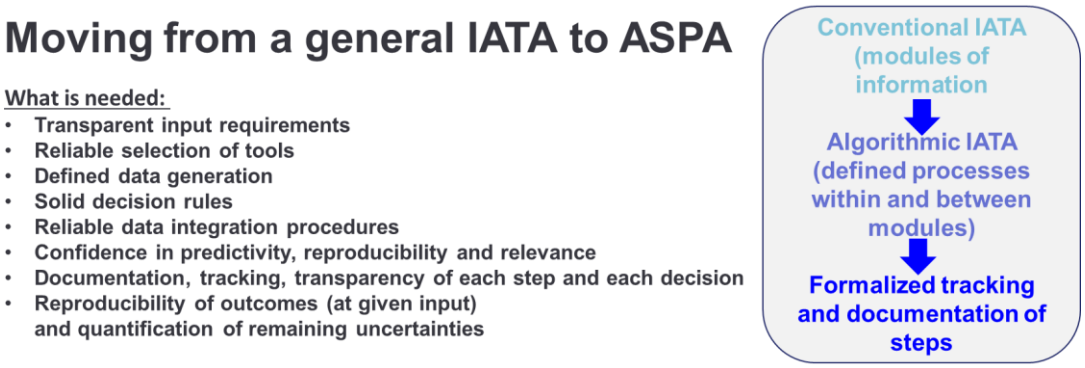

Fig. 3: Elements important to move from a generic IATA concept to ASPA.

Exemplification of ASPA by a picoxystrobin case study

An exemplification shows best how the ASPA may work, and whether it is applicable to DNT. In a recent screen, initiated by the US NIEHS, picoxystrobin emerged as a hit in some assays. Details may be found in a video recording (https://www.youtube.com/watch?v=fqk17Y3wz9o) or an accompanying publication (Magel et al., 2024).

Briefly, picoxystrobin inhibited the migration of neural crest cells in the UKN2 assay. This test (Nyffeler et al., 2017 a;b) is part of the DNT-IVB, and hit confirmation, combined with mechanistic investigations,

showed that the compound blocks mitochondrial function and may reduce cellular energy levels. A PBK model was established (including also foetal compartments), and PoD from NAM testing were converted by IVIVE to expected adverse doses. As an additional correction factor, the uneven distribution of the test compound in cell culture compartments was considered and calculated as explained earlier (Magel et al., 2024, Fisher et al., 2019). To prepare data for risk assessment, it was considered important to give a visual overview of concentration ranges that follow good reporting standards (Hartung et al., 2019) and to allow various comparisons. For instance, based on a maximal exposure, the picoxystrobin concentrations that may be reached in various maternal or foetal compartments were calculated and displayed (Fig. 4). In the same overview, the PoD concentrations obtained from various NAM are displayed. For the most relevant couple of exposure and NAM PoD, also the biokinetics correction factor was displayed and applied. In this context, the term biokinetics refers specifically to distribution phenomena of the test compound in the culture dish. It describes and quantifies differences between the nominal test concentration and predictions of actual concentrations at the intracellular target site. The VIVD model (Fisher et al., 2019) which takes into account compound logP and ionisation of the test compound, cell and medium volumes, as well as protein and lipid content of cell culture compartments, was used. Based on this modelling approach, the highest reached concentration was at least 80 times lower than the concentration required for a biological (or possibly toxic) effect (Fig. 4). When the predicted intracellular concentration that triggered toxicity was used as PoD for in vitro-to-in vivo extrapolation, a threshold human toxic dose of > 7.2 mg/kg/day was predicted.

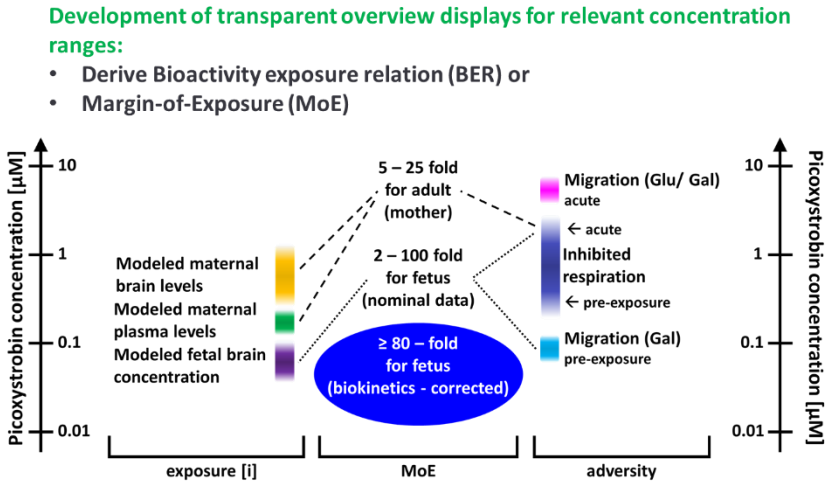

Fig. 4: Overview of data from the picoxystrobin case study and exemplification of a comparative data overview

Perspectives

It has been addressed here, how the DNT-IVB may be used for risk assessment. The DNT-IVB generates patterns of activity calls: these need to be followed up and then further assessed in an IATA process. It was suggested here to work towards a structured, algorithmically defined IATA, as exemplified by ASPA. The outcome of such an ASPA process was exemplified for picoxystrobin.

Not addressed here was how such outcomes of data integration would be used in risk assessment e.g. for setting of health-based guidance values or for classification and labelling. A final ASPA module would need to provide guidance on this. A stakeholder meeting held at the premises of the BfR in Berlin on 26-28 May 2025 discussed and defined these steps.

The picoxystrobin case study also indicated some scientific problems that need clarification in the future. For instance, the assays of the DNT-IVB are performed in a highly standardised format. This has

advantages for definition of readiness criteria, for comparability of the data and for ease of interpretation. However, the human variability (concerning genetics, metabolic states and exposure times/windows) may be larger (Suciu et al., 2023; Delp et al., 2018) than what is captured by the DNT-IVB NAM. This was exemplified here, by different PoD obtained in medium with glucose (Glu) or with galactose (Gal) (100-fold difference, i.e. going from 0.1 µM to 10 µM),, just to name a simple assay variation (Magel et al., 2024). Future studies need to explore whether and how such additional information can and should be obtained and whether it has an effect on risk assessment.

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US EPA Case-studies: Prioritisation, Weight of Evidence and Waiving in vivo DNT test studies

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New approach methodologies (NAMs) for developmental neurotoxicity (DNT) present an opportunity to evaluate compounds for potential hazard quickly and economically. The scientific consensus is that data from DNT NAMs can inform for screening and prioritisation and weight of evidence decisions for DNT. Over the past decade, EPA has developed in vitro NAMs that evaluate chemical effects on key neurodevelopmental processes and has used data from these assays to inform decisions on chemicals. This presentation covered different case studies where data from one or more DNT NAMs have informed screening and prioritisation and weight of evidence decisions for industrial compounds and pesticides. Collectively, these case studies provide insight into how Integrated Approaches to Testing and Assessment can be designed and utilized such that they benefit from EPA NAMs data. (This abstract does not represent EPA Policy).

This paper discusses the United States Environmental Protection Agency's (US EPA) experience with the DNT-IVB, specifically focusing on several different case studies involving prioritisation and weight of evidence evaluations. It includes some examples where data from the assays in the DNT-IVB were used to help support decisions about granting waivers for the in vivo DNT Guideline study. These case studies followed IATA principles, but they have not been submitted to OECD as formal IATAs. The US EPA has developed and contributed to the DNT-IVB the following assays: proliferation and apoptosis in hNP1 cells, neurite outgrowth in human neurons from FUJIFILM Cellular Dynamics International's (FCDI) and rat primary cultures, synaptogenesis and neurite maturation in rat cortical neurons, and network formation in rat cortical neurons.

The first case covered a screening and prioritisation example, a case study on per- and polyfluoroalkyl compounds (PFAS). This class of compounds has been recognised over the past decade to be a significant environmental concern where widespread contamination is resulting in broad exposure to human populations. At the same time, little is known about the health effects of PFAS compounds, including their potential to cause DNT. Epidemiological studies evaluating PFAS with adverse neurodevelopmental outcomes are equivocal, and animal studies were limited to only a few PFAS compounds out of the thousands that humans are exposed to. To develop screening level data, US EPA selected a set of ~160 compounds representing different structures from the universe of PFAS compounds and screened this set in several NAMs assays, including four of the US EPA's DNT NAMs (proliferation, apoptosis, human neurite outgrowth and network formation, as well as the respective cytotoxicity assays for each endpoint). Only about 25% of the PFAS compounds were active in these assays, and they were generally less potent than compounds that are well recognised to cause DNT in mammals (Carstens et al., 2023). When these data were compared to effects of PFAS compounds in screening assays for activity against nuclear, oestrogen and thyroid receptors, lipid peroxidation and oxidative stress, or vascular, immune, skin and lung responsiveness, as well as high-throughput transcriptomics, effects in the DNT assays were less frequent and less potent than other endpoints; in no case was the DNT point of departure (POD) the lowest

POD derived from all of these in vitro endpoints (Judson et al., 2024), suggesting that DNT may be of less concern for PFAS compounds.

Several important challenges were faced during this study. The first was that it took place during pandemic restrictions, which delayed the timeframe for completion. More importantly, compound cost, availability and stability were challenges that needed to be overcome. Key to success here was that this was a large project supported by US EPA teams that provided assistance with chemical procurement and analytical characterisation to provide information on compound stability. Lessons were learned from this case study, including that analytical support is needed when working with a large and structurally diverse compound set, as well as that structural characteristics of the PFAS compounds influenced its action. For the DNT assays, active compounds tended to have a carbon chain length >7, high carbon to fluorine (C:F) ratio and a carboxylic acid moiety (Carstens et al., 2023). The study also highlighted several benefits, including that it was possible to test ~160 compounds in <2 years during a global pandemic. This is less time than it would take to complete a single guideline DNT study, and that collecting data on this many compounds in such a short time would have been unattainable using animal methods.

The second case study focused on the use of data from two of the US EPA assays (neurite outgrowth and network formation) in a weight of evidence decision regarding a waiver of DNT guideline studies for L isomers of the herbicide glufosinate. The Agency already had data from DNT guideline study for the racemic mixture DL-glufosinate and sought to determine if the L-isomers of glufosinate acid and glufosinate ammonium had bioactivity that was similar to the racemic mixture (DL-glufosinate). US EPA used an IATA-like approach to select two assays to compare bioactivity of the L- isomers with that of the racemic mixture. Because there were changes in hippocampal structure in the DNT guideline study with DL-glufosinate, US EPA selected the human neurite outgrowth assay. Further, because there were changes in network activity in “mature” neural networks following acute exposure (Lantz et al., 2014), the Network Formation Assays was selected. There were no differences between the L-glufosinate isomers and DL-glufosinate in these assays, but these compounds also did not elicit any biological activity. Therefore, the acute network activity experiment was repeated with the L-glufosinate isomers and the DL-glufosinate, and the results of Lantz et al., 2014 were replicated. This provided additional benchmarking for results of the DNT assays. Using httk to inform IVIVE, it was demonstrated that the highest concentrations tested in the in vitro studies exceeded points of departure based on other available data for L-glufosinate. This contributed to the weight of evidence decision to waive the requirement for a new DNT guideline study for the L-isomers of glufosinate (US EPA, 2021; Dobreniecki et al., 2022).

Two significant lessons were learned from this project. First, communication between Agency regulators, Agency researchers and the registrants was key to the success of this project. This project also serves as an excellent example of the benefits of DNT NAMs for WoE decisions. It is estimated that compared to the guideline DNT study, this case study saved about 15 months of time to collect the data needed to inform the decision, used less than two dozen animals and saved nearly $1.9M (Boyd et al., 2025). In addition, this study also demonstrated that in a WoE context, negative results from the DNT NAMs help to inform decisions, especially when they are supported by proper assay controls and other in vitro and in vivo data.

The third case study involved the pesticide Dichloran (DCNA). The EPA’s Office of Pesticide Programs received a waiver request for the guideline DNT study for this pesticide, for which there were data from in vivo studies available. In the 2-year rat carcinogenicity study, neurotoxicity had been reported for DCNA, but was not reported in subchronic studies in rats and dogs. Further, there was evidence of placental and lactational transfer of DCNA, which raised concerns for potential developmental neurotoxicity. Unfortunately, the developmental and reproductive toxicology guideline studies did not include DNT groups (US EPA, 2022a). To gain additional information, available information on neurotoxicity and developmental neurotoxicity in EPA’s ToxCast database was considered. DCNA had not been tested in any DNT NAMs but was reported to be active in the acute network activity assay, and when administered equivalent doses were estimated using httk, the estimated activity was near points of departure for other endpoints. This

supported concerns about neurotoxicity and developmental neurotoxicity. Based on all of the available evidence, EPA did not grant a waiver for the Guideline DNT study for DCNA (US EPA, 2022b).

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EFSA's IATA Case Studies for hazard identification and characterisation: lessons learnt

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In June 2023, the OECD published the Initial Recommendations for evaluating the DNT In Vitro Battery (DNT IVB) data [ENV/CBC/MONO (2023)13]. A series of Integrated Approaches to Testing and Assessment (IATA) using the DNT IVB data in different regulatory frameworks and context are included in the guidance as a proof-of-concept case study.
For EFSA, the Adverse Outcome Pathways (AOP) informed IATA framework, using the DNT IVB for single chemical hazard characterisation, complemented by exposure models, is the proposed tool to integrate the DNT IVB in the overall Weight of Evidence (WoE) in the EU regulatory framework for pesticides. In these case studies, the DNT IVB demonstrated to provide relevant, high-quality data and it is trusted by the stakeholders. The DNT IVB provided information regarding the DNT potential of chemicals perturbing early cellular processes difficult to measure in vivo. The workflow was built in line with the European pesticides Regulation (EU) 283/2013 and 1107/2009 in a structured evidence-based approach integrating all the available evidence, including a systematic literature review, available guideline studies and data generated through the DNT-IVB with a transparent assessment of the uncertainty. The workflow demonstrated how to apply mechanistic information in the regulatory process of DNT hazard characterisation and allowed to arrive at the same conclusion for the existing published OECD IATA.
These case studies are the first European experience integrating the DNT IVB for DNT regulatory purposes and showed the relevance of the DNT-IVB in an AOP informed IATA for regulatory decision-making. Several observations emerged during the development process, including: (1) The DNT-IVB data is publicly available and should be used in the pesticides risk assessment in EU; but the availability of data for approved pesticides in EU is limited. (2) The EFSA AOP informed IATA framework is fit for purpose and is the current recommended approach for integrating the results of the DNT IVB (3) When a non-endorsed AOP and non-standard IATA framework exists, the development of an evidence-based AOP informed IATA, in line with a systematic approach, is resource-intensive; (4) implementation/standardisation of physiologically based kinetic (PBK) modelling for quantitative in vitro to in vivo extrapolation (QIVIVE) is a necessary next step. In addition, several elements of the AOP informed

IATA Workflow can be standardised (i.e. rule based), and this would help the uncertainty analysis and expert regulatory decision making.
EFSA is currently testing over one hundred pesticides in the DNT IVB, conducting the transferability of the assays and developing more case studies. The availability of a standard framework to develop and use AOP informed IATA for DNT will be highly beneficial for the correctness of the WoE in chemical risk assessment as well as further interpretative guidance is necessary for some of the assays. This future work will need a constant collaborative effort at an international level.

Introduction

Neurotoxicity (NT) is a perturbation to the function, structure, or chemistry of the nervous system, while DNT includes when any of these changes that occur following exposure during nervous system development (U.S. Environmental Protection Agency, 1998). The assessment of neurotoxicity (NT) and developmental neurotoxicity (DNT) is critical for evaluating risks associated with chemical substances and is embedded in the various chemical regulations, particularly pesticides. While routine clinical observation for neurotoxicity is included in standard rodent-based in vivo toxicological studies (acute, sub-chronic, chronic, reproductive, and carcinogenicity studies), specific neurotoxicity studies (OECD Test Guidelines (TG) 424, 407, 408, and optionally TG 452) and developmental neurotoxicity (OECD TG 426 and TG 443- with DNT cohort 2 A and B), are triggered “when indicated by observations in other studies OR the mode of action of the test substance. In Annex II of the Regulation No 1107/2009, DNT is considered a critical effect of particular significance and when the critical effect is judged of particular significance, an increased margin of safety shall be considered, and applied if necessary.

There are some recognised inherent difficulties of assessing NT and particularly of assessing DNT of chemicals; among them (i) toxic chemicals could affect any functional or structural component of the nervous system (NS) (e.g. sensory and motor functions, memory processes, behavioural and neurologic abnormalities) with species differences and at different developmental windows of exposure; (ii) the NS exhibits a greater degree of cellular, structural, and molecular heterogeneity than other organ systems; (iii) the NS is well known for its long developmental period, which also continues after birth, and proper development of the NS is a complex process that involves the correct spatial and temporal sequence of events needed to form the central nervous system; (iv) the long human lifespan increases exposure to various chemicals and factors able to modulate the final response; (v) NS development is more vulnerable to exposure to environmental chemicals during foetal and early postnatal periods (Rodier et al., 1995). In addition, the toxic impacts of chemical exposure can interact with other risk factors like prenatal stress, and the persistence of some chemicals in the brain over time may result in cumulative toxicity (For a recent review, please see Viviani et al., 2025)

Recognising the vulnerability of the developing brain and the importance of safeguarding it from potentially hazardous chemical exposure, DNT testing in rodent models was developed (US EPA Workshop, 1989), the first regulatory guideline appeared in 1991 (US EPA., 1991) and after that the US EPA 1998 and 2004 (US EPA., 1998; 2004) and in 2007 was internationally refined and approved under the auspices of OECD (OECD., 2007) and recently with the OECD TG 443 with DNT cohort (OECD., 2018). DNT guideline-based in vivo testing studies have been designed for screening potential chemical-induced DNT hazards measuring both behavioural (i.e. clinical signs, ontogeny of motor function, startle response, and learning and memory) and neuropathological endpoints (including neurophysiological changes, and morphometric measurements) (See NAFTA guidance 2016 for details). However, the use of the in vivo DNT Test

Guidelines has been limited compared to other guideline toxicity studies. Over these 25 years, around 250 DNT regulatory studies have been conducted on a total of around 230 chemicals (Crofton and Mundy., 2024). Potentially due to the methodological and interpretation complexity, cost and resource requirements of the DNT in vivo studies. Finally, the in vivo guideline DNT study is not one of the core studies and is only triggered if there's an indication that a chemical affects the nervous system or has a relevant mode of action (MOA).

In recognition of these challenges, in June 2023 (OECD, 2023), the OECD published the Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity In Vitro Testing Battery (DNT IVB), which is the result of a major project led by EFSA, the US EPA and academic groups over the last years to standardise a battery of 17 in vitro assays for DNT assessment. These individual assays that comprise the battery were selected based on (1) their capability to measure key neurodevelopmental processes (KNDP) and (2) their readiness criteria for regulatory use (Bal-Price et al., 2018). The purpose of the OECD document is to provide guidance on the evaluation and interpretation of results from the DNT IVB for the identification of substances with DNT potential. The DNT IVB is based on measurement of changes in key neurodevelopmental processes (KNDP) with relevant in vitro test systems representing an accessible model to assess chemicals for DNT potential. Any developmental neurotoxicant is expected to affect at least one biological process at the cellular/molecular level.

So far, the DNT IVB has been used to provide data for making decisions about chemicals in various regulatory contexts of use. For single chemical hazard characterisation, EFSA applies the Adverse Outcome Pathways (AOP) informed Integrated Approaches to Testing and Assessment (IATA) framework, using the DNT IVB, which is complemented by exposure models. This is the proposed tool to integrate the DNT IVB in the overall Weight of Evidence (WoE) in the current EU regulatory framework for pesticides. The initial case studies were developed to assess the applicability of the DNT IVB in the regulatory risk assessment of pesticides. The first IATA case studies were developed before being included in the OECD DNT IVB Guidance under development by the DNT expert group as a proof of concept on the use and interpretation of the DNT-IVB. Considering that the use of in vitro methods in isolation for risk assessment is in its infancy at EFSA, for the first case studies, pesticide active substances with also in vivo OECD TG DNT study available were prioritised (Hernandez-Jerez et al., 2021).

EFSA DNT IVB regulatory case studies

The proposed tool to integrate the DNT IVB in the overall Weight of Evidence (WoE) in the current EU regulatory framework for pesticides is the Adverse Outcome Pathways (AOP) informed IATA framework. This approach uses the DNT IVB for single chemical hazard characterisation and is complemented by exposure models (Hernandez-Jerez et al., 2021).

The EFSA Plant Protection Products and their Residues (PPR) Panel built a DNT workflow in line with the current European pesticides Regulation (EU) 283/2013 and 1107/2009 (European Commission, 2009, 2013). The framework is in line with EFSA uncertainty analysis guidance and uses a structured evidence-based approach. (Hernandez-Jerez et al., 2021) to identify the fit-for-purpose use of the DNT IVB (OECD., 2023). So far, using EFSA framework for EU pesticides decision making, the following case studies are publicly available: deltamethrin, flufenacet, and acetamiprid (OECD 2022a; OECD 2022b; OECD 2022c; Hernandez-Jerez et al., 2024).

For the first case studies, two pesticide active substances, tested in the 17 assays of the DNT-IVB, were selected: deltamethrin, a type II pyrethroid with a well characterised neurotoxic effect and a neurotoxic pesticidal mode of action, and flufenacet, a herbicide with a mode of action not related to a neurotoxic effect but for which a regulatory DNT study (i.e. OECD, 2007) was available. The AOP conceptual framework was used to organise and integrate all the evidence and contextualise the mechanistic evidence with the apical toxicity endpoints. Exposure data were used to support the dose and time concordance for the empirical support of the key event relationships (KERs) in the postulated AOP informing IATA.

A protocol was therefore developed based on EFSA Scientific Committee (SC) (2020), which included detailed information on the strategy and methods for the systematic review process used. The systematic literature review was conducted for three lines of evidence (human observational studies (HOS), experimental in vivo data in rodents and in vitro studies for deltamethrin and flufenacet. Then the evidence was appraised for risk of bias (RoB) using a tailored version of the OHAT-NTP RoB tool (https://ntp.niehs.nih.gov/whatwestudy/assessments/noncancer/riskbias). To ensure that the assessment provided reliable information for decision making, special considerations were given to the uncertainty analysis, with the identification, characterisation and evaluation of uncertainties and limitations. The uncertainty analysis was performed for each line of evidence to support conclusions on the hazard identification and characterisation using a predefined set of questions.

The stepwise approach culminated in a proposed evidence-based AOP network for deltamethrin with a probabilistic quantitative estimation of the weight of evidence (WoE) using a Bayesian network analysis. The stressor-based approach was selected because the intention was to use the AOP conceptual framework to establish a causal relationship between the prototypical chemical deltamethrin exposure and the AO (Hernandez-Jerez et al., 2021; OECD, 2022a). In a later stage, Deltamethrin AOP informed IATA was used as a starting point for developing an agnostic AOP with voltage gate sodium channel (VGSC) inhibition as a molecular initiating event (MIE) during mammalian development leading to adverse consequences in neurodevelopment evidenced as deficits in cognitive functions (Hernandez-Jerez et al., 2024). In this recent EFSA report, a robust AOP is presented (AOP 442 in AOP Wiki) mapping one of the KEs covered by the DNT IVB. The AOP developed describes an AO initiated by the binding of a stressor to the VGSC (MIE) during mammalian nervous system development. This MIE (KE1353) results in disruption of sodium channel gate kinetics (KE1977) and subsequent disruption of neuronal action potential (KE1983). This KE leads to a subsequent alteration in neurotransmission during development (KE4) that can be measured using the neural network formation assay, one of the standardised assays included in the DNT IVB. These early KEs and KERs are considered canonical based on established knowledge and methods in neurobiology. When these KEs are disrupted during development, they can modify hippocampal anatomy (KE757) and physiology (KE758), ultimately impairing certain cognitive functions (e.g. learning and memory; AO402). This second part of the AOP, starting from KE4, benefits from an existing endorsed AOP. The WoE for the entire AOP developed herein is strong due to the strong biological plausibility for all KERs, with empirical support either strong or moderate. Knowledge gaps identified include the relationship between the AO and specific critical developmental time windows, as well as the lack of quantitative relationships along the KERs up to the AO. New research is proposed to address these limitations. Several regulatory applications of this AOP have been identified for the assessment of DNT. This AOP should foster the use of data from the DNT IVB for hazard characterisation (Hernandez-Jerez et al., 2024).

As regards of flufenacet case study, a structured scientific assessment approach was employed, in line with the one used for the deltamethrin case study. This was defined by the inclusion of a predefined Systematic Review protocol for the literature (including the appraisal of the evidence with a critical appraisal tool and data extraction and analysis) and a quantitative uncertainty analysis. The AOP-informed IATA was used to integrate all the existing in vivo and in vitro data, including the DNT-IVB. The outcome of the analysis indicates that evidence is available for which a probability of at least 66% would support the identification of a DNT adverse outcome (from the OECD TG 426 available study). Regarding the analysis of the in vitro data (only data from the DNT IVB was available), there was no evidence of any DNT KE; therefore, a DNT I-B informed AOP, informed IATA, could not be developed.

Following the integration of all evidence from in vivo and in vitro studies, it is concluded that flufenacet is unlikely to be a developmental neurotoxicant in humans through a direct mechanism. The inclusion of the DNT-IVB data has been critical to contextualise the outcome of the in vivo study. This case study supports the applicability of the DNT IVB for data poor chemicals or to test the hypothesis that a substance is not

DNT through a direct neurotoxic mechanism. The negative results in the zebrafish study were consistent with the lack of effects in the DNT IVB (OECD, 2022b).

The workflow was also used recently for assessing DNT of acetamiprid following a request from the European Commission to advise on human health or the environmental impacts based on new scientific evidence presented by France during the decision-making phase.

Acetamiprid is a neonicotinoid insecticide with a well-established mode of action on nicotinic acetylcholine receptors (nAChR). Evidence indicates that the affinity of neonicotinoids for the insect nAChR is much higher than the mammalian nAChR (Casida, 2018). This identifies a known MIE of nicotinic acetylcholine receptor binding for neonicotinoid insecticides. However, an adverse outcome pathway has not yet been identified for neonicotinoid exposure. The results of the weight of evidence indicated that there are major uncertainties in the body of evidence for DNT properties of acetamiprid and further data are therefore needed to come to a more robust mechanistic understanding to enable appropriate hazard and risk assessment (Hernandez-Jerez et al., 2024).

EFSA AOP IATA's lessons learnt and future directions

From these case studies, there is consensus that the DNT IVB provides an important tool illustrating advancements in science that indicate that with increased mechanistic understanding and technological progress, other approaches become possible for assessing DNT. The DNT IVB provided information regarding the DNT potential of chemicals perturbing early cellular processes difficult to measure in vivo (Hernandez-Jerez et al., 2021).

For EFSA, the AOP-informed IATA framework is fit for purpose and is the current recommended approach for DNT hazard identification and characterisation as well as for integrating the results of the DNT IVB in EU pesticide Risk Assessment. The workflow can be used as a starting point for an international agreement in order to develop a tiered approach or Defined Approach for DNT hazard characterisation.

In 2023, a total of 476 compounds were tested in one or more of the assays of the DNT IVB and 81 compounds in all 17 assays of the DNT IVB (OECD, 2023). Since then, more data became available and there are a number of ongoing and planned efforts to fund and test additional chemicals using the current DNT-IVB by EFSA, US NIEHS and US EPA. There is an international agreement that all the data of the different projects will be made publicly available in US EPA ToxCast programs (https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadabledata). This includes raw data, processed data as well as the analysis algorithm code, this represents not only an example of good practice on data transparency but also allows a regulatory independent assessment of the data.

The public availability of DNT IVB data and publication of the Initial Recommendations on Evaluation of DNT-IVB by OECD made necessary, in line with Article 8/5 of the 1107/2009 EU pesticides regulation, the integration of the DNT IVB in the hazard and risk assessment with the available data for active substances. The integration of DNT IVB available data into the EU pesticides regulatory dossiers is expected (e.g., acetamiprid).

However, from the first EFSA case studies, several observations emerged (1) the expert judgement nature of IATAs; (2) when a non-endorsed AOP and non-standard IATA framework exists, the development of an evidence-based AOP-informed IATA, in line with a systematic approach, is resource-intensive and requires specific expertise; (3) further interpretative guidance is necessary for some of the assays; (4) implementation/standardisation of physiologically based kinetic (PBK) modelling for quantitative in vitro to in vivo extrapolation (QIVIVE) is a necessary next step.

The standardisation of several elements of the AOP informed IATA Workflow would help the uncertainty analysis and expert regulatory decision making. The availability of a standard framework to assess for DNT

will be highly beneficial for the reliability of the WoE in chemical risk assessment as well. This future work will need a constant collaborative effort at an international level.

Next step in the regulatory implementation roadmap is to define how to integrate, in a standardised approach, the DNT IVB into human health risk assessments in the various chemical regulatory frameworks. In the EU pesticide sector, the implementation of the DNT IVB represents a front running activity in testing the paradigm of using in vitro methods and Integrated Approaches to Testing and Assessments (IATAs) in the process of chemical risk assessment paving the way for the regulatory use of other Human-based New Approach Methodologies (NAMs). This forms the basis for how in vitro approaches may be integrated into regulatory frameworks (Viviani et al., 2025).

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Industry perspective on leveraging the mechanistic understanding from the DNT-IVB to optimise the development of safe pesticides

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Regulatory guideline toxicity studies rely on in vivo apical endpoints for hazard identification and subsequent risk assessment. Fit-for-purpose mechanistic data that evaluate the etiology of apical outcomes can provide insight on a hazard's human relevance. This was the light in which the DNT-IVB was used to provide mechanistic insight as a line of evidence to contextualize putative chemical-related findings from an existing in vivo DNT study. Briefly, the pesticidal active ingredient, acibenzolar-s-methyl, and its major circulating mammalian metabolite were independently tested across the entire DNT-IVB. The data analysis approach was pre-defined wherein analysis would be conducted using the US EPA ToxCast Pipeline algorithm, cytotoxicity assays integrated to ensure cell death did not confound outcomes, and in vitro-to-in vivo extrapolation (IVIVE) applied to any positive hit calls to identify relevant in vivo exposure levels. The final weight of evidence evaluation considerations include 1) reliability of in vitro bioactivity results and in vivo DNT findings of concern, 2) toxicological and biological relevance of in vitro bioactivity data with IVIVE integration, 3) consistency of evidence across in vitro assays assessing the same neurodevelopmental endpoint and with known biological relationships in the DNT adverse outcome pathway, and 4) consistency of evidence between in vitro bioactivity results and in vivo DNT findings of concern. This case study highlights the process for integrating mechanistic data from the DNT-IVB in a weight of evidence to inform uncertainties in an existing in vivo DNT study to refine toxicity endpoint selection for regulatory risk assessment.

{paper not submitted by the speaker}

Inclusion of the DNT-IVB in EU's Pesticides Risk Assessment – a European Member State Perspective

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The use of new approach methodologies (NAMs) is the way forward to move away from animal testing, however, it also challenges the current European regulatory system of the pesticide assessment and approval, where data requirements still refer to in vivo testing for most endpoints. NAMs have been used up till now to screen for specific endpoints and prioritise which substances should be further investigated and tested in higher tier studies. This is also one target use of the DNT-IVB.
Some NAM approaches are already used in regulatory assessment as full replacements to address the regulatory questions and data requirements (e.g. acute toxicity endpoints as skin and eye irritation). Others are applied to fill data gaps in integrated approaches or to provide mode of action (MoA) information. The use of the DNT-IVB is considered promising and could help (1) to address developmental neurotoxic endpoints (a triggered data requirement for pesticides related to specific mode of actions or pesticides raising concern for this endpoint) and (2) to serve as source/basis to refine a point of departure for risk assessment.
Important questions like
-How can the DNT-IVB be integrated into the hazard and risk assessment?
-How should it be weighted in the context of GHS classification?
-What is the impact of DNT-IVB results on the approval criteria for pesticides?
will be raised during the presentation and opened for discussion.
Furthermore, experiences and recommendations (e.g. trainings) from an EU Member State (MS) perspective will be reflected.

The use of new approach methodologies (NAMs) is the way forward to move away from animal testing. However, it also challenges the current regulatory system of the European pesticide assessment and approval, where data requirements still refer to *in vivo* testing for most endpoints. Up until now NAMs have been used to screen for specific endpoints and prioritise substances that should be further investigated and tested in higher tier studies. This is also one target use of the Developmental Neurotoxicity *in vitro* testing Battery (DNT-IVB).

Developmental neurotoxicity (DNT) assessment in regulatory context in EU and integration of DNT IVB (in vitro testing battery) in pesticide assessment

In Europe the Regulation (EC) 1107/2009 concerning the placing of plant protection products on the market refers to the data requirements for active substances of plant protection products in Regulation (EC)

283/2013 to address endpoints like reproductive toxicity and neurotoxicity (comment author - shortened text was taken from regulations only to depict main information).

In Section 5.6.2 of Part A of the Regulation (EC) 283/2013 the data requirements refer to developmental toxicity studies in the following way: *"The developmental toxicity studies reported, taken together with other relevant data and information on the active substance, shall be sufficient to permit the assessment of effects on embryonic and foetal development, following repeated exposure to the active substance… When indicated by observations in other studies or the mode of action of the test substance, supplementary studies or information may be required to provide information on the postnatal manifestation of effects such as* ***developmental neurotoxicity****. Such developmental toxicity studies shall always be carried out."*

In Section 5.7.1 of Part A of the Regulation (EC) 283/2013 the data requirements refer to Neurotoxicity studies in rodents in the following way: *"Neurotoxicity studies in rodents shall provide sufficient data to evaluate the potential neurotoxicity of the active substance (neurobehavioural and neuropathological effects) after single and repeated exposure. Such neurotoxicity studies in rodents shall be performed for active substances with structures that are similar or related to those capable of inducing neurotoxicity, and for active substances which induce specific indications of potential neurotoxicity, neurological signs or neuropathological lesions in toxicity studies at dose levels not associated with marked general toxicity. Performance of such studies shall also be considered for substances with a neurotoxic mode of pesticidal action."*

Communication from the Commission in the framework of the implementation of Part A of the Annex of the Commission Regulation (EU) No 283/2013 are setting out the data requirements for active substances in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market (2023/C 344/02). This commission communication presents an agreed list of studies that shall be used to address the endpoint in the data requirements. For developmental neurotoxicity assessment, currently OECD Developmental neurotoxicity Study (OECD TG 426; Chapter 3.8 in Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption) and North American Free Trade Agreement (NAFTA) Technical Working Group on Pesticides (TWG) Developmental Neurotoxicity Study Guidance Document are listed.

To address risk assessment, Regulation (EC) 1107/2009 gives the following information in Annex II about procedure and criteria for the approval of active substances, safeners and synergists: 3.6. Impact of human health*" 3.6.1. Where relevant, an ADI, AOEL and ARfD shall be established. When establishing such values an appropriate safety margin of at least 100 shall be ensured taking into account the type and severity of effects and the vulnerability of specific groups of the population. When the critical effect is judged of particular significance, such as* ***developmental neurotoxic*** *or immunotoxic effects, an increased margin of safety shall be considered, and applied if necessary."*

Since Regulation 283/2013 is currently under evaluation and redrafting, future developments cannot be considered since the above explained framework presents the situation at the time point of this workshop (October 2024).

In the future, member states of the European Union will be more often confronted with data generated from assays of the Developmental Neurotoxicity *in vitro* testing battery (DNT IVB). Several guidance documents have been published in the last years to help facilitate the use, evaluation and assessment of uncertainties of data derived from the DNT IVB (Crofton and Mundy, 2021; OECD, 2023). In addition, several case studies have been published and discussed at the European level, which can be used as a template for integration of the DNT IVB in a hazard characterisation context during assessment of pesticides (EFSA PPR panel, 2021; OECD, 2022; EFSA, 2024).

Key applications of the DNT IVB in the assessment of pesticides from a Member State 's perspective could be as follows:

- Results from DNT IVB can be used to fill data gaps when traditional in vivo data are absent or insufficient.
- The DNT IVB is considered beneficial in a weight of evidence assessment with unclear *in vivo* results.
- The DNT IVB can be used for possible refinement of uncertainty factor in the derivation of toxicological reference values.
- The DNT-IVB can indicate the relative DNT potential in comparison to the point of departure used for risk assessment.

DNT IVB in classification and labelling of pesticides

The Regulation (EC) 1272/2008 on Classification, Labelling and Packaging of substances and mixtures provides the European Commission's regulatory context on how the DNT IVB could be implemented in classification and labelling of pesticides.

The RAC Guidance Note "Addressing developmental neurotoxicity and neurotoxicity under the current CLP hazard classes" (RAC/62/2022/05) was published in September 2022 and gives following clarification: *"RAC agreed that neurotoxic effects investigated or detected at any point in the life span of the organism that had been exposed during the developmental period, covering both prenatal and postnatal developmental period until sexual maturation (determined in rats by preputial separation in males and vaginal opening in females), even if the exposure had continued also after sexual maturation, should be assessed and concluded under* ***developmental toxicity*** *whereas neurotoxic effects caused by exposure of mature animals/humans (exposure only after sexual maturation) should be assessed and concluded under STOT SE or RE."*

When again Regulation (EC) 1107/2009 (Annex II, 3.6.4) is taken into consideration, it is clearly stated that the endpoints "toxic for reproduction category 1A and 1B" are cut-off criteria for the approval/renewal of active substances, safeners and synergists. Also, a classification as toxic for reproduction category 2 would have the downstream consequence, that all groundwater metabolites of the active substance would be considered as relevant by default and should not occur ≥0.1µg/L (Sanco/221/2000 – rev.11).

Hazard categories for reproductive toxicants according to Regulation (EC) 1272/2008, according to table 3.7.1(a) of Annex I are defined in the following way (comment author - shortened text was taken from regulations only to depict main information): "… *Substances are classified for Category 1 (known or presumed human reproductive toxicant) for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility, or on development* ***in humans*** *or when there is evidence from* ***animal studies****, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans*….*Substances are classified in Category 2 (suspected human reproductive toxicant) for reproductive toxicity when there is some evidence from* ***humans or experimental animals****, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development,…"*

Classification of an active substance as toxic for reproduction clearly indicates what data is needed and therefore, in the absence of *in vivo* data, the regulatory impact will be different. Certainly, developmental neurotoxicity is quite a complex endpoint and therefore, the DNT IVB as a stand-alone assessment regarding developmental neurotoxicity seems to be insufficient to classify a pesticide as toxic for reproduction. However, the DNT IVB seems to be a suitable tool to serve as supportive information to inform on adverse effects on development (e.g. to show the key event (KE) for an observed adverse outcome was met).

Way forward to a harmonized approach on EU level

The presenting Member State came to the conclusion that the use of the DNT IVB in pesticide assessment will depend on several factors:

- The availability of DNT IVB assays from publicly available data bases is considered as crucial (e.g., ToxCastDB https://comptox.epa.gov/dashboard/ – a model would facilitate easier extraction of data).
- Further guidance on the interpretation of such assays and training for Member States to integrate results from the DNT IVB in a weight of evidence assessment of the pesticide evaluation will be needed.
- (Q)IVIVE, PBK modelling will be used as a tool to put data from the DNT IVB into quantifiable context and therefore further guidance and training for Member States will be needed.

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The regulatory use of the DNT-IVB data within IATAs for Next Generation Risk Assessment needs standardization of a comprehensive and practical workflow for uncertainty characterisation

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In vitro methodology for developmental neurotoxicity (DNT) may significantly reduce the practical limitations of in vivo approaches in terms of ethical conflicts, costs and required testing-time. These practical advantages of in vitro methodology can also facilitate their standardisation and validation process, which in turn may reduce the uncertainty in the experimental data variability. Though the uncertainty in the human population level relevance of the experimental data from in vitro versus in vivo methodology may be conceptually similar, the acceptable level of uncertainty for any new approach is still a matter of science-policy level discussion. This discussion relates to the uncertainty of biological and mechanistic coverage of the current in vivo and in vitro approaches as well as to the ethical-societal and economic needs.

However, the Globally Harmonized System (GHS) classes represent essentially positive alerts for toxicity. There are two reasons why a chemical may not be classified: 1) Ample data are available supporting that the chemical is not hazardous or 2) Insufficient data are available for classification. Thus, the principles of GHS classification would not be violated by the use of in vitro methodology, with a limited biological and mechanistic coverage of DNT. It should be possible to immediately phase in the in vitro methodology into the current classification system as long as a positive readout is sufficiently reliable.

The uncertainty related to a positive in vitro DNT result relates essentially to the two questions: 1) May some of the observed effects occur in the specific in vitro experimental cellular microenvironment only? 2) May some of the observed effects be compensated at tissue and/or organism level? However, this uncertainty should be contextualised with the human real world variability: (Epi)genetic background, diet, lifestyle, stress, infections and chemical co-exposure may favour the progression of early molecular/cellular effects to an organism level effect. This human variability is not captured within rodent in vivo experiments, though we may like to be more inclusive for various susceptible humans.

Now, the human in vitro molecular/cellular level effects may be used as risk factors for organism level effects, just like some genetic predispositions may be a risk factor, which translates to toxicity and disease in probabilistic

terms. What is an adequate level of concern for regulatory action does not need a purely scientific discussion, but a science-policy discussion. Anyway, a future classification should account for such human variability. Moreover, rather than aiming for a classification based on an in vitro battery that mechanistically fully covers the current in vivo approaches, a completely new class, based on in vitro measurable mechanistic information only, may be a scientifically more feasible goal. Considering that one set of early in vitro key events may lead to many diverse organism level adverse outcomes, also beyond DNT, a new classification system may try to overcome the classification based on observed types of effects or mechanisms and classify based on potency only. Thereby it might be easier and faster to develop a robust classification system, which would not provide different classification results with every new discovery of toxicological mechanisms and related methodology. Such a new class could be phased in and evolved over time until animal data requirements will be phased out completely.
Within this presentation, I will utilize available knowledge about current uncertainties and the conceptual considerations outlined here to argue for a new probabilistic classification approach using the DNT in vitro methodology. I will demonstrate the latter using a) the 81 chemicals with a complete DNT in vitro battery data set, b) potency thresholds derived from the current GHS classification rules, c) high throughput PBK modelling and d) available human variability data. This approach is also developed and discussed as one possible solution to the (EPAA).

In line with the European Commission work on the Roadmap to an Animal Free Regulatory System several European Horizon Projects are working on concepts and case studies for a New Approach Methodologies (NAMs) based Next Generation Risk Assessment (NGRA). These include projects within the ASPIS cluster, the Green Deal Health Cluster, and CHIASMA - INSIGHT - PINK cluster. The latter aims to integrate NGRA with Life Cycle Assessment (LCA) and the Safe and Sustainable by Design (SSbD) framework and this also requires profound adaptations of current LCA and SSbD principles. Several of these projects include DNT in vitro battery data, while other projects entirely focus on case studies for the regulatory use of the DNT in vitro battery, like those from the European Food Safety Authority (EFSA) and US Environmental Protection Agency (EPA), discussed at the specifically related OECD Workshop which this report builds upon. Published NGRA concepts show similarities in that they refer to the basic concept of hazard, exposure, risk and uncertainty characterisation. Yet, hazard assessment is based on NAM data and physiologically based kinetic (PBK) modelling, integrating experimental and computational methods within tiered workflows to optimize efficiency in data generation and assessment. Current NGRA concepts include more or less defined decision criteria (Virmani et al. 2025, manuscript in preparation).

However, a common, not yet accomplished, challenge within the work towards NGRA is the development of a standardised comprehensive and practical workflow for the characterisation of the uncertainty of NGRA results. Ideally, the uncertainty within NGRA should become transparent such that a possible reduction of uncertainty with any additional testing can be anticipated and help decide on when an acceptable uncertainty has been reached. For this purpose, the uncertainty from the use of animal test data also needs to be

accounted for. In the following text, a potential approach is sketched out theoretically which may be tested with case studies and revised within the one or other of the aforementioned ongoing scientific projects.

We may build on the WHO 2018 Guidance on Evaluating and Expressing Hazard Uncertainty, which builds on empirical toxicological animal and human data. It allows replacing the standard deterministic extrapolation factors (often 10 x 10 = 100) for animal to human sensitivity differences and human variability, by data based, generic, compound independent probabilistic extrapolation factors, i.e. extrapolation factor distributions. The WHO framework allows for assessing the reference dose with specific parameters for the incidence of the effect in the population and the confidence intervals considered. While there are some advantages to the approach that incorporate a high level of statistical rigor and transparency, some stakeholders prefer that only the most conservative settings are applied to ensure maximal human health protection. The Guidance also provides a statistical framework for the use of such probabilistic factors and an Excel tool (APROBA) to derive human reference values, including its uncertainty, i.e. confidence interval. Importantly the approach allows quantifying the relative uncertainty contribution stemming from a) the Benchmark Dose (BMD) modelling of the Point of Departure (PoD) derived from the experiment, b) the extrapolation from the experimental data to the assessment target, i.e. humans and c) the uncertainty of the assessment target.

For NGRA this approach could be adapted via two steps, as depicted in figure 1 and figure 2:

Figure 1: Replacing the probabilistic animal to human extrapolation factors by a reversed dosimetry (PBK) model for the extrapolation from an in vitro Benchmark Concentration (BMC) to a human external dose, including a confidence interval for the resulting BMD. Human variability may be modelled either by using the available WHO 2018 data based models or by adapting the PBK modelling such that it covers human physiological diversity. In addition, a new hazard class is needed to exploit the full scientific and regulatory potential of NAMs. Such a new class would not necessarily need to predict the current GHS class for reproductive (R) or specific target organ toxicity (STOT), but represent a new class with a NAM science based categorisation for low, medium and high concern. Toxicodynamic BMC values from scientifically validated NAMs and outputs form PBK models could provide the basis for defining the category boundaries of such a new class. The class could be phased into regulation and with increasingly comprehensive and robust NAM data sets, over time, this might reduce the regulatory dependency on animal data based GHS classes. Respective work is ongoing inter alia within the DESIGNATHON challenge of the European Partnership for Alternatives to Animal Testing (EPAA).

safety assessment	data	assessment variable	extrapolation from model to assessment target	variability of assessment target	reference value	classification
Traditional	rodent data (rarely dog or else)	usually NOAEL rarely BMD [+ BMDL/U]	interspecies usually default factor 10 rarely probabilistic [+LCL/UCL], e.g. WHO 2017	human variability usually default factor 10 rarely probabilistic [+LCL/UCL], e.g. WHO 2017	usually human safe dose rarely human dose (HD) protective for x% of population [with y% prob.]	effect type: Reproductive toxicity effect potency: STOT RE & potency for R-SCL (mixture classification)
Next Generation	**human in vitro / in silico data**	**default** BMC [+ BMDL/U]	**default probabilistic reversed dosimetry (PBK) extrapolation** [incl. uncertainty from input data & model parameter]	**default probabilistic** human variability, e.g. from PBK and/or historical WHO data	**default** human dose HD protective for x% of population [with y% prob.]	effect potency: **probabilistic, revised*** STOT SE & RE

BMC: benchmark concentration , BMD: benchmark dose, BMDL: benchmark dose lower confidence limit, BMDU: benchmark dose upper confidence limit, HD: human dose, LCL: lower confidence limit, NOAEL: no observed adverse effect level , PBK: physiologically based kinetic , R: reproductive toxicity class, RE: repeated exposure , SCL: specific concentration limits , SE: single exposure , STOT: specific target organ toxicity , UCL: upper confidence limit , WHO: world health organization

** new classification system in discussion, e.g. within EPAA Designathon, i.a. revised STOT SE & RE & CR, ED potency boundaries extrapolated by PBK to in vitro concentrations for use with NAM data: https://single-market-economy.ec.europa.eu/calls-expression-interest/epaa-designathon-human-systemic-toxicity_en*

Fig. 1 Adaptation of the traditional safety assessment approach to a NAM based Next Generation Safety Assessment (adaptations highlighted via bold text).

Figure 2: The WHO 2018 uncertainty framework could be adapted from a probabilistic animal data based approach to a probabilistic NAM data based approach (as outlined in more detail in Figure 1). Thereby, the relative contribution (%) of the uncertainty could be transparently represented for a) BMC modelling (upper and lower confidence limit), b) reversed dosimetry (PBK) modelling and c) human variability modelling. This quantitative uncertainty could be amended by a characterisation of qualitative uncertainties. Different types of quantifiable and qualitative uncertainties from the use of DNT NAM data and animal data were published (Paparella et al., 2020), further discussed, revised and summarised within a background document for the OECD Working Party for Hazard Assessment (WPHA) for use within IATA case studies. This document may serve to identify and discuss major qualitative uncertainties and how they may shift the relative contributions (percent) of the quantitative uncertainty estimates provided via the adapted WHO 2018 framework. Ideally, this would be accomplished via an Expert Knowledge Elicitation (EKE) process (EFSA, 2024), which integrates different experts' opinions on the scientifically appropriate shifts. It may be carried out via visualisation of uncertainty distributions shifts by the individual experts, mathematical integration of these individual distributions, moderated exchange of arguments within the group for adequate shifts and refinements of the experts' inputs, such that finally the variability of experts' opinions is democratically visualised in the group's result.

Next, another EKE approach could be used to identify and discuss which of the NAM data-based uncertainties could be reduced by which type of additional testing and what would be the remaining uncertainty after additional testing by applying value of information (VOI) analysis. This discussion needs due considerations of the uncertainties from the use of animal test data, which correspond to the uncertainties from the use of the NAM data.

After each of the assessment steps in the workflow, the uncertainty contributions from bmc modelling, reversed dosimetry extrapolation, human variability modelling finally need to be integrated for its overall impact on the health-based guidance value and classification.

Such an overview may build the basis for a textual conclusion on an acceptable overall uncertainty.

Workflow for uncertainty characterization	A uncertainty of experimental data	B uncertainty for extrapolation from model to assessment target	C uncertainty of assessment target	uncertainty of resulting health based guidance value	uncertainty of classification
assessment variable	bmc [nM]	reversed dosimetry (PBK) estimated Health Based Guidance Value [mg/kg bw day]	human variability [GSD]	health based guidance value	effect potency: STOT SE & RE, R potency for SCLs
reference to modelling approach	e.g. frequentist models or Bayesian Network models	e.g. httk or any refined model	e.g. PBK or WHO 2018 human variability approach	integration of columns A, B, C	integration of columns A, B, C (consider potency borders instead of exposure)
quantifiable uncertainty	model uncertainty: bmcl/bmcu	model parameter uncertainty: P05, P50, P95 values	e.g. WHO 2018: lognormal distributed log(GSD) P50 = 0.324, P05 = 0.151, P95 = 0.697		
% contribution of quantifiable uncertainty (total 100%)	%	%	%	-	-
Expert Knowledge Elicitation (EKE): which is the major qualifiable uncertainty	select arguments from section on **reliability** within WPHA background table on uncertainty	select arguments from section on **relevance** within WHPA background table on uncertainty	e.g. WHO 2017: limitations in historical background data:	integration of columns A, B, C	integration of columns A, B, C
EKE: % contribution of overall uncertainty	%	%	%	-	-
EKE: which are the major corresponding uncertainties from standard animal DNT testing?	select arguments from section on **reliability** within WPHA background table on uncertainty	select arguments from section on **reliability** within WPHA background table on uncertainty	select arguments from section on **reliability** within background table on uncertainty	integration of columns A, B, C	integration of columns A, B, C
EKE: If any, which elements of the major uncertainty could be reduced by which type of testing (in silico, in vitro or in vivo)?					
EKE: what is the expected remaining uncertainty after additional testing [%].	%	%	%	integration of columns A, B, C	integration of columns A, B, C
Conclusion on acceptable overall uncertainty within current IATA		text		text	text

IATA: Integrated approaches to testing and assessment, EKE: expert knowledge elicitation, PBK: Physiologically based kinetics, STOT: specific target organ toxicity, SE: single exposure, R: reproductive toxicity class, RE: repeated exposure, SCL: specific concentration limits, - = not relevant: bmc: benchmark concentration, WHO: world health organization

Fig. 2: Adaptation of the WHO 2028 framework for an expert based modification of relative quantitative uncertainty contributions (%) from the three modelling steps (bmc, reversed dosimetry, human variability), possibly supporting standardised, traceable conclusions for acceptable uncertainty of NGRA results.

The suggestion outlined here represents a first draft which requires further specifications, inter alia: Which reversed dosimetry (PBK) models shall be used, how they need to be validated and how they may include human variability. Additionally, the mathematics for probabilistically integrating the NGRA relevant model outputs to derive health-based guidance values and hazard categories needs review and specifications.

Importantly, the complete workflow, including the EKE, needs to be tested and revised based on real case studies. While the EKE may be labour intensive and not perfectly robust, a series of case studies may provide a basis for an improved standardisation of a comprehensive and practical workflow for uncertainty characterisation.

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DNT-IVB data application for screening chemicals: industry's perspective

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A battery of in vitro assays is being evaluated to screen substances for developmental neurotoxicity (DNT) potential. This battery is based on new approach methods (NAMs) for key neurodevelopmental events (e.g., neuronal differentiation and migration, synaptogenesis, myelination). From an industry perspective, the DNT in vitro battery (IVB) offers promise as a screening approach; however, there are limitations with respect to domain of applicability, assay/battery reliability, coverage of adequate biological space, and the need for a decision framework. For a decision framework, industry supports contextualising DNT IVB data using an Integrated Approach to Testing and Assessment (IATA) with integration of additional information such as read-across/QSAR data, pharmacokinetics (in vitro-to-in vivo extrapolation), data from tissue-level models (e.g., brain organoids) and possibly alternative in vivo assessments (e.g., zebrafish, planaria) before deciding whether additional mammalian toxicity studies are needed. This presentation focused on an IATA approach to examine the DNT potential of a case study chemical, metformin. The goal of this presentation was not to indict the case study chemical as a potential developmental neurotoxicant (metformin is a negative reference chemical for the DNT IVB) but to work through the steps of the IATA approach to illustrate its utility and identify some points to consider when integrating data streams into a DNT assessment as well as critical questions that require resolution in a decision framework and/or DNT IVB guidance.

The case study chemical, metformin, has been identified as a negative reference chemical in the DNT IVB. Metformin is a drug used to treat type 2 diabetes mellitus, polycystic ovary syndrome and gestational diabetes (approved by the European Medicines Agency and US Food and Drug Administration, although the last two uses are considered "off label" by US FDA). A treatment for gestational diabetes is important as approximately 10% of pregnant women are affected and this condition poses additional health risks for both the mother and child. Metformin improves the body's response to insulin, improving pregnancy outcomes and there is agreement that the therapeutic benefits outweigh the risks. Doses of metformin begin at 500 mg/day (once per day) and increase to doses up to 2500 mg/day (twice daily dosing) with doses titrated for glycemic control.

To integrate data to assess DNT potential, an initial tiered testing framework has been proposed (personal communication; K. Crofton). The proposed tiered testing framework integrates exposure information from high-throughput (HTP) exposure modelling at the lowest tier, progressing to HTP in vitro-to-in vivo extrapolation (IVIVE) then to refined assessments using specific physiologically based kinetic (PBK) models, species extrapolation modelling, and finally, in vivo pharmacokinetic data with PBK models for species extrapolation at the highest tier. For hazard characterisation, tiers begin with computational and other HTP bioactivity assessments (Tier 0), progress to DNT IVB data (Tier 1), confirmatory/orthogonal in vitro testing (Tier 2), complex models (Tier 3), and specialized animal testing (Tier 4). Across the tiers,

hazard data are integrated with exposure/PBK IVIVE data to determine if there is an acceptable margin of exposure (MoE). If an acceptable MoE is achieved, the assessment can be concluded; however, if the MoE is insufficient, higher tiered assessments are required.

To apply this framework, exposure to pregnant women must first be established. Based on its therapeutic uses, metformin was a candidate for DNT screening as it can be used during pregnancy. For Tier 0, a computational assessment of metformin was undertaken using both publicly available and in-house QSAR models. There was a positive prediction for potential interactions with mitochondria at Complex I, which is consistent with a proposed mode-of-action for metformin. We consider mitochondrial inhibition to be a relevant mode-of-action for DNT assessments due to the importance of mitochondria in development and in maintenance of neuronal membrane potential.

For Tier 1, metformin was evaluated in all 17 of the DNT IVB assays with data publicly available in the ToxCast and ICE databases. While negative in most assays, metformin was positive in the cortical synaptogenesis assay for two endpoints – decreased branch point count (BPCount) and decreased neurite length. The lowest bioactivity point of departure (PoD) without flags for data uncertainty was 12.03 µM, the AC50 for BPCount. In the Tier 2 confirmatory assessment, additional assay data were not collected. Instead, bioactivity values for other HTP assays in ToxCast were examined to indicate the likelihood that DNT positive results were specific. Metformin had six positive hits in the ToxCast database (run in 413 assays) with the next closest PoD equal to 53.39 µM (concentration at activity cut-off, ACC) for ATG_mPXR_XSP1. With these data, the specificity of effects on BPCount is somewhat unclear, while bioactivity in other HTP assays occurred at higher concentrations and the effect on BPCount was seen below cytotoxicity concentrations, there were no bioactivity patterns, including a lack of effects on related endpoints (e.g., synapse count, signalling in microelectrode array).

Next, a PBK model was developed for metformin use during pregnancy using GastroPlus™ (Simulations Plus, Lancaster, CA). The PBK model was anchored using experimental data from the scientific literature. The model was used to predict plasma time course data for metformin after 28-day repeated oral exposure at 2500 mg/day starting at gestation week 10 in pregnant women weighing 70 kg. Results were verified by additional literature reports. Using this model, foetal blood levels (~46 µM) exceeded bioactive concentrations in the BPCount assay (12.03 µM), indicating an inadequate MoE for DNT IVB endpoints. It is important to note that foetal brain concentrations of metformin were not determined in this exercise.

In the Tier 3 hazard assessment, information on bioactivity of metformin in more complex in vitro DNT assays was sought. There were numerous published reports of metformin's use in brain organoid, neurosphere or brain slice models; however, in many of these publications, metformin was used at high concentrations and/or to treat underlying neurological alterations (e.g., hypoxic injury, periventricular heterotopia, diabetic neurotoxicity), making it difficult to discern potential effects on normal neurodevelopmental processes.

In the Tier 4 hazard assessment, similar scientific reports were identified in which metformin was evaluated in a variety of in vivo rat studies in which neurodevelopmental disorders were induced (e.g., anaesthesia-induced DNT, foetal alcohol spectrum disorder, autism spectrum disorder). In each case, metformin was neuroprotective and/or attenuated the neuronal effects induced in rats by other treatments. In addition, three human epidemiology studies were identified that examined potential effects of metformin on neurodevelopment. There were no neurodevelopmental effects identified in children born to mothers treated with metformin with or without insulin during pregnancy. One study showed a trend for weaker language performance in children from metformin- or insulin-treated mothers, but these data were preliminary and were not reported in other studies (Tertti et al., 2015). It is important to note that these studies used small sample sizes (<150 subjects) and did not evaluate children older than age 2.

In conducting the metformin case study, it was recognized that the tiered testing framework is a useful tool, but guidance is needed to use the DNT IVB assay results and this framework successfully. First, guidance on the interpretation of the DNT IVB would be helpful (i.e., is any positive result in 17 assays a meaningful

finding? What about assays like microelectrode array and neurite outgrowth/synaptogenesis assays that have multiple endpoints – is a single positive hit meaningful? Should potency be used to prioritise positive hits? With 17 assays, how will the false discovery rate be controlled across the battery?). In addition, it would be beneficial to determine the database (i.e., ToxCast, ICE) and PoD value (i.e., AC50, BMD, ACC) to use and define what constitutes a sufficient MoE. Lastly, with the metformin case study, a weight-of-evidence assessment does not support DNT despite positive results in the DNT IVB cortical synaptogenesis assay at concentrations that can be achieved in foetal blood at therapeutic doses to the mother. Based on this result, the specificity of the DNT IVB should be evaluated with additional case studies, especially 'agreed upon' negative reference chemicals. Lastly, the performance and feasibility of the tiered decision framework should be evaluated with multiple DNT case study chemicals, both positive and negative, to evaluate data integration, examine outcomes and improve efficiency.

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3 Exposure assessment and Quantitative In Vitro to In Vivo Extrapolation (QIVIVE)

Towards quantitative in vitro to in vivo extrapolation of DNT IVB data: principles and considerations for regulatory application

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To allow a quantitative interpretation of the developmental neurotoxicity in vitro battery (DNT IVB) data and its integration in the chemical hazard and risk assessment process, the application of physiologically based kinetic (PBK) modelling is considered essential, allowing a so-called quantitative in vitro to in vivo extrapolation (QIVIVE). This QIVIVE entails the translation of in vitro effect concentrations into equivalent external (e.g., oral) exposure levels. Examples of QIVIVE have been described in the scientific literature, but no harmonized approach nor guidance is available, and QIVIVE is generally not yet applied in regulatory hazard and risk assessment. In this presentation, the principles of PBK modelling-facilitated QIVIVE are presented, and the different steps of the approach are discussed, including the selection of the PBK model, its parameterization, and the selection of the relevant in vitro dose metric for translation (related to the in vitro distribution kinetics of the test item). Considerations are provided regarding kinetic data requirements, assessment of uncertainties and on the reporting of the QIVIVE, tailored to pesticide active substances in Europe.

Quantitative in vitro to in vivo extrapolation (QIVIVE) has been described as '*the process of estimating the environmental exposures to a chemical that could produce target tissue exposures in humans equivalent to those associated with effects in an in vitro toxicity test (e.g., an EC50, a Benchmark concentration, or an interaction threshold identified by a biologically based dose-response model for the toxicity pathway of concern)*' (Yoon et al., 2012). An 'environmental exposure' can be considered as any external exposure and is often called an 'external equivalent dose'. Furthermore, besides for humans, QIVIVE can also be performed for animals. Typically, physiologically based kinetic (PBK) modelling is the tool used to make a

translation from an external exposure to a target tissue exposure (Yoon et al., 2012; Blaauboer, 2001; 2003; 2010; Louisse et al., 2017; Chang et al., 2022).

QIVIVE approaches described in the scientific literature that link an external exposure to an internal exposure with a PBK model use a forward or reverse dosimetry approach. With a forward dosimetry QIVIVE approach, a PBK model is used to estimate at (a) given external exposure scenario(s) the related estimated internal exposure(s) (e.g., a plasma or a target tissue concentration). The internal exposure(s) is/are then compared to effect concentrations in the vitro toxicity assay of interest for a quantitative contextualisation of the in vitro toxicity data. External exposure selected as starting point can, for example, be an available health-based guidance value (HBGV) or a given exposure estimate. With a reverse dosimetry QIVIVE approach, a PBK model is used to estimate at (a) given internal exposure(s) (e.g., a plasma or target tissue concentration), the related external exposure(s). The internal exposure used as starting point for the QIVIVE is based on effect concentrations of (an) in vitro toxicity assay(s) of interest. For the quantitative contextualisation of the in vitro toxicity data, the QIVIVE-based external exposure(s) is/are then compared to an external exposure of interest, such as an HBGV or a given exposure estimate. Also, in various proof-of-principle studies, obtained external equivalent doses have been compared to NOAELs/LOAELs/BMDLs obtained from animal studies.

The principles of forward and reverse dosimetry-based QIVIVE examples described in the literature are highly similar, mainly differing in the starting point of the assessment (i.e., external exposure for forward dosimetry and internal exposure for reverse dosimetry). In reverse dosimetry QIVIVE approaches described in the scientific literature, the PBK model itself is typically applied in a forward dosimetry manner, i.e., a range of internal exposures is predicted related to a range of external exposures, and the external exposure is then derived related to the internal exposure that is used as starting point for the QIVIVE. In both forward and reverse dosimetry QIVIVE approaches, the PBK model allows to make a link between a concentration in vitro and a related external exposure. An important step of the QIVIVE is to make a link between the concentrations applied in the in vitro assays (and the related effect and/or no effect concentrations), i.e., the in vitro exposure metrics, and the internal dose metrics used in the PBK modelling. In the first QIVIVE example described in the scientific literature, in which neurotoxicity dose levels were predicted based on effect concentrations obtained in an in vitro test system based on SHSY5Y cells, the nominal concentration in vitro was linked to the total brain concentration described by the PBK model (Dejongh et al., 1999). In a second QIVIVE publication in the scientific literature, in which developmental toxicity dose levels were predicted based on effect concentrations obtained in an in vitro test system based on ES-D3 cells, the unbound (free) concentration in vitro was linked to the unbound concentration of the chemical in the plasma (Verwei et al., 2006). In the high throughput QIVIVE approaches published by the US-EPA (Rotroff et al., 2010; Wetmore et al., 2012) nominal in vitro concentrations are linked to total (bound and unbound) concentrations in plasma. When cellular (or cell-associated) concentrations have been determined, QIVIVE can also be based on the linking of the cell-associated concentration to the tissue concentration of interest (Mielke et al., 2017; Fragki et al., 2023).

Currently, there is no generally agreed harmonised approach on how an in vitro (effect) concentration should be linked to an internal dose metric, and the most adequate approach may differ from one case to the other, e.g., related to chemical characteristics and/or the nature and location of the molecular target (related to the molecular initiating event (MIE)) of the chemical (Groothuis et al., 2015; Mielke et al., 2019; Rietjens et al., 2019).

It is generally assumed that the unbound concentration at the target (an extracellular target (e.g., certain membrane receptors), a target in the cytoplasm or the nucleus (e.g., certain cytosolic/nuclear receptors), or a target in an organelle (e.g., certain mitochondrial respiratory chain complexes)) is expected to relate to the toxicological effect of the substance (Pelkonen et al., 2008). For highly-permeable chemicals interacting with plasma membrane receptor(s), the unbound concentration at the site of action is assumed equal to the blood or plasma unbound chemical concentrations (Guo et al., 2018; Chu et al., 2013). This assumption may not be valid when chemical targets are intra- or subcellular, especially for chemicals with

transporter-mediated disposition, as the activity of transporters at the tissue-blood barrier can result in "asymmetry" between the tissue and blood unbound chemical concentrations (Guo et al., 2018; Chu et al., 2013). When the location of the molecular target is not known, the selection of the most relevant exposure metric is not straightforward. If the target of the chemical is an extracellular receptor, the unbound concentration in the medium may be considered the most relevant in vitro exposure metric. In the absence of transporter-mediated disposition, this may also hold true for chemicals that have a cytosolic or nuclear receptor in the cell as their target, assuming that the unbound concentration outside the cell is (at equilibrium) equal to the unbound concentration inside the cell (Pelkonen et al., 2008). In those cases, the unbound chemical concentration in the interstitial fluid surrounding the cells can be considered as the relevant exposure metric in vivo. Generally, most (low-tier) PBK models do not describe concentrations in the interstitial fluid. Using the unbound blood or plasma concentration for the QIVIVE would assume that the unbound concentration in blood or plasma is (at equilibrium) equal to the unbound concentration in the interstitial fluid. This may be a reasonable assumption, but may lead to an overestimation of unbound interstitial concentrations in the brain, for which the blood-brain-barrier may limit the transfer of chemicals to the brain tissue (Pardridge, 2005). QIVIVE based on the linking of the nominal concentration in vitro to the total blood or plasma concentration in vivo can be considered to provide a conservative estimate of the external equivalent exposure doses, as the protein concentration in in vitro culture media is generally lower than the protein concentration in blood or plasma, with a typically higher unbound concentration in vitro compared to the unbound concentration in blood or plasma (Punt et al., 2021). However, one should be cautious using this approach for chemicals that have chemical characteristics that make their in vitro testing cumbersome, resulting in large uncertainty in the in vitro exposure, i.e., no to limited exposure of the test system to the test item. When assuming the intracellular unbound concentration (at equilibrium) to equal to the unbound concentration outside the cell (in the cell culture medium in vitro and in the interstitial fluid in vivo), one must consider that in certain cases the equilibrium may get disturbed, e.g., in case of cytotoxicity. For those cases, it has been suggested that instead of the unbound medium concentration, the total cell concentration may provide a more adequate in vitro exposure metric for QIVIVE (Mielke et al., 2019). QIVIVE based on total cell concentration was shown to provide better estimations of ibuprofen liver toxicity than when based on the unbound concentration in the medium (Mielke et al., 2017; 2019). So far, limited examples are available that performed QIVIVE based on total cell concentration (Mielke et al., 2017; Fragki et al., 2023).

Although various QIVIVE examples have been described in the scientific literature, there is, so far, limited application in regulatory hazard and risk assessment. In the EU pesticide hazard and risk assessment, two QIVIVE approaches have been evaluated; 1) in the peer review of the active substance deltamethrin, a QIVIVE approach submitted by the notifier was evaluated and related uncertainties identified[1]; 2) for acetamiprid, a working group of the Scientific Panel on Plant Protection Products and their Residues (PPR Panel) of the European Food Safety Authority (EFSA) assessed whether in vitro DNT data could be extrapolated to external equivalent doses using PBK modelling-facilitated QIVIVE, but concluded that the available kinetic data were considered too limited to parameterise a PBK model for the QIVIVE (EFSA, 2024). The application of QIVIVE for the quantitative interpretation of in vitro data on mitochondrial effects in the brain was performed in an EFSA-funded project on the applications of New Approach Methodologies (NAMs) for the risk assessment of tebufenpyrad, providing important insights into application of QIVIVE approaches for regulatory hazard and risk assessment and related uncertainties (Henri et al., 2022).

Currently, a working group of the PPR Panel is working on a Scientific Opinion providing recommendations on the application of physiologically based kinetic (PBK) modelling for the QIVIVE of developmental neurotoxicity in vitro battery (DNT IVB) data for pesticide active substances in the EU[2]. To allow its use in the regulatory hazard and risk assessment, transparent reporting related to the different steps of the

[1] https://www.efsa.europa.eu/sites/default/files/2023-12/minutes-ppr-mammalian-toxicity.pdf

[2] https://www.efsa.europa.eu/sites/default/files/2024-12/4th%20wg-ppr-qivive-dnt-ivb-minutes.pdf

QIVIVE is considered essential, i.e., related to 1) the selection of the in vitro exposure metric for QIVIVE, 2) the selection/development of a PBK model to be used for QIVIVE, and 3) the link between the in vitro exposure metric to an internal dose metric in the PBK model. For each step, different choices can be made. As there are no generally agreed harmonised approaches for these steps, it is of utmost importance to transparently report on the approach used and to clearly substantiate the choices made. To provide more insight into the impact of making certain choices in the steps of the QIVIVE, evaluating the outcomes of different scenarios may provide important information to support decision making.

1) It is noted that there is at present no general agreed approach on the selection of the in vitro point of departure for the QIVIVE related to the effect size (e.g., certain benchmark response, which may differ from one assay to the other), so the selection of the in vitro point of departure is to be substantiated as well. Regarding the in vitro exposure metric, it is acknowledged that in current practice, often only information is available about nominal concentrations applied in the test system. Dependent on the chemical characteristics and the toxicological target, a nominal concentration may lead to relevant or non-relevant in vitro exposure metrics for QIVIVE (Groothuis et al., 2015). For each QIVIVE, considerations of the chemical characteristics and the impact on the relevance of the nominal concentration for QIVIVE need to be discussed. Knowledge on the in vitro distribution kinetics of the test item (as discussed by Nynke Kramer in the OECD meeting of October 2024) and related insight into the unbound concentration available in the test system and/or the cell-associated concentration allows for a more robust QIVIVE.

2) Regarding the selection of a PBK model for the QIVIVE, a tiered approach is recommended to be used (as discussed by Cecilia Tan in the OECD meeting of October 2024), considering that a low tier PBK model would provide a conservative estimate of the internal concentration used for QIVIVE and that to using a higher tier PBK modelling approach adequate kinetic data should be available for model parameterisation. Regarding model development and evaluation, reporting templates (OECD, 2021) are to be used. Besides that, specific attention should be given to the quality of the kinetic data used for model parameterisation. Given that there are no specific test guidelines available for deriving kinetic data for PBK model parameterisation, each study providing kinetic data for model parameterisation should be critically assessed. Also, in case various kinetic studies for a given chemical of interest are available, all studies must be critically assessed, and it must be well substantiated what data are used for model development and/or model evaluation and what studies are not used.

3) Regarding the linking of the in vitro exposure metric to an internal dose metric in the PBK model, , some initial considerations are provided in this section. The approach used in the QIVIVE should be well described and substantiated. Furthermore, providing results related to different approaches may provide important information to support decision making.

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Maternal exposure and development neurotoxicity effects in offspring: tiered modelling approaches

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Developmental neurotoxicity (DNT) uniquely involves evaluation of potential adverse effects on foetuses or nursing infants resulting from maternal exposure, differing from typical evaluation of toxicological effects that affect the exposed individual. Applying quantitative in vitro to in vivo extrapolation (QIVIVE) to the DNT in vitro battery (IVB) data requires linking an in vitro point of departure (POD) to the in vivo concentration in the target tissue, such as the developing brain during specific developmental windows. After selecting an appropriate in vivo dose metric, a physiologically based kinetic (PBK) model is used for QIVIVE, converting the in vitro POD to an external exposure level. Depending on the dose metric, a gestational or lactational component may be added to a PBK model to predict chemical concentrations in foetuses or nursing infants from maternal exposures. However, this addition is unnecessary if free maternal plasma concentration can convatively represent foetal or infant brain concentration, given that the blood-brain barrier and placenta effectively prevent the chemical from reaching the foetal or infant brain. The complexity of the model should align with the regulatory purpose and acceptable uncertainty levels. Sufficient data availability is crucial for accurate model parameterisation, and undue model complexity without reliable data can lead to unreliable predictions. A tiered modelling framework for QIVIVE analysis is proposed to guide PBK model selection, ranging from high-throughput adult-only models to gestational/lactational models. Estimating a margin of exposure based on lower tier QIVIVE results and potential human exposure levels may also guide the decision on needing a higher tier model. Additionally, it is important to recognize that some risk assessment applications, such as priority ranking or read-across purposes, may not require a PBK model. Alternative integrated testing approaches considering toxicodynamic and toxicokinetic evidence can be suitable without relying on QIVIVE.

The role of physiologically based kinetic modelsin quantitative in vitro to in vivo extrapolation

Applying quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) to convert developmental neurotoxicity (DNT) *in vitro* battery (IVB) data to external exposure levels first requires linking an *in vitro* point of departure (POD) to the *in vivo* concentration at the target tissue, such as the developing brain during specific developmental windows, or to an appropriate surrogate measure, such as maternal plasma concentration. Next, a physiologically based kinetic (PBK) model is typically used to convert the selected *in vivo* dose

metric, equivalent to the *in vitro* POD, into an external exposure level. PBK models are mathematical representations describing the absorption, distribution, metabolism, and excretion (ADME) of a chemical in an organism. These models incorporate physiological and anatomical parameters (such as body weight, tissue volumes, blood flow rates) and chemical-specific ADME factors (such as absorption rate, plasma protein binding, partition coefficients, metabolic and excretion constants) to simulate a chemical's disposition and biotransformation in the body following exposure.

For DNT assessment, when the developmental window occurs after birth and involves direct chemical exposure to infants or young children, both exposure and adverse effects pertain to the same individual. In contrast, other scenarios involve maternal exposure with DNT effects occurring in the fetus or nursing infants. In these cases, a gestational or lactational component may be incorporated into a PBK model to predict chemical concentrations in fetuses or nursing infants from maternal exposures. However, a simpler model structure without such addition may suffice if total maternal plasma concentration can conservatively approximate free foetal or infant brain or plasma concentration, considering the protective roles of the blood-brain barrier (BBB) and placenta in limiting chemical transfer across these barriers.

Selective barriers: blood brain barrier and placental barrier

The BBB is a vital component of the neurovascular unit, and its primary role is to regulate the brain's microenvironment to ensure proper neuronal function (Goasdoue et al., 2017). It is a highly selective barrier between the systemic circulation and the brain parenchyma, controlling the passage of molecules between these compartments (Abbott et al., 2006). Specialized transport systems on the barrier's luminal and abluminal membranes actively regulate the movement of nutrients, ions, and other essential compounds into and out of the brain (Abbott et al., 2006). The BBB also effectively restricts the entry of potentially harmful substances, particularly hydrophilic molecules, due to the presence of tight junctions between brain endothelial cells (Abbott et al., 2006; Rhea et al., 2019). Furthermore, the BBB functions as a metabolic barrier through intracellular and extracellular enzymes that degrade or modify certain molecules, further limiting their access to the brain (Abbott et al., 2006, Chaulagain et al., 2023). It is estimated that over 98% of small molecular drugs and all large molecules, such as monoclonal antibodies and gene therapeutics, cannot cross the BBB (Pardridge, 2005). The BBB becomes functional as soon as it is formed, serving as a protective barrier even during the early stages of embryogenesis (Abbott et al., 2010; Chaulagain et al., 2023). Tight junction proteins, such as occludin and claudin-5, are expressed in the developing human brain as early as 12 weeks of gestation (Virgintino et al., 2000). Efflux transporters, including P-glycoprotein (P-gp), are also present in the foetal BBB (Goasdoue et al., 2017). This evidence further indicates early barrier functionality.

Various *in vitro* methods have been developed to estimate BBB permeability, ranging from 2D systems to 3D organoids and microfluidic models (Chaulagain et al., 2023). Transwell systems and co-cultures are examples of 2D models, which involve culturing brain endothelial cells on a permeable membrane. Using stem cells or primary cells, 3D organoid models resemble brain tissue and include key BBB functions. Microfluidic models involve microfluidic chips with microchannels lined by brain endothelial cells, simulating the microvasculature of the brain. There are also *in silico* methods for predicting BBB permeability (such as Kumar et al., 2022; Wang et al., 2018), designed to screen compounds during the early stages of drug development. Even though they currently cannot provide the values needed for a PBK model to accurately predict brain concentrations, these *in vitro* and *in silico* tools are valuable for estimating BBB permeability and may help guide decisions on whether a higher tier model is needed.

The placental barrier is a selective interface between the maternal and foetal circulations. It plays a crucial role in gas exchange, nutrient and water transfer, immune protection, and hormone secretion, all of which are vital for foetal development (Griffiths and Campbell, 2015). The placental barrier also has several mechanisms that protect the fetus from harmful substances (Griffiths and Campbell, 2015). The syncytiotrophoblast layer that covers the placenta villi serves as the primary physical barrier. In addition,

to reach foetal blood, chemicals must also traverse the foetal connective tissues and the endothelium of foetal capillaries. The ability of a chemical to cross the placental barrier depends on its physiochemical properties (Griffiths and Campbell, 2015; Giaginis et al., 2009; Mathiesen et al., 2021). Larger molecules, particularly those exceeding 500 Da, often exhibit incomplete placental transfer. Hydrophilic, highly polar, and ionized molecules are less likely to cross the placental barrier. Finally, chemicals bound to maternal plasma proteins are less likely to cross the placenta. The placenta can function as a metabolic barrier that expresses various enzymes and transporters. Key enzymes, including those of the cytochrome P450 family, have been shown to metabolize compounds, such as benzo[α]pyrene, aflatoxin B1, and certain pesticides (Myllynen, 2005; Griffiths and Campbell, 2015; Giaginis et al., 2009). The placenta can also metabolize through conjugation reactions, such as sulfation and glucuronidation (Mathiesen et al., 2021). The placenta also expresses efflux transporters, including P-gp and breast cancer resistance protein, which actively pump certain chemicals, such as digoxin and dexamethasone, back into maternal circulation (Griffiths and Campbell, 2015).

A variety of *in vitro*, *ex vivo*, and *in silico* methods are available to provide insights into the protective ability of the placental barrier. *In vitro* methods involve cell cultures to investigate placental transfer at the cellular and molecular levels (Cherubini et al., 2021). Commonly used tools include trophoblast cell lines (such as BeWo and JEG-3), immortalized trophoblast cell lines (such as HTR-8/SVneo), and placental explants. *Ex vivo* approaches use the human placental perfusion model, which maintains the placenta's structural integrity and functional aspects (Giaginis et al., 2009; Zabel et al., 2022; Cherubini et al., 2021). The dual perfusion techniques, where both maternal and foetal circulations are perfused, enable the evaluation of transfer rates, metabolism, and impact on placental function. The single-sided perfusion techniques, which perfuses only the maternal side, can be used to study chemical released by the placenta. In silico techniques (Giaginis et al., 2009) can predict placental transfer based on the chemical structure and properties, such as molecular size, polarity, and lipophilicity.

A tiered modeling framework

A tiered framework is proposed to guide the selection of PBK models in QIVIVE analysis of DNT IVB data. The types of models range from high-throughput models to more detailed gestational and lactational models. Lower tier models prioritise simplicity and practicality, offering rapid and relevant answers; while higher tier models, though resource-intensive, aim for greater fidelity to the system when necessary. Model selection along this continuum should be guided, in part, by the intended application, as higher tier models do not perform better than their lower tier counterparts in all situations. Tier 0 models are generic models with a limited number of compartments. These models are parameterised using high-throughput *in vitro* data and physiochemical properties to predict steady state concentration in maternal plasma. These models are usually implemented for chemical screening and prioritisation purposes, especially for data-poor chemicals. Tier 1 models are more refined to address specific needs, such as predicting concentrations of metabolites or target tissue concentrations. Tier 0 and Tier 1 models are also appropriate for conducting QIVIVE for juveniles directly exposed to chemicals with DNT potential. These tiers are also suitable when evidence suggests maternal plasma concentrations are equal to or greater than foetal or infant plasma/brain concentrations. Tier 2 models are gestational models that predict plasma concentrations in the fetus; or lactational models that predict plasma concentrations in nursing infants. Tier 3 models build upon Tier 2 to predict brain concentrations in the fetus or nursing infants.

It is important to reiterate that a higher tier model is not inherently better. Instead, higher tiers require more data to ensure reliable predictions. If data are insufficient, lower-tier models with fewer parameters may be preferable, as they offer less uncertainty in parameter estimates. Attempting to parameterize complex models with significant uncertainties can result in unreliable predictions, as in the case of predicting plasma or brain concentrations in the fetus and nursing infants. Developing gestational and lactational PBK models is widely recognized as extremely challenging, even for pharmaceutical agents. Several knowledge gaps contribute to the limited number of published gestational models (Lu et al., 2012; Thepaut et al., 2023;

Chaphekar et al., 2021): (1) ADME data are often sparse or unavailable, particularly regarding pregnancy-specific and fetus-specific parameters; (2) processes such as enzyme ontogeny, active transport mechanisms, and other biochemical factors in the fetus are not fully understood, making accurate modeling difficult; (3) the physiological and anatomical changes during pregnancy are highly dynamic and complex; (4) while animal data can provide valuable insights, extrapolating findings to humans can be challenging during pregnancy due to differences in placental structure and function. Similarly, significant gaps make the development of lactational models challenging (Chaphekar et al., 2021; Jones et al., 2023; Nauwelaerts et al., 2021): (1) variability in milk composition can be influenced by age of the neonate, preterm versus term delivery, time course of each feeding session, and the duration of lactation; (2) reliable data on a key parameter, milk-to-plasma ratio, are often lacking, particularly for chemicals that bind to transporters; (3) lactational models require the integration of maternal and neonatal PBK models, but significant uncertainties remain regarding critical factors such as milk production rates and infant feeding patterns.

Each tier of the PBK model carries a certain level of uncertainty, reflecting the inherent challenges of simplifying complex biological systems and limited availability of data. However, the uncertainty does not mean that these models are unusable or unreliable. As British statistician George Box famously stated, "*All models are wrong, some are useful*." No model can perfectly replicate the complexity of the system it is designed to simulate. The value of a model is not in its perfection but in its ability to address specific scientific questions or provide insights into system behaviour. This parsimony principle also applies to PBK modelling, where a PBK model should be as simple as possible while still capable of achieving the intended purpose. The complexity of a PBK model is often constrained by the availability of reliable data to parameterise it or evaluate its predictive ability. Essential parameters must sufficiently represent the target species' physiology and the chemical's key ADME characteristics. An overly detailed or comprehensive model without robust supporting data can result in unreliable predictions and undermine its utility in any application. Further, it may also create a false sense of confidence in its ability to predict granular outcomes. The key is to transparently recognise the limitations of a model while leveraging its strengths to address the scientific questions.

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Deltamethrin - PBK/IVIVE strategy to consider developmental neurotoxicity (DNT) in vitro data for human health risk assessment

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Deltamethrin (DLT) has been selected as one of the case study compounds to be investigated by EFSA to demonstrate how the newly established DNT in vitro battery (DNT IVB) test data can be used within an integrated approach to testing and assessment (IATA). DLT is a pyrethroid with a well-characterised neurotoxic mode of action. Furthermore, a DNT in vivo study conducted in accordance with OECD Test Guideline 426 is available. No evidence of DNT was reported in this study.

The lowest benchmark concentrations (BMCs) for specific hits in the DNT IVB for deltamethrin were 0.5 µM (BMC50 rat neuronal network formation) and 0.6 µM (BMC30 human oligodendrocyte differentiation). To put this into perspective, to possible human exposure, we developed a PBPK model to estimate human foetal brain concentration at a daily intake of 0.01 mg deltamethrin/kg body weight. This is the acceptable daily intake (ADI) for deltamethrin and therefore reflects a worst-case exposure scenario after dietary exposure to deltamethrin established by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2010.

In a first step, a physiologically-based kinetic (PBK) model was developed for rats and validated on in vivo rat toxicokinetic data of DLT over a broad range of doses. It was then translated to humans, and human foetal plasma concentrations of DLT were successfully simulated by integration of a maternal-foetal PBPK model. Subsequently, the model was used to estimate human foetal brain exposures across various scenarios with variations of key model assumptions and parameters (e.g. blood-brain and blood-placenta barrier partitioning) to address uncertainties. Model calculations from a so-called “realistic data-driven approach” up to “worst case scenarios” were conducted and the predicted foetal brain concentrations were compared to the lowest in vitro BMCs from the DNT IVB. This resulted in margins of safety from 80 to 34349, with a safety margin for the “realistic data driven approach” of 28250.

The presented results show that DLT concentrations in the human foetal brain are highly unlikely to reach concentrations associated with in vitro findings under human dietary exposure conditions. Therefore, the new in vitro DNT results are considered to have no impact on the current risk assessment approach.

Introduction

Deltamethrin (DLT) has been selected as one of the case study compounds to be investigated by EFSA to demonstrate how the newly established DNT *in vitro* battery (DNT IVB) test data can be used within an integrated approach to testing and assessment (IATA). DLT is a pyrethroid with a well-characterised neurotoxic mode of action. Furthermore, a DNT *in vivo* study conducted according to OECD Test Guideline 426 is available. No evidence of DNT was reported in this study.

Results

Within the EFSA IATA case study it was reported that DLT has a DNT-specific and concentration-dependent activity in the rat neuronal network formation assay (rNNF; BMC_{50} 0.5 µM), human neuronal network formation assay (hNNF, BMC_{50} 4.1 µM), oligodendrocyte differentiation (NPC5; BMC_{30} 0.6 µM) and neural crest cell migration assay (UKN2; BMC_{25} 18.4 µM) (EFSA PPR Panel 2021). The same set of raw data was re-analysed by Blum et al. 2023 and uploaded to the EPA ToxCast database resulting in different conclusions for most of the individual assays (hit vs. no hit, specificity, BMC value) emphasising the need for an aligned data analysis approach. An independent 2nd testing campaign with a subset of assays from the DNT IVB (NPC assays 2-5) could only confirm the inhibition of oligodendrocyte differentiation after DLT exposure as specific hit with a BMC ≈ 0.5 µM.

Another aspect that becomes apparent during this 2nd testing campaign was that DLT starts to precipitate in the NPC assay medium between 0.25 and 0.74 µM indicating that the effects on oligodendrocyte differentiation might be biased by test substance precipitation. In addition, this raises the question, if precipitation occurred also in other DNT IVB assays. OECD Guidelines for other *in vitro* methods recommend excluding concentrations which show precipitation (e.g. OECD 456) or test only one concentration that is producing a precipitation (e.g. OECD 487) to avoid artifactual effects resulting from the precipitate adhering to the cells or being taken up by the cells. A clear guidance for how to deal with effects at precipitating concentrations for the interpretation of DNT IVB data is currently missing but *in vitro* effects at precipitating concentrations should be generally interpreted with caution.

To put the DNT IVB results into perspective to possible human exposure we developed a PBPK model to estimate human foetal brain concentration at a daily intake of 0.01 mg DLT/kg body weight. This is the acceptable daily intake (ADI) for DLT established by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2010 and therefore reflects the real worst-case exposure scenario after dietary exposure in humans.

The DLT physiologically-based kinetic (PBK) model was established in PK-Sim® (www.open-systems-pharmacology.org). The physicochemical properties, in vitro and in vivo data for deltamethrin were used as input parameters to develop a rat PBK model. It was validated on *in vivo* rat toxicokinetic data of DLT over a broad range of doses. It was then translated to humans, and human foetal plasma concentrations of DLT were successfully simulated by integration of a maternal-foetal PBPK model. Subsequently, the model was used to estimate human foetal brain exposures across various scenarios with variations of key model assumptions and parameters (e.g. blood-brain and blood-placenta barrier partitioning) to address uncertainties (Maass et al., 2023). Major learnings during the PBPK model development were that availability of rat in vivo data was very important for the fitting of several parameters (e.g. lipophilicity). Highly lipophilic compounds are not fully covered by PK-Sim and are not in the applicability domain of several in silico tools to predict BBB or BPB penetration. However, implementation of different scenarios is a good approach to address the uncertainty of modelling aspects and input parameters. Model calculations from a so-called “realistic data-driven approach” up to “worst case scenarios” were conducted and the predicted foetal brain concentrations were compared to the *in vitro* BMCs from the DNT IVB. This resulted in margins of safety from 80 to 34349, with a safety margin for the “realistic data driven approach” of 28250, considering an *in vitro* BMC of 0.5 µM.

The presented results show that DLT concentrations in the human foetal brain are highly unlikely to reach concentrations associated with *in vitro* findings under human dietary exposure conditions. Therefore, the *in vitro* DNT results are considered to have no impact on the current risk assessment approach.

The PBK model and its results were submitted within the currently ongoing EU renewal process for DLT and assessed by EFSA during the pesticide peer review meetings in June 2023. EFSA disagreed with the conclusion above because the experts considered the overall uncertainty in the PBK model predictions as too high to use this study in its present form in the weight-of-evidence assessment for the DNT assessment. Main uncertainties identified for the PBPK model were bias in data selection, fitting and scaling of parameters values and foetal plasma: maternal plasma ratios, etc. However, it was noted that some uncertainties may be covered by the described worst-case scenarios. No further details were provided.

Conclusion

The deltamethrin case study highlights that there is the clear need to define general recommendations to allow the use of PBK modelling approaches for the quantitative interpretation of the DNT IVB data in an IATA for DNT. In addition, certain technical aspects of the DNT IVB (e.g. acceptability criteria, data analysis) require further alignment and guidance before its regulatory implementation.

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QIVIVE in developmental neurotoxicity: The role of in vitro distribution kinetics

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Physiologically based kinetic (PBK) models are promising tools in next generation risk assessment. They can be used to extrapolate in vitro effective concentrations in the developmental neurotoxicity in vitro testing battery (DNT-IVB) to bioequivalent doses in pregnant women, a procedure referred to as quantitative in vitro-in vivo extrapolation (QIVIVE). These models consist of a series of mass balance differential equations simulating transport of chemical between tissues over time. Using physiological and chemical-specific input parameters, such as tissue volume and tissue-blood partition coefficients, these models can simulate the extent to which chemicals are absorbed, distributed, metabolised and excreted (ADME) in and out of the body, including the developing foetus. For QIVIVE, the PBK model is used to calculate the external dose of the parent compound to which a pregnant woman needs to be exposed to reach levels of the bioactive chemical in the foetal blood represented by the nominal in vitro effect concentration. However, these nominal concentrations ignore the fact that ADME processes occur in vitro assay and that these processes affect the accumulation of a test chemical in cells and thus the observed in vitro effect concentration. Chemicals differentially evaporate, metabolise and bind to serum constituents such as albumin, well plate plastic and cells. The fraction of chemical at the target site in cells can be significantly different between chemicals and between in vitro assays of the DNT-IVB, despite nominal effect concentrations being similar. This hampers the extrapolation of DNT-IVB effect concentrations to bioequivalent doses in pregnant women. In this presentation, the distribution of DNT chemicals in the different systems of the DNT-IVB is discussed and methods to model and analytically measure concentrations in cells in vitro is presented.

{paper not submitted by the speaker}

4 Tiered testing, Additional Assays and Non-Mammalian Animal Models– What do they have to offer to the DNT-IVB?

The EFSA DNT-RAP Project - Laboratory Transferability and Accessibility of the DNT-IVB Test Methods

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Chemical exposure during pregnancy can disrupt key neurodevelopmental processes (KNDPs), impair brain function, and lead to developmental neurotoxicity (DNT). Currently, regulatory DNT testing relies exclusively on rat in vivo guideline studies (OECD TG 426 and TG 443 with DNT cohort 2A and B). However, according to most of the EU chemicals legislation, DNT testing is triggered by evidence of adult neurotoxicity or endocrine disruption properties. These in vivo studies are resource-intensive, requiring significant time, money, and animal use. Under the guidance of the European Food Safety Authority (EFSA) and the US Environmental Protection Agency (US EPA) and supported by the OECD, a DNT in vitro battery (DNT IVB) has been developed, consisting of assays that model critical KNDPs, including progenitor cell proliferation, radial glia migration, neuronal and oligodendrocyte migration and differentiation, neurite outgrowth, synaptogenesis, neural network formation, and apoptosis. The DNT IVB has emerged as a critical tool for identifying compounds with DNT potential. A key prerequisite for regulatory acceptance of the DNT IVB is the demonstration of its transferability to a naïve laboratory and interlaboratory reproducibility data generation. This requires a lab-to-lab

transfer from test developers to a naïve laboratory to: (a) validate the reproducibility of the test methods in a naïve setting and (b) ensure the assays are accessible to stakeholders, such as industry and regulatory agencies. If the DNT IVB becomes a regulatory data requirement, testing capacity will need to expand significantly beyond the current academic infrastructure. DNT RAP2, an EFSA procurement project, is assessing the feasibility of transferring all DNT IVB assays to a naïve laboratory. This includes: (i) optimizing standard operating procedures (SOPs) to improve transferability and reproducibility, (ii) transferring and implementing the optimized SOPs in the naïve laboratory, and (iii) conducting blinded testing of DNT-positive and DNT-negative chemicals in both the developer and naïve laboratories. Validation of these test methods will enhance confidence in the battery, reduce uncertainties in its regulatory application, and ensure its commercial availability, paving the way for a more sustainable and efficient approach to DNT regulatory assessment.

Introduction

The assessment of neurotoxicity (NT) and developmental neurotoxicity (DNT) is critical for evaluating risks associated with chemical substances, particularly pesticides (Jurewicz and Hanke, 2008; Voorhees et al., 2017). While routine testing for neurotoxicity is included in standard rodent-based in vivo toxicological studies (acute, sub-chronic, chronic, reproductive, and carcinogenicity studies), specific neurotoxicity studies (OECD Test Guidelines (TG) 407, 408, and optionally TG 452) are not routinely performed. Moreover, targeted DNT studies, such as those outlined in OECD TG 426 and TG 443 (with DNT cohort 2 A and B), are typically only required when triggered by specific evidence, such as observed neurotoxic effects, endocrine disruption concerns, or a known mode of action. The importance of NT and DNT testing is highlighted by the vulnerability of the developing nervous system to chemical stressors (Jurewicz and Hanke, 2008; Rice and Barone, 2000). Early-life exposure to neurotoxic chemicals is linked to the emergence of neurodevelopmental disorders such as ADHD, autism, or loss of IQ. These complex effects expose the limitations of the classical rodent in vivo DNT studies, which face challenges such as low throughput, high resource requirements, and limited human relevance. This underscores the urgent need for innovative approaches to assess chemical risks during development. Human-based New Approach Methodologies (NAMs) have emerged as effective tools to address these challenges by providing valid and reliable mechanistic data for chemical hazard assessment while reducing reliance on traditional animal studies (Viviani et al., 2025). Over the past decade, significant progress has been made in developing NAMs for DNT risk assessment, driven by initiatives from the EFSA, US EPA and the OECD in collaboration with academic experts (Sachana et al, 2019; 2021; Bal-Price et al., 2015; 2018; Fritsche et al., 2015; 2018; Masjosthusmann et al.,2018). A cornerstone of these efforts has been the development of DNT NAMs, their integration into a DNT in vitro testing battery (DNT IVB), and the publication of guidance documents that demonstrate the utility of the battery within Integrated Approaches to Testing and Assessment (IATA) for DNT (Masjosthusmann et al., 2020; Blum et al.,2023; Koch et al., 2022; Harrill et al., 208; Shafer et al., 2019). The OECD's "Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery" outline the DNT IVB assays, establish criteria for evaluating the DNT relevance of data, address uncertainties and biological coverage, and assist in determining the degree of certainty in findings to support their use in regulatory hazard/risk assessments (OECD, 2023). Before regulatory application, NAMs must undergo rigorous validation to ensure

reproducibility and transferability across laboratories (Hartung, 2010; Leist et al., 2012). The DNT RAP2 project, initiated by EFSA, seeks to validate the DNT IVB assays, supporting EFSA's commitment to reducing animal testing while improving the precision and efficiency of chemical risk assessments. By establishing the reliability and applicability of DNT NAMs, this validation study represents a pivotal step toward their regulatory acceptance, facilitating the transition to a more comprehensive, human-relevant, and sustainable approach to DNT hazard evaluation.

Main objectives and study phases of the DNT RAP 2 project

The DNT RAP2 project aims to optimise and standardise the standard operating procedures (SOPs) for the DNT IVB assays and evaluate their transferability to a naïve laboratory, specifically the DNTOX GmbH. These efforts focus on ensuring that all 17 assays, as defined in OECD (2023), meet robust criteria for reproducibility and accessibility. The project prioritises the use of commercially available cell systems, particularly human induced pluripotent stem cells (hiPSCs), to integrate formal quality systems for cell banks and quality controls. Key objectives include optimising the DNT IVB assays and their SOPs to address limitations that hinder transferability and reproducibility, enhancing clarity and standardisation across laboratories. This includes addressing any factors that may hinder transferability, such as proprietary elements or inconsistencies in data analysis. The optimised SOPs will be transferred to the naïve laboratory for implementation, with further refinements based on their findings. Additionally, 10 compounds, classified as either "DNT hit" or "no hit," will be tested in both developer and naïve laboratories to evaluate reproducibility and the success of the method transfer based on the concordance of results between the developer and naïve laboratories. Through these efforts, the project seeks to demonstrate the robustness of the DNT IVB assays across diverse laboratory settings as a prerequisite for regulatory application. The DNT RAP2 transferability study is structured into consecutive phases designed to optimise and implement the DNT IVB assays in a naïve laboratory, while ensuring their accessibility, transferability, and reproducibility. Initially, test systems will be optimised to address issues with availability and usability. Subsequently, the naïve laboratory will review and refine the SOPs to enhance clarity and transferability. Once finalised, the assays will be transferred to the naïve laboratory for implementation. Performance evaluations will follow, using reference chemicals specific to each assay, to determine the success of the transfer and broader applicability. Finally, under the DNT RAP1 project, another EFSA procurement, 116 chemicals, including pesticides and DNT-negative substances, will be tested in the DNTOX laboratories under blinding conditions using the transferred DNT IVB assays. Specifics of the study phases are outlined in the following paragraphs:

a. Investigating strategies to ensure accessibility of the DNT IVB test systems: The DNT RAP2 project focuses on ensuring the accessibility and effective transferability of the DNT IVB assays. It involves the refinement of all 17 DNT IVB assays, which encompass eight key neurodevelopmental processes (KNDPs) across developmental stages. The test methods are human or rat cell-based and encompass different cell systems (e.g. hiPSCs, primary cells, genome engineered neural cell lines) (OECD, 2023). The goal is to transfer these assays from the developer laboratories to a naïve laboratory for comparative reference compound testing, while addressing challenges related to cell system accessibility and reproducibility. Key activities include refining differentiation protocols for generating the final test systems and their incorporation into updated SOPs. Specific emphasis is placed on assays like NPC1, NPC2-5, UKN5, and hNP1, where uncertainties with regards to cell system accessibility have been identified. The project will also evaluate and update acceptance criteria to account for changes in test systems, experimental protocols, and data analysis methods. Any modifications to SOPs and test methods will be systematically documented. Through this work, the project aims to ensure the accessibility, reproducibility, and transferability of all DNT IVB assays, paving the way for a broader acceptance of the DNT IVB in testing chemicals for DNT.

b. Optimisation of the SOPs of the 17 DNT IVB assays during the transferability study: DNT RAP2 focuses on optimising the SOPs for the 17 DNT IVB assays during their transfer to a naïve laboratory. For

assays requiring differentiation or neural induction of hiPSCs, such as UKN2, UKN5, and NPC1, detailed protocols will be integrated into the updated SOPs. The optimisation process also aims to standardise the assays to improve reproducibility and reduce operator errors. This includes harmonising plate layouts, test compound concentrations, biological and technical replicates, and data acquisition and analysis procedures. Specific emphasis is placed on providing detailed guidance for assays involving high-content imaging (HCI) and micro-electrode arrays (MEA) to ensure their reproducibility across laboratories using different devices. Additionally, acceptability criteria, including solvent control values, positive control performance, and variability measures, will be refined. The SOPs will be restructured to ensure consistency in style and content, minimising ambiguities and facilitating their use in different laboratories. All revisions and improvements will be reported and approved by EFSA and JRC, ensuring that the optimised SOPs are comprehensive and transferable.

c. Consecutive transfer of the DNT IVB assays to a naïve laboratory: The DNT IVB assays will be transferred sequentially to a naïve laboratory, including all protocols needed to generate the final test systems, such as differentiation and neural induction protocols. Test systems will be adapted from those described in OECD 2023 to improve accessibility. Assay transfers will begin with NPC1, UKN4, UKN2, and UKN5, followed by NPC2-5. US-EPA assays will be transferred later as the US-EPA personnel will handle pesticide testing in the DNT RAP1 project themselves. Key preparatory steps include establishing test systems, generating quality-controlled cell banks, and optimising data analysis protocols for compatibility with the naïve laboratory's equipment. Close collaboration between the naïve laboratory, developer laboratories, and EFSA is essential.

d. Testing strategy of DNT reference chemicals across laboratories: In the DNT RAP2 project, both developer laboratories and the naïve laboratory will test the same 10 chemicals (six DNT positive and four negative chemicals), selected based on historical data available at the developer laboratories and recommendations for negative chemicals (Martin et al., 2022). Testing will be unblinded to ensure appropriate concentration ranges are used, and each chemical will be tested in at least three independent biological replicates. Despite the availability of historical data, developer laboratories will retest these chemicals as part of the project. Some test systems will be modified for accessibility. For assays such as NPC1-5 and hNP1, developer laboratories have already generated data using new cell systems, and both the naïve and developer laboratories will test chemicals using these updated systems. For other assays, reference chemicals will be tested by both the developer and naïve laboratories using the same test systems as described in OECD 2023.

Final evaluation of the transferability and performance of the DNT IVB assays: Changes in test systems can influence the response to environmental chemicals, making it essential to evaluate the transferability of DNT IVB assays using comparable data generated in both the developer and naïve laboratories with the new test systems. The success of the transferability study will not rely on comparisons with historical data but instead on consistent compound classifications ('DNT hit,' 'non-specific hit,' or 'no hit') according to the assay-specific prediction models highlighted in OECD 2023. Raw data will inform on the performance of endpoint-specific controls, dynamic ranges, and inter-/intra-experimental variability, ensuring compliance with the published acceptability criteria. If these criteria are unmet, evaluations will determine whether differences arise from either changes in test systems or assay performance in the naïve laboratory. If new test systems are responsible, the final SOPs will incorporate updated acceptability criteria.

Conclusion

The DNT RAP2 project represents a pivotal step in advancing the regulatory acceptance of the DNT IVB as a reliable and human-relevant tool for DNT hazard assessment. By addressing challenges related to accessibility, reproducibility, and laboratory transferability of the DNT IVB assays, the project lays the groundwork for broader application of NAMs in regulatory frameworks such as IATAs. The optimisation of

SOPs, testing of reference chemicals, and validation of new test systems in both developer and naïve laboratories are essential for ensuring the robustness of the DNT IVB assays. Successful implementation will not only reduce reliance on animal testing but also enhance the efficiency and precision of chemical risk evaluations, aligning with EFSA's vision for a more sustainable and scientifically advanced approach to DNT testing based on mechanistic information. This initiative provides a foundation for integrating innovative in vitro methods into regulatory hazard assessments, bridging the gap between academic research and practical regulatory application.

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Establishment of, data generation from, and comparison to existing data from the developmental neurotoxicity in vitro battery

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Prevalence of neurodevelopmental disorders has increased in recent decades. The precise cause of this increase has not been identified; however, it has been speculated that environmental factors (e.g., exposure in utero to compounds in the maternal diet) may contribute. As such, the need to comprehensively evaluate potential developmental neurotoxicity (DNT) of compounds is paramount. At present, there is a paucity of data on DNT potential for most commercially available compounds. Furthermore, regulatory agencies worldwide do not require DNT testing unless there is indication for potential DNT from an in vivo animal study. Moreover, the current regulatory accepted paradigm for in vivo DNT testing (i.e., OECD 426) is time intensive, can produce ambiguous results, and is not cost-effective.

In response to the need for more efficient evaluation of DNT potential, an international effort was initiated to develop more rapid in vitro new approach methods (NAMs) that capture fundamental neurodevelopmental processes. The culmination of this effort was a publication by the Organisation for Economic Co-operation and Development (OECD) on initial recommendations on the application of data from these in vitro NAMs, now deemed the DNT in vitro battery (IVB). However, for these initial recommendations to be accepted into an OECD guidance document, several steps were outlined by international stakeholders which included the transfer to and implementation of the DNT IVB assays in new laboratories.

As part of this transfer and implementation step, the U.S. Food and Drug Administration (FDA) Human Foods Program (HFP) has initiated transfer of select DNT IVB assays from the U.S. Environmental Protection Agency (US EPA) and the Leibniz Research Institute for Environmental Medicine. Importantly, this transfer is not one-to-one, i.e., direct technology transfer. As such, there are several considerations and challenges that have impacted this process; this presentation will focus on two of these challenges: cell models and data analysis.

First, of the DNT IVB assays that FDA HFP selected to transfer, only one of the cell models utilized was commercially available. Moreover, the laboratories do not have access to primary animal-derived cells necessary for select assays. As such, each approach must be adapted for a new cell model, which can potentially have a significant impact on the performance

of an assay. Second, the OECD initial recommendations advise that the EPA ToxCast pipeline be applied for data analysis. FDA HFP has implemented a concentration-response modeling pipeline akin to ToxCast; however, there is no consensus on across-laboratory comparisons, particularly if the response threshold is different between laboratories. Therefore, it has been difficult to determine whether FDA HFP observes appropriate compound responses in transferred DNT IVB assays. Challenges aside, FDA HFP has successfully transferred and evaluated performance compounds in multiple DNT IVB assays to date, which will also be discussed in this presentation.

In response to the need for more efficient evaluation of developmental neurotoxicity (DNT) hazard potential, an international effort was initiated to develop more rapid *in vitro* new approach methods (NAMs) that capture fundamental neurodevelopmental processes (Sachana et al., 2019). Culmination of this effort was a publication by the Organisation for Economic Co-operation and Development (OECD) on initial recommendations on the application of data from these *in vitro* NAMs, now deemed the DNT *in vitro* battery (IVB) (OECD, 2023). However, for these initial recommendations to be accepted as an official guidance document, several steps were outlined by international stakeholders which included the transfer and implementation of DNT IVB assays in new laboratories. As part of this transfer and implementation step, the U.S. Food and Drug Administration (FDA) Human Foods Program (HFP) has initiated establishment of select DNT IVB assays developed at the U.S. Environmental Protection Agency (US EPA) and the Leibniz Research Institute for Environmental Medicine (IUF). Importantly, this establishment was not a direct technology transfer. As such, several considerations and challenges impacted this process.

The first challenge encountered was cell model selection. Of the cell models utilised in US EPA and IUF DNT IVB assays, only one, iCell GlutaNeurons, was commercially available. This cell model is utilized in the hN initiation neurite outgrowth assay developed at EPA[3]. As the cell model was available, FDA HFP elected to establish this assay first. Notably, however, new cell models have been selected for other in-progress assay establishments. For the hN initiation assay, FDA HFP applied a similar cell culture and chemical exposure schema as US EPA. In addition, similar neuronal cell markers were utilised to identify neurons and neurites for high-content imaging (HCI). Two pertinent differences were (1) HCI-technology employed, confocal versus epifluorescence, and (2) cell viability endpoint measured, adenosine triphosphate (ATP)-quantification versus neuron count, for FDA HFP and US EPA, respectively.

Identification of performance compounds was the next challenge. Performance compounds are selected not necessarily because of human-relevant DNT, but because of demonstrated activity or inactivity in an assay. Application of these compounds in the newly established assay demonstrates the new laboratories capability to perform the assay. Suggested performance compounds for the hN initiation assay were provided in the initial recommendations (see Appendix B.9 Table 5.5.1) (OECD, 2023). This led to a subsequent challenge, data analysis. To be included in the DNT IVB, selected assays had data that were analysed in the U.S. EPA ToxCast Pipeline (OECD, 2023). Therefore, FDA HFP opted to apply the same analysis procedure (Sheffield et al.,2022). Unfortunately, none of the suggested performance compounds had data analysed in this manner. US EPA, however, kindly provided the raw data for the performance compounds from the original publication (Druwe et al., 2016), which FDA HFP then analysed.

The final challenge was how to compare data across laboratories. There is currently no consensus on how to make these comparisons. Consequently, FDA HFP elected to compare the binary activity hit call (active versus inactive), the activity concentration at cutoff (ACC), and the activity concentration at 50% maximal activity (AC50) across laboratories. As an example, a single endpoint, neurite length, for the hN initiation

assay was compared between FDA HFP and US EPA. Importantly, a response threshold or cutoff equal to 30% activity was set for both institutions. Of the ten anticipated active compounds FDA HFP detected 9/10, while EPA detected 7/10 for this endpoint. For common active compounds, ACC and AC50 values were all within an order of magnitude. Moreover, the two anticipated inactive compounds were inactive across both institutions.

Finally, concordance was assessed across all human neurite outgrowth assays – this included four institutions and five different cell models. Apart from FDA HFP and US EPA iCell GlutaNeuron hN initiation assay data, all other data were obtained from the U.S. EPA CompTox Chemicals Dashboard v2.4.1. For this assessment, only the AC50 values for the neurite length endpoint were compared, as the response threshold varied for data captured from the dashboard. Three common performance compounds were evaluated across all institutions and were anticipated to be active. Only one compound (methylmercury (II) chloride) was detected by every institution. However, for the other two, one (lead (II) acetate trihydrate) was detected by a single institution, while the other (trans-retinoic acid) failed to be detected by one institution.

In conclusion, FDA HFP established the hN initiation neurite outgrowth assay developed at EPA. Capability to perform the assay was demonstrated – 16 performance compounds, 12 anticipated to be active and 4 anticipated to be inactive, were evaluated and only 1 anticipated active (lead(II) acetate trihydrate) failed to be detected. Common normalisation and concentration-response modelling approaches were applied to compare across laboratories. FDA HFP and US EPA assay data were comparable and effective concentrations, i.e., ACC or AC50, very similar. There was limited data, however, to do a complete concordance analysis for all human neurite outgrowth assays.

Next steps are for FDA HFP to continue establishment of other DNT IVB assays. This will involve as previously mentioned selection of new cell models and performance compounds. Furthermore, FDA HFP will also continue to refine data analysis in line with updates to the U.S. EPA ToxCast Pipeline. In addition, NAMs in development at other institutions will also be implemented – this will include but is not limited to a multiplex proliferation and apoptosis assay in human induced pluripotent stem cells, as well as a three-dimensional brain organoid neural network formation assay.

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Tiered testing and considerations for integrating additional assays and models to the DNT-IVB

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Currently, Developmental Neurotoxicity (DNT) testing is not routinely required for pesticides or any of the low to high-production tonnage chemicals. One reason for the lack of mandatory DNT testing is the resource-intensiveness and the high uncertainty in interpretation of the current OECD guideline studies. Therefore, the European Food Safety Authority and the United States (US) Environmental Protection Agency (EPA) have teamed up with academic laboratories to set up an in vitro test battery (IVB) based mainly on human cells for faster, cheaper, and more reliable assessment of compounds` DNT potential. In 2023, the OECD published initial recommendations on evaluation of data from this DNT IVB. For practical implementation, a tiered testing approach containing the DNT IVB is warranted. Such a tiered testing will streamline a DNT testing paradigm and make it suitable for broader application beyond pesticides. Tiered testing is a stepwise approach that combines existing information on both exposure and hazard with the goal of obtaining uncertainty levels acceptable to the regulatory need. All available data on exposure and hazard are used to assess risk based on the margin of exposure (MOE), the ratio of hazard and exposure estimates, and used in an overall weight-of-evidence (WoE) framework. Overall, use of this tiered testing framework for DNT should be driven by problem formulation based on regulatory needs using the IATA framework. The required amount of confidence in, the information will vary based on problem formulation. Regulatory needs can be diverse (e.g., prioritisation, new chemicals, pesticide registration; see Section on Target Uses in the Initial Recommendations for more details, OECD 2023). If there is insufficient information or unacceptable uncertainty that hampers a fit-for-purpose regulatory assessment, the next tier of testing should be required. It is important to note that this framework provides a possible workflow but should not be used as a prescriptive set of required steps. Regulatory requirements and existing data will dictate how the framework is employed.

The framework is supposed to consist of 5 tiers supporting the IATA framework:

Tier 0 - Computational/HTP Bioactivity: Tier 0 data could be used for prioritising chemicals for testing in Tier 1, based on indications of possible developmental neurotoxic effects coupled with and/or modelling potential human exposure.

Tier 1 - DNT IVB (OECD, 2023): This battery was designed to screen chemicals for activity on fundamental neurodevelopment processes using assays that include human and rat neural cell cultures.
Tier 2 - Confirmatory/Orthogonal Assays: increasing the number of Tier 1 replicates/test concentrations; use of orthogonal assays; testing in more than one species.
Tier 3 - Complex models: brain region-specific complex 3D model; zebrafish embryo or other alternative species; measurement of specific MIEs or KEs; model barrier kinetics (blood-brain-barrier, placental barrier); inclusion of metabolic competency.
Tier 4 - Specialised animal testing: either the OECD/EPA guideline studies or modified animal testing.
This talk will explain this provisional DNT tiered testing framework, discuss the usefulness of the different tiers and propose how it is used for decision-making.

Introduction

There is a large data gap for testing thousands of chemicals for their potential to interfere with human brain development. One reason for this void in data concerning developmental neurotoxicity (DNT) is the resource-intensity of the current DNT OECD guideline studies. Therefore, an international community has been developing a DNT testing in vitro battery (IVB) for regulatory application (OECD, 2023). There are two peculiarities of the DNT IVB; first, it was set up on the basis of basic neurobiological knowledge and second, it has been developed in an international research team consisting of regulatory toxicologists and academic toxicological scientists (Bal-Price et al., 2015). This tandem of researchers and regulators has led to a substantial, well characterised product, i.e. the DNT IVB. For making the DNT IVB available to the market, the contract research organization (CRO) DNTOX GmbH with its location in Düsseldorf, Germany, was founded (www.dntox.de). DNTOX offers in vitro DNT testing services to agencies and industry.

Possible uses of the DNT IVB in the chemicals framework

Once the IVB was proposed (Crofton and Mundy, 2021), it has demonstrated its regulatory utility in different context of use with several case studies (OECD, 2022a; b; c). After the OECD published initial recommendations on the evaluation of data from the DNT IVB (OECD, 2023) and the tests are in the process of being commercially available, the next question is how to apply the DNT IVB across different jurisdictions ad regulatory frameworks. The current cases studies used a weight-of-evidence (WoE) framework where all available data on exposure and hazard are used to assess risk based on the margin of exposure (MoE).

Another possible option is to integrate the DNT IVB into a DNT tiered testing strategy. Such a tiered approach should organize toxicological assessments to maximize efficiency and minimize the use of animals. It involves a hierarchy (tiers) of tests, starting with those that use existing information or simple biological methods before moving onto tests using cells and eventually live animals only as necessary (https://www.efsa.europa.eu/en/glossary/tiered-approach). There are several examples that concern tiered approaches. For example, the `Risk assessment of combined exposure to multiple chemicals: a WHO/IPCS framework` (Meek et al., 2011) is an example of tiered exposure and hazard considerations with regards to chemical mixtures. Moreover, Leonard and Tan (Leonard and Tan, 2019) published `Tiered

approaches for screening and prioritizing chemicals through integration of pharmacokinetics and exposure information with in vitro dose-response data`. A third publication by Andersen and co-workers (Andersen et al., 2019) proposed `Developing Context Appropriate Toxicity Testing Approaches Using New Alternative methods (NAMs)`. Some of them are embedded in current legislation frameworks, e.g. in the strategy to investigate EATS-related endocrine activity in the context of the ED assessment (Andersson et al., 2018) or the EFSA genotoxicity assessment of chemical mixtures (EFSA Scientific Committee et al., 2019) just to name a few.

Considerations on tiered testing approaches for DNT

Concerning the DNT IVB, the questions were if the battery can be embedded in a tiered approach? How could such an approach look like in the case of DNT? How would we deal with high-throughput screening assays in a tiered approach? Do complex 3D in vitro models or whole organisms like the zebrafish embryo pose an added value to the DNT testing strategy?

It is important to note that the use of a tiered testing framework for DNT should be driven by a problem formulation approach based on regulatory needs using the Integrated Approaches to Testing and Assessment (IATA) framework (OECD, 2017; Sachana and Leinala, 2017; Sakuratani et al., 2018). The required amount of, and confidence in the information will vary based on the regulatory need for DNT information. Specific criteria for such use need to be developed by regulatory bodies who will determine the acceptability of each tier based on their needs.

Here we have explored the current knowledge on assessment of DNT using available DNT models (animals and non-animal) and we have developed an aspirational tiered approach for DNT risk assessment aiming to compile the current knowledge on DNT sources of information for hazard and exposure and guide further research. A draft document suggested by Kevin Crofton and Bill Mundy that mainly built on the previously mentioned publications was utilized as the basis of the current proposal for a tiered testing approach for DNT (see Sue Marty in these proceedings). In addition, a provisional tiered testing approach for DNT was previously suggested by Masjosthusmann et al. (Masjosthusmann et al., 2020) in the European Food Safety Authority (EFSA) external scientific report on the `Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity`. In the latter, also exposure-driven approach, the issue of species extrapolation was highlighted (Figure 1A).

For the OECD workshop “Critical Innovations in Pesticide Safety Testing and Chemical Risk Assessment for DNT” in October[3], these tiered testing proposals for DNT were discussed during the meeting resulting in the draft tiered testing framework for DNT risk assessment suggested in Fig. 1B.

This draft should be seen as an attempt of integrating the current knowledge on DNT in a tiered testing framework. It is not intended to guide the use of the DNT IVB in human health risk assessment. The aim is to define where future research could help to develop additional assays and models to the DNT IVB and to embed them into a tiered testing approach for DNT assessment. There are various uncertainties in this proposal, among them, (1) a systematic analysis of all DNT available methods and sources of information has not been done; (2) the predictive capacity of each of the tiers has not been defined; (3) for some of the tiers readiness criteria for regulatory use have not been discussed or demonstrated (e.g., tier 1 TK, QSAR models or in vivo targeted studies); (4) the entire workflow in this strategy has never been employed for any case study.

Further discussion among stakeholders, including the assay developers and regulatory communities will require to make changes and further define some of the tiers. In addition, future advancements on DNT

[3] https://www.oecd.org/en/events/2024/10/workshop-critical-innovations-in-pesticides-safety-testing-and-chemical-risk-assessment-for-developmental-neurotoxicity.html

models (e.g. omics technologies, complex in vitro organ systems, alternative species) will also drive changes in the methods used in the different tiers.

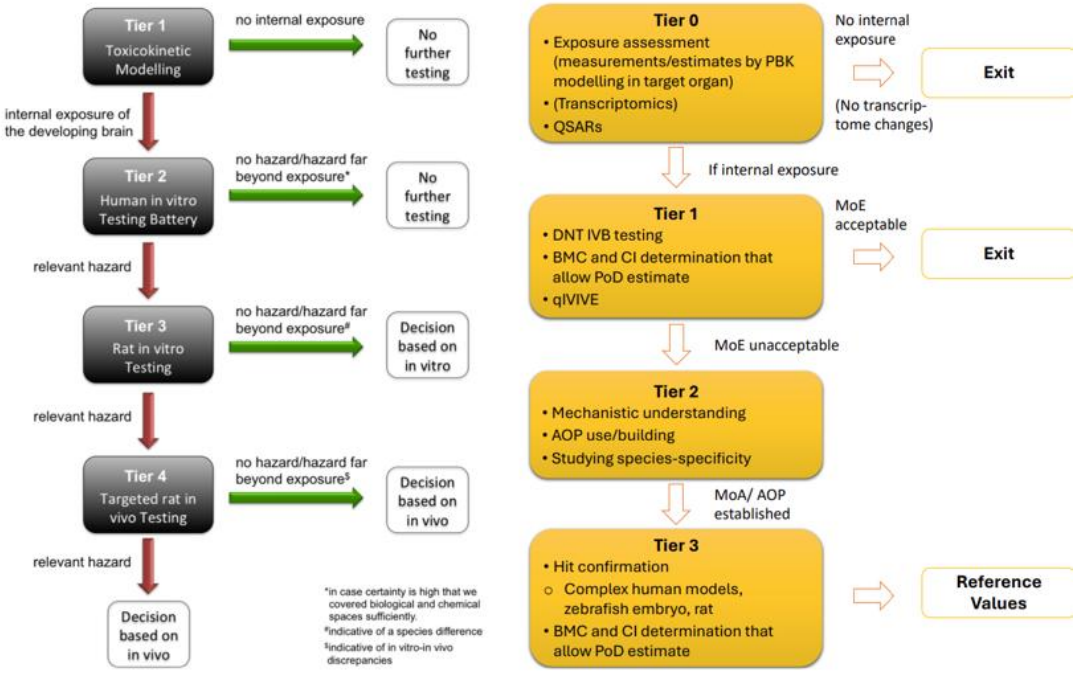

Fig. 1: Proposed Tiered Approaches for DNT containing DNT IVB evaluation. A. A tiered testing approach was suggested in Masjosthusmann et al. (2020). B. The current tiered approach explored at the October 2025 OECD workshop for the embedding of the DNT IVB into tiered approach. For explanation, please see text.

In the breakout session group of the Workshop, there was an unanimous opinion that for a risk assessment problem formulation, exposure assessment is crucial to understand if a MoE is acceptable or not. In case regulations allow, in a Tier 0, exposure assessment can be supported by actual compound measurements and/or physiology-based kinetic (PBK) modelling. An acceptable MoE might lead to an exit already in Tier 0, whereas an unacceptable MoE will guide into Tier 1. In parallel, hazard assessment might be supported by other computational tools like quantitative structure activity relationships (QSARs) already in Tier 0. However, the group felt that the QSARs are not far enough developed to predict DNT for decision-making, yet might contribute valuable data to the over-all procedure. In Tier 1 DNT IVB testing will lead to an in vitro PoD by estimating benchmark concentrations (BMCs). Due to the complementary nature of the IVB assays they themselves will not be performed in a tiered fashion but rather all 17 assays should be run in parallel leading to 17 individual PoDs according to the respective prediction models (OECD, 2023). In Tier 1 also quantitative in vitro to in vivo extrapolation (qIVIVE) should be performed. qIVIVE will enable an understanding of the intracellular instead of the nominal PoD concentration and relate it to the estimated in vivo exposure data. The lowest PoD across the IVB will then determine the MoE. In addition to the BMC, the uncertainty of the PoD is assessed by calculating the 95% CI around the BMC (Keßel et al., 2023). Depending on the MoE and the related uncertainty (both acceptable), the tiered approach might be exited at Tier 1. This means that the confidence in the DNT IVB at this point is high enough to take a decision. This is the case because the uncertainties concerning the biological coverage (applicability domain) of the DNT IVB are very well described. Identified uncertainties concern nicotinic acetylcholine receptor modulation, interference with myelination beyond oligodendrocyte development, meddling with microglia

and endocrine modes of action (MoAs) not covered by the DNT IVB. These uncertainties can be described and potentially reduced with the help of the scientific literature. If the MoE is unacceptable, mechanistic understanding of the compound needs improvement in Tier 2. This can be achieved by different means including testing in mechanism-based high throughput assays (Jeong et al., 2022), by cell painting (Cimini et al., 2023; Nyffeler et al., 2023) or by performing (high throughput) transcriptomics (Harrill et al., 2024). The cell-based data from the DNT IVB in combination with the e.g. omics data will inform on the MoA and on the question if there is already an adverse outcome pathway (AOP) available as was exemplified earlier for different compound classes (Klose et al., 2021, 2022, 2023). In case there is no AOP available for this MoA, it needs to be built. Also, in Tier 2 understanding of species-specificity needs to be established. The species-specificity might influence the higher complexity hit confirmation in Tier 3 as a MoA not applicable to other species than humans cannot be tested in any non-human system. Species conformity can be tested with e.g. rat test systems corresponding in endpoint evaluation and developmental timing to human test systems of the DNT IVB as was exemplified earlier (Baumann et al., 2016; Masjosthusmann et al., 2019; Klose et al., 2021; Saavedra et al., 2021). In case the most sensitive endpoint in the DNT IVB was established with a rat test system, it will be advisable here to inquire if the observed effect is applicable to humans by studying the observed effect in respective human test systems. Tier 3 is thought to be optional, yet preferred, for hit confirmation in more complex model systems. There are several options for such a hit confirmation ranging from more complex human in vitro models like BrainSpheres or brain organoids to zebrafish embryos or targeted/behavioural rat in vivo evaluations. Here, each model system has its own strengths and limitations (see Table 1). For the more complex human induced pluripotent stem cell (hiPSC)-derived models, like BrainSpheres (Pamies, Barreras, et al., 2017; Hartmann et al., 2023) or brain organoids, they confer the correct species of protection, human beings. While the brain organoids are more variable due to their anatomical features, they are now established tools for disease modelling of a large variety of human diseases (Eichmüller and Knoblich, 2022). With a lower degree of variability, also BrainSpheres can be used to model complex human diseases phenotypes, which lack human-representative animal models (Modafferi et al., 2021; Kapr et al., 2024). Endpoints evaluated with the BrainSpheres in Kapr et al. (Kapr et al., 2024) refer to the DNT IVB testing endpoints. For adding immunological competence to the BrainSpheres, human induced pluripotent stem cell-derived microglia cells are currently being added to the spheres within the EFSA Brain Health, the Horizon Europe CHIASMA and SCAHT (Swiss Centre for Applied Human Toxicology) projects. For using these complex human models in Tier 3 of the DNT testing, quality control of hiPSC has to be followed (Pamies et al., 2017; Tigges et al., 2021), for the respective endpoints biological plausibility and biological applicability needs to be shown, and an understanding of developmental timing will help data interpretation. In case these points are already tackled, complex human models might already be used for hit confirmation of the DNT IVB.

Table 1: Features of models for DNT Tier 3 testing

	BrainSpheres	Brain Organoids	Zebrafish Embryo	Rat in vivo
Human	+	+	-	-
Complexity	++	+++	+++	++++
Variability	+	+++	+	++
Human Disease Modelling	+	+	(+)	(+)
Immunocompetence	(+)	(+)	+	+
Behavior	-	-	+	+

The use of the zebrafish embryo for DNT evaluation has also been on the rise (Tal et al., 2024) as it might be used as a screening tool and an alternative to mammalian animal testing. Despite its definition as a

non-animal model in the European Union, it is undoubtful that the zebrafish indeed is a vertebrate animal and hence viewed in an ethically critical way by some stakeholders. Its advantages are clearly its complexity and the ability to perform distinguished behavioural tasks even with a zebrafish embryo. However, species differences in behaviours between humans and zebrafish have not been intensively studied. For gaining confidence in the zebrafish embryo model as a Tier 3 testing strategy, understanding chemicals` MoA and mapping to AOPs will be crucial. Also, more understanding on the zebrafish embryo compound kinetics, its reproducibility and predictive value needs to be gained. This is especially crucial as it is a different, aquatic species that, similar to the rodent, does not necessarily reflect full human physiology. Interpretation of a positive hit in the in vitro battery that is not supported by the zebrafish would right now be very difficult. Clearly more data and mechanistic understanding on such conflicting cases is needed. Zebrafish neurospheres (established within PARC by the German Federal Institute for Risk Assessment (BfR)) as an in vitro model for matching the human neurosphere model of the DNT IVB might be helpful in the future for facilitating the understanding of species-specific aspects.

Alternatively, targeted or if needed DNT TG in vivo or behavioural tests using the rat might be performed as a Tier 3 confirmatory testing. Here, the species-overarching experiments of Tier 2 will be leading the way as targeted in vivo rat testing would be based on the MoA identified with the IVB. As the full OECD DNT TG 426 (OECD, 2007) has a vast number of uncertainties including high variability (Paparella et al., 2020), targeted testing should be more focused and hence less prone to variable results. However, even if in vitro MoA between human and rodent show similar results, it is uncertain if the two species display the same similarities on the organism level.

With the mechanistic understanding in place, PoDs will also be derived from testing the chemicals in the more complex models. A comparison of PoD between Tier 1 and Tier 3 testing has to be done and a final decision taken by expert knowledge.

Thoughts on integration of high throughput transcriptomics into the DNT testing approach

Another option might be the use of high throughput transcriptomics in Tier 0 using the DNT IVB test systems leaning on a concept previously developed by Harrill and co-workers (Harrill et al., 2019). As there are redundant test systems for the 17 assays of the DNT IVB, it would boil down to 8 test systems needed for the transcriptomics analyses. In case there are transcriptome changes, one could proceed with the functional DNT IVB assays that indicated effects by transcriptional alterations. If this additional Tier 0 test based on transcriptomics might be time and cost saving can be calculated and adopted or not. However, in case there are no changes on the transcriptional levels, functional DNT IVB testing could be waived. Currently, there are uncertainties in using transcriptomics data in the risk assessment process. These include linking gene changes to adverse/homeostatic effects, link to AOPs, definition of definite thresholds, understanding time-related responses, dealing with transcriptome platform-specific effects, batch effects, data interpretation, validation of transcriptomic approaches just to name a few. There are ongoing efforts exploring how high-throughput transcriptomics can be incorporated into chemical prioritisation, mode-of-action analysis, and point-of-departure estimation by the US-EPA (Reardon et al., 2023).

Conclusion

The DNT IVB is, at its current status, ready to be used in the regulatory arena (i.e. screening and prioritisation, as part of a weight of evidence approach in single chemical hazard assessments). As research will proceed, a tiered testing approach is one possibility of the DNT IVB use in the chemicals framework of the EU in the future. Current uncertainties concerning most of the tiers might be tackled by research in the future.

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High-throughput Phenotypic Profiling with Cell Painting as a potential first-tier DNT Screen

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Imaging-based high-throughput phenotypic profiling (HTPP) with the Cell Painting assay is an efficient and cost-effective approach for characterising the bioactivity of chemicals. HTPP is compatible with a multitude of human-derived in vitro cell models, including those relevant for assessing developmental neurotoxicity (DNT) hazard. The HTPP approach uses a combination of fluoroprobes that label organelles and sub-cellular structures that are present in most eukaryotic cell types, including the nucleus, nucleolus, endoplasmic reticulum, Golgi apparatus, plasma membrane, actin cytoskeleton and mitochondria. The HTPP approach has been applied to hNP1 neuroprogenitor cells, which are a component of the OECD DNT in vitro battery (DNT-IVB). A collection of 282 chemicals, including many that have been screened in the DNT-IVB, have been screened in eight-point dilution series in the hNP1 HTPP assay. Results from the hNP1 HTPP assay will be compared to those obtained from a high-throughput multiplexed version of the hNP1 apoptosis and proliferation assay as well as other assays from the OECD DNT in vitro battery to determine if the HTPP assay has similar sensitivity and to assess its potential as a first-tier test for DNT hazard.

Introduction

As a Potential First-Tier DNT Screen. Outside of the context of in vitro developmental neurotoxicity (DNT) screening, high-throughput phenotypic profiling (HTPP) with the Cell Painting assay has been proposed as a chemical bioactivity screening assay in the first tier of a New Approach Methods (NAMs)-based tiered hazard evaluation approach (Thomas et al., 2019). Cell Painting is a high-content imaging assay where a variety of organelles (nucleus, nucleolus, endoplasmic reticulum, Golgi apparatus, actin cytoskeleton, plasma membrane, mitochondria) are labelled with a combination of fluorescent probes (Bray et al., 2016). Cells are rapidly imaged and a large number (many hundreds) of phenotypic features are measured that quantitatively describe the morphology of individual cells. Numerical feature data can then be aggregated to the well level and modelled for a variety of purposes including benchmark dose modelling for identifying phenotype altering concentrations (PACs) as in in vitro points-of-departure and comparison of phenotypic profiles for chemical grouping and mechanistic inference where suitable reference chemicals for a molecular mechanism of action are available (Nyffeler et al., 2023). The Cell Painting assay has been applied to numerous human-derived cell types (Gustafsdottir et al., 2013; Warchal et al., 2020; Willis et al., 2020) and used to evaluate the effects of industrial chemicals, pharmaceuticals, nanoparticles and genetic manipulations (i.e. knockdown or over expression) on cell morphology (Alijagic et al., 2023; Haghighi et al., 2022; Nyffeler et al., 2023). Notably, Cell Painting has been successfully applied to a human neuroprogenitor cell model, hNP1 cells (Culbreth et al., 2021). These cells are currently used as part of

the DNT in vitro testing battery (DNT-IVB) described (Sachana et al., 2019) and the OECD initial recommendations for evaluation of data from the developmental neurotoxicity (DNT) in vitro testing battery (OECD 2023).

Within the DNT-IVB, hNP1 cells are used to evaluate chemical effects on neuroprogenitor cell proliferation and apoptosis using two separate 96-well format assays. Both proliferation and apoptosis are critical processes of nervous system development and when modelled in vitro these processes are accompanied by marked changes in cellular morphology as they occur. One could hypothesize that the Cell Painting assay conducted in hNP1 cells could detect chemicals that affect either proliferation or apoptosis (or both) and also detect chemicals with biological activity that is not associated with these processes but could be indicative of DNT hazard. In addition, it is expected that the efficiency of DNT screening in neuroprogenitor cells would be increased with Cell Painting due to the 384-well assay format and ability to detect different types of biological activity in a single assay.

Results

Preliminary data from a screen of 282 DNT-relevant chemicals in hNP1 neuroprogenitor cells using the Cell Painting assay included many chemicals previously tested in the 96-well versions of the apoptosis and proliferation assays. The chemicals were screened in 8-point concentration series across four biological replicates (i.e. independent cultures) with one technical replicate well per treatment per replicate. As part of the Cell Painting data analysis procedure, treatments that produced a >= 50% decrease in relative cell count compared to vehicle control were considered cytotoxic and were removed from concentration-response modelling of the Cell Painting data. In addition, an imaging-based cell viability assay based on propidium-iodide (PI) dye exclusion was run in parallel with Cell Painting. A lowest observable effect concentration (LOEC) was determined based on the percentage of PI-positive cells and treatments above the LOEC were also excluded from concentration-response modelling of the Cell Painting data. Chemicals that had less than 5 non-cytotoxic test concentrations were flagged as overtly cytotoxic and a PAC from the Cell Painting assay was not determined. There were 18 chemicals in the set flagged as overtly cytotoxic including several cytoskeletal toxicants (i.e. colchicine, paclitaxel) and organotin compounds. Of the remaining 264 chemicals, 94 (35.6%) were active only in the Cell Painting assay, 71 (26.9%) were active in both Cell Painting and the cell viability assay and 98 (37.1%) were active in neither the Cell Painting or cell viability assay. Of the 71 chemicals active in both the Cell Painting and cell viability assays, the PAC from Cell Painting was always more potent than the benchmark concentration determined from the cell viability assay. This demonstrated that in hNP1 cells the Cell Painting assay was more sensitive than the cell viability assay. This is consistent with observations in other human-derived cell types where this assay has been applied (Nyffeler et al., 2023).

The results from the Cell Painting assay were compared to results from a novel 384-well format multiplexed proliferation and apoptosis assay conducted in hNP1 cells using the same set of 282 test chemicals. The performance of this 384-well multiplexed assay in terms of identifying active and inactive chemicals for effects on proliferation and apoptosis compared favourably with the original 96-well assays from the DNT-IVB. The Cell Painting assay was also predictive of activity in the proliferation and apoptosis assay endpoints from the 384-well multiplexed assay. Of note, a subset of chemicals was identified as active in the Cell Painting assay and inactive in both the proliferation and apoptosis assay endpoints. Identification of this group of chemicals supported the hypothesis that Cell Painting can detect bioactivity in hNP1 cells that is not associated with effects on either proliferation or apoptosis. For most chemicals, the PAC from the Cell Painting assay was within 1 order of magnitude of the AC50 of the most sensitive endpoint from the 384-well multiplexed assay, with the Cell Painting assay being slightly more sensitive in terms of in vitro potency estimates. There were seven chemicals where the PAC was > 1 order of magnitude more sensitive than the multiplexed proliferation and apoptosis assay, but only 1 chemical where the opposite was true.

Conclusions

Overall conclusions were: 1) Cell Painting can be applied to hNP1 neuroprogenitor cells for chemical bioactivity screening, 2) chemical effect on cell morphology can be detected with Cell Painting at concentrations below the onset of cytotoxicity, 3) bioactivity in proliferation and apoptosis assays conducted in hNP1 cells are predictive of bioactivity in the Cell Painting assay conducted in that same cell type, 4) in hNP1 cells, chemicals active in both the proliferation & apoptosis multiplexed assay and Cell Painting had comparable potencies, 5) a subset of chemicals was active in Cell Painting and not the proliferation & apoptosis assay, indicating that the biological effects of those chemicals may be independent of effects on these key neurodevelopmental processes and 6) that Cell Painting has the potential to increase efficient of DNT hazard screening in hNP1 cells and reveal bioactivity not previously captured using proliferation and apoptosis as an endpoint.

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3D human stem cell-derived models for developmental neurotoxicity studies

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Recent advances in stem cell technology have enabled the development of 3D brain Microphysiological Systems (neural spheroids, organoids, assembloids, and organoid-on-chip) from induced Pluripotent Stem Cells (iPSCs), providing human-relevant models to study neurodevelopmental disorders such as autism spectrum disorders. These models help explore chemical effects on the key events of neural development, such as synaptogenesis, myelination, and neuroinflammation, enhanced by integrated iPSC-derived microglia and CRISPR/Cas9 gene editing. Also, using iPSC from different donors allows us to address gene environmental interaction and sex differences in vitro. Additionally, Organoid Intelligence (OI) merges organoid-based systems with AI and brain-computer interfaces toward biocomputing and novel DNT testing paradigms focused on learning and memory.

Introduction

The human brain's intricate complexity poses significant challenges in developing accurate, human-relevant models to study its development and assess potential toxicological impacts. Challenges and limitations of traditional animal-based developmental neurotoxicity (DNT) testing methods have been extensively discussed in the recent decade, pushing the DNT field to develop human-relevant predictive alternatives – New Approach Methodologies. Recent advances in stem cell technology have piloted a new era of human brain modelling by developing three-dimensional (3D) models derived from induced pluripotent stem cells (iPSCs). These models promise to more closely recapitulate human physiology in a dish, which might lead to a better understanding of neurodevelopmental disorders by enabling a detailed study of the mechanisms underlying these conditions in human-relevant and yet complex environments. The applications for these models in DNT are also rising.

3D models can vary in complexity, cellular composition, histoarchitecture, and functionality, from spheroids to organoids, assembloids, and organoid-on-chip, which can be covered with an umbrella term – brain Microphysiological Systems (bMPS).

bMPS, which consists of different types of neurons (e.g., excitatory, inhibitory), astrocytes, myelinating oligodendrocytes, and microglia, may address all the key events of neural development, covered in the DNT in vitro test battery, which can, eventually, lead to harmonized DNT test, representing all assays in one model. This makes 3D bMPS an attractive tool to consider as a higher tier in the battery as of now and eventually might help to screen the chemicals at earlier stages of testing. To achieve this, further harmonization is needed, and more chemicals need to be tested to demonstrate the feasibility of bMPS for DNT testing. The advances and challenges of bMPS are summarised in Figure 1.

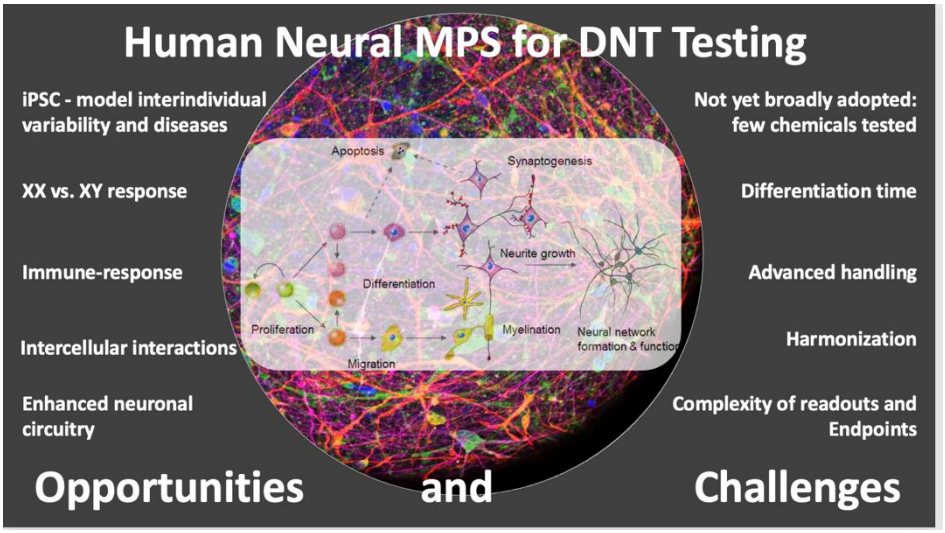

Fig. 1. Opportunities and Challenges of brain Microphysiological Systems, with the main advantage that bMPS can cover all key events of neural development outlined in the current in vitro DNT test battery. Illustration of key events is from (Aschner et al., 2016).

Integrating these 3D brain models into DNT regulatory testing frameworks could enhance neurotoxicity assessments' predictivity and human relevance. Ongoing research efforts are focused on refining these models to balance complexity with practicality, ensuring they are robust and reproducible for high-throughput testing scenarios.

Exemplified 3D bMPS applications

At the Johns Hopkins Center for Alternatives to Animal Testing, we developed such 3D bMPS ((Pamies et al., 2017) and demonstrated the applicability and usefulness in a variety of applications from disease modelling (autism, Parkinson's, SYNGAP1-RD, leukodystrophies), studying cancer and infectious diseases, chemical and drug testing, modelling gene environmental interactions, use for biomarker discovery and model more complex brain functions, such as synaptic plasticity and long-term potentiation towards development of a model of learning and memory.

Acceleration of testing with reporter lines

By engineering human induced pluripotent stem cells (iPSCs) with fluorescent reporter genes, researchers can visualise and quantify specific cell types and cellular events in real time, providing a powerful tool for high-throughput screening and mechanistic studies. Since one of the bMPS challenges is the complexity of the model and standardization, we developed several reporter lines to study the key events of neural development in live bMPS over time (Romero et al., 2023). The use of reporter lines overcomes the technical challenges of Immunohistochemistry (variable antibody affinity, issues of antibody penetration throughout the tissue, and fixation of the sample at a given time point, leading to the termination of an experiment). By targeting specific genes involved in neurodevelopmental processes, we introduced fluorescent reporter cassettes that visualise and track cell types and cellular events of interest. For example, our PLP1:GFP reporter line, which labels oligodendrocytes with a green fluorescent protein (GFP), allows us to monitor the process of oligodendrogenesis and myelination in real time (Romero et al., 2023). Similarly, our SYP:GFP reporter line, which labels synaptic vesicles with GFP, enables the assessment of synaptogenesis and synaptic function.

Long-term exposures to cover all key events from the in vitro test battery

Prolonged shelf-life of the bMPS allows using these models for longitudinal studies as well as washout, and recovery experiments. We used domoic acid, a well-known and documented acute neurotoxin, with less understood low-dose long-term DNT effects, to demonstrate how long-term exposures can be addressed in bMPS and how all key events can be covered in one model. While we did not find any domoic acid effects on viability, proliferation, and differentiation, we observed changes in oligodendrocyte morphology and excitability of neuronal networks.

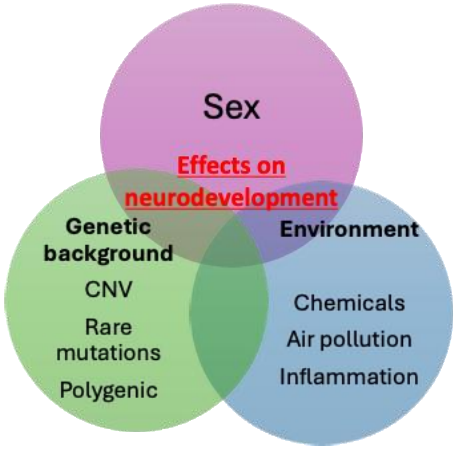

Fig. 2. Gene x Sex x Environment Interaction paradigm to model Autism with iPSC-derived bMPS

Gene Environmental Interactions

The increasing prevalence of neurodevelopmental disorders, such as autism spectrum disorder (ASD), has become a major public health concern in recent years. While genetic factors play a significant role in the aetiology of these disorders, the rapid rise in incidence suggests that environmental factors may also contribute to their development. It is now widely recognized that the interplay between genetic susceptibility and environmental exposures, known as gene-environment interactions (GxE), is a critical determinant of neurodevelopmental outcomes (Keil-Stietz and Lein, 2022).

iPSC-derived models provide a great tool to study not only the harmful effects of chemicals on brain development but also their interaction with the genome. Recently, we used CRISPR/Cas9-induced CHD8 heterozygous KO iPSC to investigate its synergy with the pesticide chlorpyrifos (CPF). CPF exposure to CHD8 heterozygous KO organoids further reduced CHD8 protein levels, disrupted neurotransmitter balance, and altered metabolic markers associated with ASD (Modafferi et al., 2021).

Currently, we are expanding the portfolio of genetic and environmental components by generating organoids from individuals with specific genetic risk factors (from male and female donors), such as copy number variations (CNVs) associated with ASD. We are part of a multi-disciplinary team investigating the complex relationships between genetics, sex, and environmental factors in the context of ASD (Figure 2).

Increase complexity by introducing immune-competence, addressing sex differences

In addition to genetic factors, sex is another important determinant of neurodevelopmental outcomes. Epidemiological studies have consistently shown that ASD and other neurodevelopmental disorders are more prevalent in males than females (Maenner et al., 2023). However, the biological mechanisms underlying these sex differences remain poorly understood. To address this gap, we are investigating the effects of sex hormones on neurodevelopment using iPSC-derived brain organoids from male and female donors.

Microglia are essential for brain health and homeostasis throughout development. As the primary innate immune cells of the central nervous system (CNS), they actively shape neurodevelopment, influencing key processes beyond immune defence. Current in vitro DNT test battery lacks neuroinflammation as an endpoint and immune-competent bMPS can overcome this limitation. We introduced microglia into the bMPS (Figure 3) to model neuroinflammation in response to environmental stress.

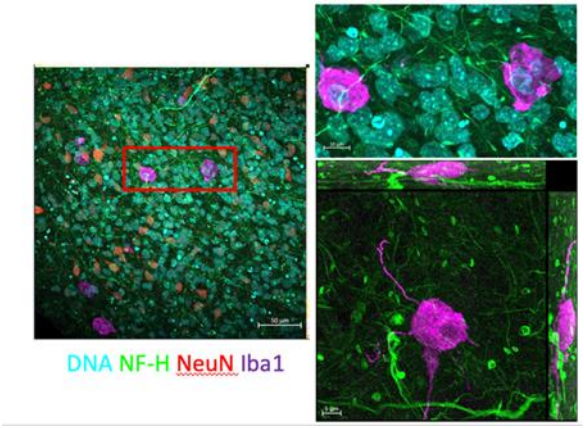

Fig. 3. Immune-competent bMPS, expressing neuronal (neurofilament, (green), NeuN (red)) and microglia-specific Iba1 (pink). Nuclei are stained with Hoechst 33342.

Addressing Cognitive Functions: Enhancing Brain Organoid Complexity for Learning and Memory Research

We recently developed a concept of Organoid Intelligence (O) and its application for DNT testing (Din et al., 2024). The integration of bMPS with synaptic plasticity, short and long-term potentiation, and depression with more complex learning models, such as reservoir computing and reinforcement learning, presents new avenues for probing network dynamics, modeling disease states, and further exploring the chemical effects on these fundamental processes of neural development (Smirnova et al., 2023). Reservoir computing, a machine learning approach designed for processing complex temporal patterns, has been applied to decode spontaneous and stimulus-driven neuronal firing patterns in brain organoids (Cai et al., 2023). Reinforcement learning algorithms, which enable systems to adapt their responses based on reward-based feedback, are now being tested on organoid-based networks, providing a framework for studying cognitive function in vitro.

GCCP

Good Cell Culture Practice (GCCP) is fundamental to ensuring the reliability, reproducibility, and scientific validity of in vitro models, particularly as iPSC-derived organoids are increasingly used for DNT testing (Pamies et al., 2020). As these models become more complex, incorporating multiple cell types and functional readouts, rigorous adherence to GCCP principles is critical to maintaining standardization across laboratories and ensuring data integrity. The scaling up of iPSC-derived organoid technologies for high-throughput screening applications demands careful characterisation, quality control, and harmonization of protocols to minimize batch-to-batch variability and enhance the predictive power of these models in regulatory toxicology.

Conclusions

In summary, the evolution of 3D brain cultures represents a significant stride toward more ethical and accurate DNT testing methodologies. By closely matching human neural development and function, these

models hold the potential to be broadly used for neurotoxicity testing and regulatory decision making. This will lead to better protection of human health from neurotoxic environmental exposures.

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The added value of non-mammalian animal models to fill the gaps in developmental neurotoxicity

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(presentation: 25min; technical Q&A: 5min)

In support of the Government of Canada's continued efforts to replace, reduce or refine the use of vertebrate animal testing in chemical risk assessment, the National Research Council (NRC) of Canada and Health Canada began collaborating in 2018 to develop a refined zebrafish embryo/larval model to potentially replace rodent models for the generation of hazard data intended for use in chemical risk assessments. The model has been developed to generate a comprehensive data set that includes morphological, behavioural, transcriptomic and toxicokinetic data. Importantly, zebrafish behavioural assays can and are being used for developmental neurotoxicity testing and international collaborative efforts to develop globally harmonised zebrafish models for regulatory use in chemical risk assessment are underway. Health Canada and NRC Canada joined the OECD DNT in vitro battery (IVB) Zebrafish Expert Group in 2019 in which 4 laboratories, including the zebrafish facility of NRC Canada, tested a common list of reference chemicals using the light dark transition test (LDTT) behavioural assay. The goal was to standardise the methodology of the assay and to assess the added value that this assay could have in the OECD DNT IVB evaluation of pesticides for developmental neurotoxicity. A recent update of the study findings was presented in April, 2024 to the OECD DNT IVB group. The results of the interlaboratory study indicate some discordance in the DNT results, which was likely due to a combination of factors, including individualised laboratory application of the LDTT through use of in-house protocols. Additionally, the results for the DNT test were subtle, which may have been due to the exposure paradigm of the protocol and some of the test compound solubility issues. This suggests that additional testing and refinements to the protocol may be needed to increase the robustness of this specific behavioural response. As well, an Integrated Approach to Testing and Assessment (IATA) case study of parathion is ongoing to demonstrate the value of the refined zebrafish model along with publicly available information (i.e., in vitro models and mammalian models) to predict human health effects. The end goal is an evaluation of the robustness of the zebrafish model for predicting human health developmental neurotoxicity. The results thus far suggest promise for use of the evolving refined zebrafish model as a potential alternative to the

rodent model for use in predicting human health effects, including developmental neurotoxicity and chemical risk assessments. Further cross validation of the zebrafish DNT model between labs will be required to optimize the model.

Introduction

In furtherance of activities within its Healthy Environments and Consumer Safety Branch, Health Canada (HC) is currently working with the National Research Council of Canada (NRC) to perform proof of concept Integrated Approaches to Testing and Assessment (IATA) of chemicals using the Zebrafish Embryo comprehensive toxicity testing platform. The platform has and is currently being developed for potential use as an integrated platform for chemical risk assessment in Canada.

The initial studies were based on the use of the OECD Test 236: Fish Embryo Toxicity (FET) test. The studies conducted at the NRC assessed the use of the FET based models (ZET- Zebrafish Embryo Toxicity) in which chemical exposure was performed from 0 to 5 days post-fertilisation (dpf). In addition, an internal model, known as the General and Behavioural Toxicity (GBT) assay, has been developed in which larvae are exposed to the compound being tested from 3 to 5 dpf (Achenbach et al., 2020). Following exposure of zebrafish larvae to test substances selected by Health Canada the models were initially used to assess toxicity by evaluating the phenotypic and behavioural effects of chemical exposure. In addition, further model development has led to the evaluation of compound absorption, metabolism and elimination (AME) by the zebrafish larvae, along with transcriptomic analysis methods that were developed in order to evaluate the sub-phenotypic activation of toxicity related pathways linked to endocrine disruption.

Cross-Validation Studies

One of the challenges that lie with moving the Zebrafish toxicity models towards standardised testing for risk assessment are the variation in methodological approaches between labs. The majority of exposure and analysis paradigms that have been developed and performed in a vast array of research labs, often differ with respect to their exposure timelines and data analysis. An example lies in the difference between the standardised OECD Test 236, where larvae are exposed to the compound being tested from 0 to 4 dpf, and the currently described ZET assay where the larvae are exposed from 0 to 5 dpf. These differences are important due to further body development by 5 dpf, along with increases in both the baseline larval behaviour and their stimulus responses at 5 dpf. Another example includes differences between static exposure to the compound being tested and daily renewal of the bath solution. Given the differences in the chemical stability and uptake kinetics/metabolism between compounds, this can lead to a significant difference in the toxic profiles generated. A recent study led by the National Institute of Environmental Health Sciences (NIEHS) in the United States, performed a comparison of different chemical exposure approaches between 3 labs and found that, while ~79% of the chemicals that were tested showed the same toxicity profiles between the labs, the remaining 21% did show different potencies between the labs (Hamm et al., 2024).

These differences, along with a number of others, make it somewhat difficult to directly compare and interpret the findings from the different assays. In order to evaluate if a standardised approach for zebrafish toxicological testing could produce the same results between labs, 4 groups performed a cross-validation analysis of the effects of chemical exposure to known neurotoxicants on the standard behaviour of zebrafish larvae following exposure from 0 to 5 dpf. The research labs, that included, an academic (Oregon State University), government (NRC) and two commercial (ZeClincs & Biobide) laboratories, refined their individual testing platforms in order to cross validate a matched protocol. The initial cross validation was designed to evaluate the effect of known neurotoxic chemicals on larval behaviour at concentrations below

the levels that induce visual phenotypes. As the assessment occurred below the visual phenotypic effects, the behavioural assessment was done at a concentration in which the potential neurotoxic effect did not overlap with other phenotypic effects of the compounds being tested. This potentially makes this assay a sensitive readout of the neurotoxic effects of compounds. The findings of the study, following the standardisation of the protocol, showed that 27 of the 28 compounds showed effects in the behavioural light/dark transition assay in at least one lab. For all four labs 13 compounds were found to be active in 4 labs; 3 were active in 3 out of 4 labs and 6 were active in 2 out of the 4 labs. While the majority of the compounds showed a notable effect on behaviour across the labs, there was a notable number of compounds that showed a smaller effect on behaviour in a limited number of labs. This may indicate a level of variability between labs or these differences may be found for compounds in which the concentrations in which there are visible phenotypic effects on the larvae overlap with the neurotoxic effects that would alter the behavioural patterns of larvae. These findings will require further testing to resolve the cause of the differences between the labs.

Further Development of the Zebrafish Platform

The toxicity testing studies conducted at the NRC that began in 2018 have demonstrated the strength of the zebrafish larval toxicity testing models for evaluating the visual and behavioural phenotypic and toxicological effects of known toxicants. In addition, studies have been developed to assess the absorption, metabolism and excretion (AME) of the compounds tested with the zebrafish larval models. Importantly, the evaluation of the AME effects/activity of known and potential toxicants is often overlooked. As such, it has recently been identified in international meetings as a data gap in the use of both cell lines and zebrafish as NAM models. We have found that the larval zebrafish can absorb and metabolise the toxicants tested in a similar way to that of mammals (Achenbach et al., 2022).

Importantly, the NRC has also developed testing models that assess the effects of compound exposure on the transcriptome of the larvae. The transcriptomic work is designed to predict endocrine disruption, general toxicity and for the comparison of the transcriptomic and phenotypic dose-response points of departure for concordance. The transcriptomic models were developed through the development of a novel bioinformatics model that will provide a high-throughput, automated analysis model that can be used to further assess the omics pathways involved in the response to toxicant exposure. This has made significant progress in the development of bioinformatics approaches (Morash et al., 2023; Morash et al., 2024). Transcriptomic Points of Departure (PODs) have been found to be significantly lower than the EC20 values. Chemical exposures resulted in a number of Differentially Expressed Genes (DEGs) and significantly populated GO/KEGG/REAC-term pathways.

The larval models thus serve as high-throughput testing platforms that can produce more data than can be obtained from cell line testing alone as they provide a means for evaluating systemic toxicity, an extremely informative and robust approach for toxicity testing. Importantly, the testing platform that has been developed is now past the pilot level developmental stage and is considered to be at a level where it has the potential for use as a new approach method (NAM) in the regulatory context in Canada. In order for HC to proceed with the development of the zebrafish platform as a model for use in regulatory applications, further testing of additional chemicals along with a cross-validation of the results with internationally recognised labs is required. Currently, cross-validation of toxicological testing for substances are underway with Oregon State University with a focus on comparison of approaches for toxicological testing and transcriptomic evaluation. Plans are in place to continue and expand the validation of the model with the US EPA and NIEHS. Additionally, compounds that were tested in an inter-laboratory validation of the zebrafish as a developmental model under the NIEHS Systematic Evaluation of the Application of the Zebrafish in Toxicology (SEAZIT) initiative will be tested for the purpose of cross-validation of the platform currently under the validation phase of testing (Hamm et al., 2024).

Validation Phase of Zebrafish Models

Health Canada is conducting evaluation for 'proof of concept' of the Zebrafish Embryo Toxicity models. IATA Case studies are underway to predict risk to human health and the environment so as to identity gaps in biology not aligned with human biology, areas of uncertainty to predict risk and domains of applicability. Health Canada will continue to test proof of concept of the ZET platform using IATA case studies on compounds used in the development phase of the ZET in which the ZET was refined with additional testing platforms for toxicokinetics, behaviour and gene expression.

As areas of uncertainty are identified and addressed with each case study, the validation process is expected to move the refined ZET towards regulatory application in Health Canada for the risk assessment of new substances.

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Developing new assays to predict glia-related Key Neurodevelopmental Processes (KNDPs) with transcriptomics data support

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Epidemiological data indicate that toxicant exposures contribute substantially to neurobehavioral deficits and diseases, with an estimated cost of >€150 billion /year in Europe, emphasising the advantages of developing New Approach Methodologies (NAMs) for testing and identifying toxic hazards, getting a mechanistic understanding to explore causal relationships and controlling toxicant exposure. New knowledge of risk factors and their underlying mechanisms of action is likely to be vital to minimise new cases of neurodevelopmental disorders and cognitive deficits. The European Parliament as well as international regulatory bodies are focusing efforts on developing NAMs that will decrease time and cost in chemical hazard assessment without the use of animals. International efforts have led to the development of the developmental neurotoxicity (DNT) In Vitro Battery (DNT IVB), and of an OECD recommendations document to interpret the data for use in regulatory decisions. However, the substantial involvement of glial cells (i.e. astrocytes, oligodendrocytes and microglia) in key neurodevelopmental processes (KNDPs) such as migration, synaptogenesis, myelination and neural network formation is not currently reflected in the DNT IVB and point to clear biological uncertainties. Additionally, the roles glial cells play in neurodevelopment are far from being fully recognised in Adverse Outcome Pathways (AOPs), since they are mostly confined to the KE “neuroinflammation", e.g. AOP13, AOP17 (https://aopwiki.org/aops). In the BRAIN HEALTH project, we aim to expand the current DNT IVB by reducing existing uncertainties on the role of glial cells for DNT, by refining and developing NAMs addressing glial cell development and function. This presentation will provide a short introduction into DNT, give some examples on the importance of glia cells for brain development and expand on the need for incorporating glia cell presence and function into the current DNT IVB. Moreover, it will be laid out how the BRAIN HEALTH project tackles this issue from different angles by

utilising functional assays using High Content Imaging, single cell and bulk transcriptomics up to refining and developing novel AOPs.

Developing new assays to predict glia cell-related key neurodevelopmental processes (KNDPs) with transcriptomics data support

Epidemiological data indicate that toxicant exposures contribute substantially to neurobehavioral deficits and diseases, with an estimated cost of >€150 billion /year in Europe (Bellanger et al., 2015), emphasising the advantages of developing New Approach Methodologies (NAMs) for testing and identifying toxic hazards, getting a mechanistic understanding to explore causal relationships and controlling toxicant exposures. New knowledge of risk factors and their underlying mechanisms of action is vital to minimise new cases of neurodevelopmental disorders and cognitive deficits. The European Parliament as well as international regulatory bodies, are focusing efforts on developing NAMs that will decrease time and cost in chemical hazard assessment, eventually leading to discontinued animal use. International efforts have led to the establishment of the developmental neurotoxicity (DNT) In Vitro Battery (DNT IVB), and the publication of initial recommendations by the OECD to interpret the DNT IVB data for use in regulatory decisions. However, the involvement of glial cells (i.e. astrocytes, oligodendrocytes and microglia) in key neurodevelopmental processes (KNDPs) such as migration, synaptogenesis, myelination and neural network formation is not partly reflected in the DNT IVB and points to potential biological uncertainties. Additionally, the roles glial cells play in neurodevelopment are far from being fully recognised in Adverse Outcome Pathways (AOPs). Brain Health aims to expand the current DNT IVB by closing existing biological uncertainties on the role of glial cells in DNT, by refining and developing NAMs addressing glial cell development and function. The NAMs that will be developed will be based on Key Events (KE) as identified in the AOP framework. Testing of EFSA relevant toxicants and model compounds targeting glia cell function using single cell and bulk transcriptomics in addition to functional analyses associated with KEs will be performed to support Next Generation Risk Assessment (NGRA).

Chemical exposure during brain development

The brain is susceptible to chemical exposure both during development and after it has fully matured. The embryonic and foetal phases of development differ fundamentally, with the embryonic phase encompassing organogenesis, while the foetal phase involves further growth and maturation. Developmental disruptions in either phase can have long-lasting consequences that extend well beyond the period of chemical exposure, potentially leading to DNT. Such effects have been linked to neurodevelopmental disorders, including attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders, later in life. In a recent study, over 350,000 chemicals and mixtures of chemicals were identified as registered for production and potentially in use (Wang et al., 2020). Despite the potentially harmful impacts of chemicals on the developing brain (Grandjean and Landrigan, 2006), only around 230 substances have been tested for DNT using OECD Test Guideline (TG) studies. (OECD, 2008; Makris et al., 2009; Crofton and Mundy, 2021; Tal et al., 2024; Crofton and Mundy, 2024). A recent EFSA review effort compiled for the first time the current extensive list of chemicals with guideline-based or DNT-similar studies based on government sources and resulted in a list of 253 DNT studies conducted with 229 chemicals (Crofton and Mundy, 2024) pointing to a clear current regulatory testing gap.

Roles of glia cells in brain development

It is now well known that glia cells (e.g., astrocytes, microglia, and oligodendrocytes) contribute to and support neurodevelopment in diverse ways. For example, astrocytes contribute not only to synaptic transmission, learning, and memory (reviewed in Di Castro and Volterra, 2022) but also play critical roles

in regulating extracellular ion homeostasis, neurotransmitter uptake, metabolic support, and blood-brain barrier formation. During development, astrocytes help guide neuronal migration and axon pathfinding, and they release signalling molecules that influence synapse formation and maturation. Astrocytes also participate in neuroinflammatory responses, often working in coordination with microglia.

In addition, microglia, which are the primary effectors of neuroinflammation, have roles that extend far beyond immune surveillance. During neurodevelopment, microglia are involved in key processes such as the regulation of neural progenitor cell proliferation and differentiation, support developmental neuronal apoptosis, modulate synaptogenesis, and are involved in activity-dependent synaptic pruning, thereby shaping neural circuits (Paolicelli et al., 2022). Additionally, microglia contribute to the regulation of myelination by influencing oligodendrocyte precursor cell (OPC) differentiation and clearance of myelin debris.

Oligodendrocytes, on the other hand, form the myelin sheath around axons, facilitating rapid signal conduction, and also provide metabolic support to neurons. Beyond their classical role, oligodendrocytes have been increasingly recognised for their immunomodulatory properties and potential interactions with microglia and astrocytes during both development and injury.

Given the essential and multifaceted roles of glial cells, particularly astrocytes and microglia in neurodevelopment, chemical insults that disrupt glial cell function can significantly impair neuronal maturation and circuit formation. As such, glial cells represent a critical target in the context of DNT.

Regulatory DNT testing and NAMs

To protect children's brains, regulatory agencies need fast, affordable, versatile, ethical constraint-free NAMs that can accurately evaluate substance toxicity in line with the Chemical Strategy Sustainability goals of the European Commission (EC, 2020), the 3Rs-Principle (EUSAAT - European Society for Alternatives to Animal Testing, accessed January 2025) and the US EPA to close biological uncertainties related to DNT potential of untested compounds. The NAMs should allow both hazard identification as well as hazard characterisation and be able to contribute to NGRA. Despite the complexity of the developing brain, KNDPs have been identified that determine healthy brain development. For testing if a substance exerts adverse effects on the developing brain without using whole animals, these KNDPs are mimicked by assays in relevant test systems in vitro (Figure 1).

There are biological uncertainties in coverage of KNDPs that have been acknowledged (Crofton and Mundy, 2021). To clarify the regulatory relevance of these biological uncertainties in identifying human developmental neurotoxicants two approaches are ongoing under the Brain Health project (1) development of robust AOPs containing glial-related KEs and (2) development of NAMs measuring glial-related KEs. Some of these uncertainties are currently addressed by setting up test systems or test methods for e.g. radial glia function, astrocyte development, myelination, and microglia by the Brain Health collaborators. Here, 2D and 3D (BrainSpheres) mixed neuronal/glia test systems are in focus. Currently, the differentiated 2D mixed cultures consist of neuronal subpopulations and astrocytes (Lislien et al., 2025), while the 3D BrainSpheres include a more complex architecture of diverse populations of excitatory and inhibitory neurons (glutamatergic, GABAergic, dopaminergic, cholinergic and serotonergic) alongside with astrocytes and oligodendrocytes (Pamies et al., 2017; Chesnut et al., 2021). Additionally, optional protocols allow for the inclusion of microglia. To address biological uncertainties in DNT IVB, Brain Health will develop and implement test methods targeting the following endpoints, each justified by specific scientific rationale (Figure 1):

A. Neural Progenitor Cell (NPC, including radial glia) proliferation of different developmental stages

B. Astrocyte development and function

C. Synaptogenesis (astroglia and microglia contribution, species)

D. Central nervous system myelination (astroglia and microglia contribution)

E. Neural Network Formation (astroglia and microglia contribution, species)

F. Microglia presence (microglia addition to existing test systems for synaptogenesis, neural network formation, myelination).

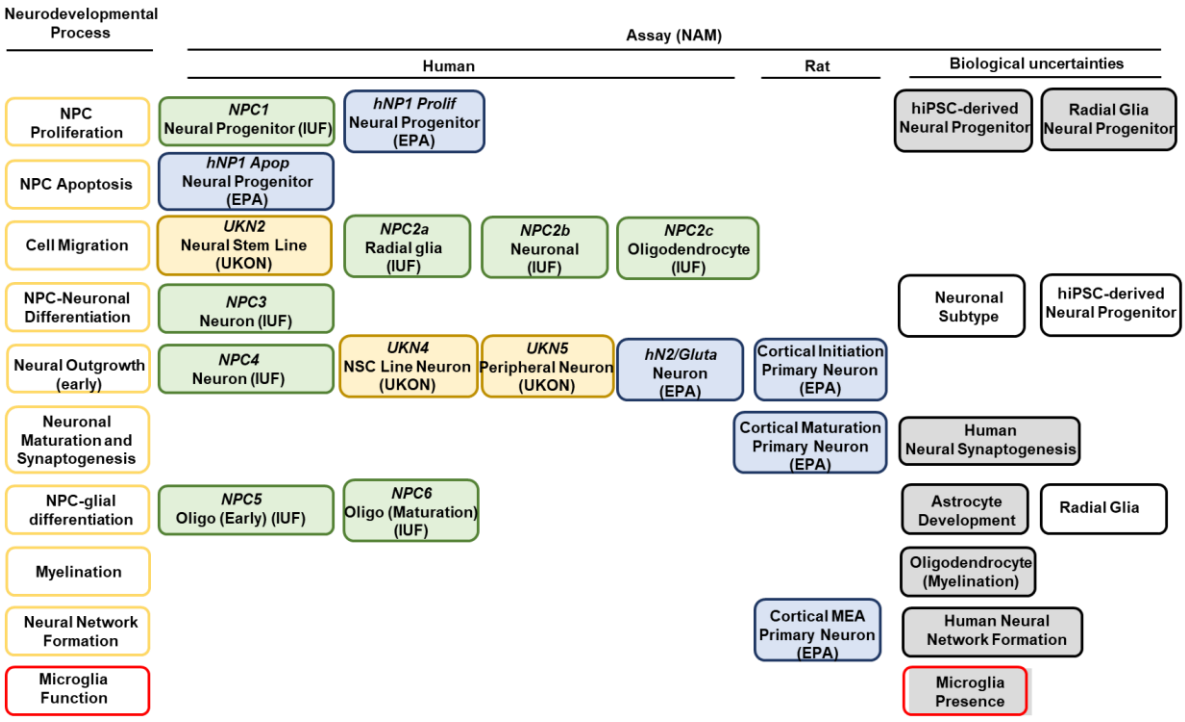

Fig. 1: Assays in the current DNT IVB and identified biological uncertainties (adapted and modified from Masjosthusmann et al., 2020). Assays are grouped according to the neurodevelopmental process evaluated (rows) and test system used (columns). Assays which need further development, and not included in the current DNT IVB, are identified as biological uncertainties. Microglia are not part of the current DNT IVB (lower part, red squares) and will be covered in the Brain Health project. Each assay is represented as a box which lists the test method name (italics), the test system (cell type used), and the home institution of the developer (green boxes, IUF =Leibniz Research Institute for Environmental medicine.; yellow boxes, UKON =University of Konstanz; blue boxes, EPA= US Environmental Protection Agency). Grey coloured squares represent DNT IVB assays Brain Health will further develop.

Transcriptomics

Cell's function and identity are precisely controlled by transcriptional processes. By combining the readouts (i.e. functional and structural endpoints) of in vitro test methods with transcriptomic analyses, it is possible to derive specific gene signatures that can support putative KEs leading to an Adverse Outcome (Glaab et al., 2021). Transcriptomics have the potential to support the identification of novel KEs (including molecular initiating events, MIE), when combined with phenotypic data from functional assays specific for glial cell function. The Brain Health project will perform single cell transcriptomics to thoroughly characterise 2D and 3D cultures undergoing differentiation to study glia development and function, including their interactions with neurons. Bulk transcriptomic analysis and cell deconvolution methods will deliver signatures associated with perturbed pathways due to exposure to relevant toxicants. Additionally, a test

battery of model compounds specifically targeting glia cells and their role in synaptogenesis, myelination and NNF will be tested in the NAMs. This will further guide the development of putative AOPs and an AOP network incorporating glia-specific MIEs and KEs.

Adverse Outcome Pathways

The current number of DNT AOPs is very limited, and even more limited when considering only OECD endorsed AOPs in the AOP Wiki. Glial cells are not sufficiently taken into account in DNT, considering their importance in neurodevelopment. Endorsed AOPs and AOPs under development can be refined by integrating KEs and KE relationships that take glial cell development and functions into account, whether directly driving neurotoxicity or only being modulatory. For example, astrocytes are critical for glutamate re-uptake, therefore, their dysfunction might, at least partially, explain the KE “glutamate dyshomeostasis” in AOP17. Some AOPs currently under development have begun to consider oligodendrocytes; however, greater emphasis should be placed on characterising KEs related to all glial cell types. Moreover, proposed KEs involving glial cell function can be substantiated by incorporating novel NAMs within the DNT IVB framework. Therefore, developing and refining DNT methods that support these proposed KEs is essential to the refinement of AOP networks for DNT. Dysfunction of glial cells contributes to the pathogenesis of neurodevelopmental disorders (Zhou et al., 2024), however KEs linked to their impaired development and diseases are mostly lacking in the AOP-Wiki (Jaylet et al., 2024). Addressing these biological uncertainties will enhance scientific confidence among regulatory agencies, facilitating the application of NAMs for DNT in a regulatory context.

Conclusion

Glial cells are essential for brain development, yet their role in DNT remains unclear due to the lack of glia-related DNT mechanisms of action from OECD TG regulatory testing and their underrepresentation in in vitro NAMs. Chemical disruptions affecting glial cells could significantly impact neurodevelopment, highlighting the need to integrate glial-related KEs into DNT assessments. Expanding the NAM portfolio to include glial cell functions, unravelling chemical mechanisms using transcriptomics, and incorporating findings into AOPs could refine the current NAM DNT testing strategy. Addressing these uncertainties will enhance regulatory confidence in NAMs and improve chemical safety evaluations for neurodevelopmental health.

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Microglia for Studying Developmental Neurotoxicity

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New approach methodologies are being evaluated for their utility to inform developmental neurotoxicity (DNT) hazard instead of using animals. Although a broad battery of in vitro assays representing key processes of neurodevelopment has been developed, the biological coverage of glial cell components, specifically microglia, remains sparse. Microglia play several critical roles in brain development, such as regulating neural growth and synaptic pruning, modulating inflammatory responses, and building a functional blood-brain-barrier (BBB). Disruption of microglia in development has been linked to several adverse outcomes, such as BBB leakage or long-lasting behavioural impairments in rodent models. Moreover, aberrant microglia have been implicated in various neurodevelopmental and neurodegenerative diseases. This presentation will explore the potential for microglia to serve as a key 'sensor' for predicting chemical effects on neurodevelopment and discuss current efforts to integrate microglia into the in vitro battery to address biological uncertainty.

Background

There is growing evidence that the immune system plays a critical role in normal brain development and function, such as blood-brain-barrier formation, neurogenesis, cell migration, synaptic formation and remodelling, and myelination. Moreover, perturbation of resident immune cells in the developing brain, such as microglia, have been found to disrupt key neurodevelopmental processes and to be linked to neurodevelopmental disorders outcomes, emphasising the importance of immune cell function as a critical biological process in neurodevelopment. The goal of this presentation was to review current NAMs to study microglia in the developing brain thereby filling a critical gap in developmental neurotoxicity assessment.

Many studies have examined the role of microglia in neurodevelopment; however, there are a limited number of studies that have examined how microglia may influence responses to environmental chemicals during development. Indeed, immune cells in the brain, such as microglia, have been found to activate in response to known developmental neurotoxicants. For example, Gassowska-Dobrowolska et al. (2023) found that prenatal and neonatal exposure to lead resulted in significant increases in pre-inflammatory cytokines in offspring and pathological changes in microglial cells in a rat model. Moreover, microglia have been found to be sensitive to maternal exposure to endocrine-disrupting chemicals in development (Bruce-Keller et al., 2000; Vegeto et al., 2001), resulting in sex-specific effects on microglia colonisation of the brain (Rebuli et al., 2016) and learning and memory impairments in offspring (Huo et al., 2024). Microglial activation and microglial depletion in the developing mouse embryo have been found to impair blood brain barrier permeability, suggesting that BBB formation may be a key adverse outcome of microglial dysfunction in development (Ronaldson and David, 2020, Naphade et al., 2023).

Assaying microglia function in vitro

There is currently a lack of standardised assessment of neuroimmune function in traditional toxicology guideline testing; however, assays have been developed to measure immune response in neuro-relevant cell lines. Microglial assays using the BV-2 cell line have been developed for toxicological screening, including assays measuring: 1) phagocytosis using high-content imaging and fluorescent active cell sorting, 2) cytokine expression or inflammasome activation, and 3) microglial bioenergetics assay such as the seahorse XF cell mitochondrial stress test (Agilent technologies) (McPherson et al., 2023; Bowen et al., 2020; Childers et al., 2021). These assays have been used to assess perturbations in microglia from environmental chemical exposure. For example, bromated flame retardants were found to alter mitochondrial respiration, phagocytic function, and inflammasome activation in BV-2 cells (Bowen et al., 2020). More work is needed to understand the relevance of measuring immune response in the BV-2 cell line for assessment developmental neurotoxicity, considering the BV-2 cell may be a more relevant model for adult microglia. Human primary microglial cells, such as induced pluripotent stem cells or human-derived tissue sources, are currently being explored in immune response assays and may offer species and developmental relevance.

Developing a high-throughout microglia cytokine detection platform

Efforts are underway to develop high-throughput methods to detect microglial activation from chemical exposure for neurotoxicity assessment. For example, Menghang Xia and colleagues at NCATS/ NIEHS are developing a high-throughput cytokine detection platform using a human microglial cell line (Yang et al., in preparation). One challenge facing in vitro assay development in this field is that commonly used in vitro microglial cell lines do not recapitulate the complexity of microglial functions in vivo, such as release of TNF-alpha in response to an immunological insult like lipopolysaccharide (LPS) stimulation (Timmerman et al., 2018). This group sought to identify an appropriate in vitro microglia model to mimic in vivo conditions for screening environmental chemicals. They evaluated cytokine profiles from four microglial cells lines, human induced pluripotent stem cell-derived microglia (hiMGC), HMC3, P10354-IM, and BV2 and found that the cytokine response from the hiMG cells most closely recapitulated in vivo response profiles of cytokines interleukin 6 and TNF-alpha. Based on this finding, the same group developed a homogeneous time-resolved fluorescence (HTRF) assay platform in a 1536-well plate format for high-throughput screening. Preliminary screening of a subset of chemicals, including chemicals with evidence of in vivo DNT, indicated that the assay successfully detected changes in cytokine activity. These preliminary findings offer a promising NAM for screening and evaluating neuroinflammatory effects of environmental chemicals.

Computational modelling approaches to screen for microglia developmental toxicants

Computational models have also been explored as a promising NAM to screen for developmental neurotoxicants. Considering BBB dysregulation has been linked to neuroinflammation and neurodevelopmental defects (Haruwaka et al., 2019, Zhao et al., 2022, Bittle and Stevens, 2018), one emerging hypothesis is that microglia play a central role in neurovascular patterning. Knudsen and colleagues developed an agent-based model of blood-brain barrier morphogenesis using CompuCell 3D software (Naphade et al., 2023). This prototype computational neurovascular unit model comprises five cell types, including endothelial tip cells, endothelial stalk cells, pericytes, microglia, and neural stem cells. It was found that depletion of microglial cells in the model reduced vascular arborization and recapitulated less microvessel density in the subventricular plexus at E14.5 in a mouse model. Moreover, disruptions of targets CSF-1 or VEGF-C altered microglial activation states (resting M0 state versus activated M1 state), which may serve as a promising biomarker for BBB dysfunction (Hattori et al., 2023). Future model development is needed to enhance the translation of the model for predicting putative developmental neurotoxicants. Additionally, ongoing work is focusing on developing a reference chemical set for potential

microglia developmental toxicants using literature mining and computational approaches to integrate relevant in vitro toxicological screening data from the ToxCast database (Feshuk et al., 2024).

Conclusion

Understanding immune cell interactions to chemical stressors in neurodevelopment may inform a critical gap in current developmental neurotoxicity assessments. In addition to high-throughput in vitro assay development, complex tissue models that integrate neural and immune development should be considered in the future for toxicological screening. For example, zebrafish may be an effective model for evaluating immune responses in the developing brain, considering factors such as an intact biological system, rapid development, highly conserved immune responses, tissue transparency, and behavioural readout potential. Similarly, brain organoids may offer advantages for studying neuroimmune responses to environmental chemicals, including human relevance (organoids derived from human pluripotent stem cells), cellular organisation and complexity, and the ability to measure immune and/or neurodevelopmental processes.

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Evaluation of neurotoxicity for pesticide-related compounds in human iPS cell-derived neurons using microelectrode array: Japanese experience

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New approach methodologies (NAMs) are expected to provide information to identify potential hazard of developmental neurotoxicity (DNT) for a large number of chemicals. Multi-electrode array (MEA) system, a functional assessment of the in vitro testing battery, is widely used to measure neural network activity. To date, acute and chronic MEA assays have been used to evaluate neurotoxicity and DNT potential using rat cortical neurons. In addition, human iPSC-derived neurons can be used to detect DNT potential using MEA. However, MEA protocol and vendor differences should be investigated for regulatory considerations.

Here, we focused on neural activity using iPSC neurons with acute exposure to positive and negative DNT compounds and tried to compare our acute datasets with chronic datasets from a literature review. We found that the acute assay can detect similar chemicals when evaluating activity associated with chronic treatment. We also found that our acute assay can identify chemicals based on mode of action, while the chronic assay provides information including developmental and cytotoxic effects.

Next, we performed principal component analysis (PCA) using the firing rate and network burst rate in the MEA datasets. The PCA revealed distinct responses to pesticides that inhibit acetylcholinesterase, GABA receptors, and Na^+ channel openers, indicating that the mechanism of action of pesticides can be estimated using this MEA method.

Taken together, both MEA analyses can provide hazard identification and prioritization for chemicals with DNT potential. Future studies should investigate when and how MEA can be used in the DNT-IVB scheme. It would be necessary to consider exposure with the in vitro data.

Introduction

New Approach Methodologies (NAMs) are expected to provide information to identify potential developmental neurotoxicity (DNT) hazards for a large number of chemicals. In Japan, multi-electrode array (MEA) system, which is one of the functional assays in the DNT in vitro testing battery, is widely used to measure neural network activity using rat cortical neurons as well as human iPSC-derived neurons. However, MEA protocol and vendor-to-vendor differences have not been well understood toward regulatory considerations.

Effects of pesticides on DNT assessment using MEA system

To use the MEA system for DNT assessment, we focused on neural network activity using iPSC neurons (SynFire neurons in NeuCyte, Inc) with acute exposure to positive and negative DNT compounds. A spike was counted when the extracellularly recorded signal exceeded a threshold of ± 5.3 σ, where σ was the standard deviation of the baseline noise. Network bursts were detected using a four-step method (Ishibashi et al., 2023). We found that exposure to pesticides caused significant changes in at least one of the MEA parameters, particularly in total spikes and bursts.

We then performed principal component analysis (PCA) on the MEA parameters, including firing rate and network burst rate. The PCA revealed distinct responses to pesticides that inhibit acetylcholinesterase, GABA receptors, and Na channel openers, indicating that the mode of action (MoA) of pesticides can be inferred using this MEA method.

We also compared our acute data sets with chronic data sets from a literature review (Bartmann et al., 2023). We found that the acute assay can detect similar chemicals, such as deltamethrin, when evaluating the activity associated with chronic treatment. We also found that our acute assay can identify chemicals based on mode of action, while the chronic assay provides information such as developmental and cytotoxic effects. In addition, we applied a machine learning approach to improve the analysis of MEA data using 10 compounds. The neural network model trained on optimised parameters of each test compound was used to predict neurotoxicity potential (=excitation/inhibition). The potential was predicted for the untrained data set based on the optimal operating point of the model calculated from each compound data set used for training. As a result, the MoA was accurately predicted for all compounds. As expected, the vehicle was classified as "negative".

Thus, our data suggest that MEA data using iPSC neurons can provide hazard identification and prioritisation for chemicals with DNT potential. Exposure would need to be considered with the in vitro data. Future studies should investigate when and how MEA can be used to prioritise chemicals and contextualise chemical risk assessment in the DNT-IVB scheme.

Quality control of MEA data

There are several issues to be addressed to ensure reliable and reproducible MEA data using the iPSC neurons. One of the issues is to ensure a stable supply of iPSC neurons. The quality of neurons should be assessed by excitation/inhibition balance (E/I balance) and morphology (aggregation, etc.). In addition, MEA baseline criteria (number of active electrodes, number of bursts, etc.) and assay timing are critical to gain confidence. To ensure multi-site data, positive and negative compounds should be selected to verify the response (GABA, AMPA, NMDA, etc).

Since there are still vendor differences in iPSC neurons, typical MEA patterns and analysis should be checked for each iPSC neuron.

Toxicokinetics

The blood-brain barrier (BBB) plays an important role as a biological barrier by regulating molecular transport between circulating blood and brain parenchyma. We have successfully developed brain microvascular endothelial-like cells from human iPSCs (Yamada et al., 2024).

To address the impact of toxicokinetics, we investigated the combination of neurons with an iPSC-derived blood-brain barrier (BBB) model. Using a microfluidic system, we successfully developed an integrated system combining the BBB and MEA platforms.

In the near future, this two-organ micro-physiological system with on-chip perfusion system could improve the predictive accuracy of DNT and help identify chemicals with DNT hazard potential.

Points to consider DNT-NAMs

It is important to consider NAMs, such as human relevant models, in a regulatory context. DNT-IVB assays are very useful to provide mechanistic data for compounds with potential DNT hazard identification and risk assessment. As we have accumulated many datasets from "traditional" animal testing and chemical risk assessment, there is still a gap between DNT-NAMs and current risk assessment. To date, Threshold of Toxicological Concern (TTC) and Ames QSAR are mainly accepted in Japan. The objective of risk assessment is focused on setting reference values (such as ADI, TDI) for risk management. Although some studies have shown the usefulness of other techniques, such as in vitro data and PBPK, examples of NAMs application are very limited. To use NAMs for regulatory considerations, quantitative evaluation by NAM should be established. Further studies should be conducted to fill the gap to understand how to use DNT-NAMs in the regulatory context.

Conclusion

These data suggest that this integrated approach could improve the predictive accuracy of DNT and aid in the identification of chemicals with DNT hazard potential. Further discussions are needed on how and when the in vitro DNT battery can be used to prioritise chemicals and contextualise chemical risk assessment.

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5 Summary of the break-out groups' discussions and Next steps

Summary of the break-out groups' discussions

Participants engaged in breakout groups to address specific topics. On day two, the workshop participants were split into four groups, and they addressed the questions listed in Annex B.

Breakout group 1: Use the DNT-IVB data as complementary information and part of the wight of evidence (WoE) in an IATA. What are the next steps for standardisation?

The first group's discussion focused on the integration of DNT-IVB data into the DNT Weight of Evidence (WoE) assessment of pesticides as part of an IATA for DNT Risk Assessment problem formulation. Through the course of the discussion, both EFSA and US EPA agreed that the DNT IVB in several case studies demonstrated to provide relevant, high-quality data on information regarding DNT potential of chemicals that are perturbing early cellular processes and that are difficult to measure in vivo. It was agreed by the breakout group 1 participants that the available DNT IVB data are useful for providing mechanistic understanding of DNT potential of pesticides when integrated into a WoE approach. The public availability of DNT IVB data makes necessary the integration of the DNT IVB in the hazard and risk assessment as part of available data for the pesticides active substances (e.g. in line with the Article 8/5 of the 1107/2009 EU pesticides regulation). In 2023, a total of 476 compounds were tested in one or more of the assays of the DNT IVB and 81 compounds in all the 17 assays of the DNT IVB (OECD., 2023). Since then, more data became available and there are a number of ongoing and planned efforts to fund and test additional chemicals using the current DNT IVB by EFSA, US NIEHS and US EPA.

A standardised IATA framework template for DNT and further interpretative guidance of the data were highlighted as important elements for robust and reproducible WoE decisions. The participants of the group highlighted the importance of identifying, characterising, and evaluating uncertainties for the different lines of evidence that could be used for assessing DNT (e.g. in vivo test guideline and non-test guideline, DNT IVB, structural alerts…) and how to possibly reduce them and emphasised the need to refer to any existing guidance and develop specific guidance for uncertainties related to DNT, covering practical approaches to identification, characterisation, and evaluation of uncertainties. The group acknowledged the work of another OECD group on QIVIVE guidance for DNT and suggested the application of this guidance to estimate internal doses. There is an OECD project to develop an IATA framework template specific to DNT that will cover problem formulations, data models, data interpretation, and aims to promote standardised reporting and documentation of the DNT in vitro battery data, including individual DNT-IVB assays and whole summary data by considering all available information sources and literature data. The timeline for the completion of the DNT IATA framework depends on the group's work pace and the incorporation of feedback from case studies and regulatory bodies. A first draft of the IATA framework template was made available to the participants of the workshop.

Overall, the group considered that the uncertainty analysis and WoE assessment approach in the context of IATA should be further standardised by developing a dedicated DNT IATA framework template and associated guidance.

Breakout group 2: Moving forward to an agreed-tiered testing strategy. What is still missing?

The second group discussed tiered testing and its potential application within the IATA framework when considering DNT-IVB data. The proposed structure for tiered testing includes five tiers, with the top three tiers reaching agreement between the participants, whereas tiers four and five were the subject of detailed discussion. The Tiers are there to provide information, and you don't proceed to the next tier (or exit the assessment) until you have that information. Tier Zero aims to provide the following information:

- Estimate of either administered dose and/or estimate of internal exposure
- Information to inform IVIVE
  - Hepatic clearance
  - Fraction bound in plasma
  - Gut absorption / bioavailability
- Flagging Information: Generated from in silico models to understand or interpret data, including
  - Hydrophilic stability
  - Metabolic stability
  - In vitro disposition kinetics
  - Physicochemical properties (QSAR approaches)
  - Neurotoxicity QSAR (if available)
  - Cramer class

Tier One aims to provide information on data and outcomes from a complete set of DNT IVB assays, in vitro points of departure, chemical stability in the test system, and their conjunction with additional weighted evidence information. The participants discussed the potential for uniform dose ranging across all assays, the need for reproducibility and transferability of the DNT IVB, and potentially contextualising DNT compared to other relevant organ/systems toxicities.

Tier Two covers information relevant to margin of exposure to inform human risk, bioactivity to in vivo effect comparisons, and comparison to existing animal data for hazard evaluation in certain circumstances.

Higher Tiers (3, 4, and 5) address complex models and assays that are not part of the tier One testing battery, resembling more of a toolbox that aims to be used to obtain mechanistic information and/or confirm or populate an AOP. The strengths and limitations of complex models, the role of 3D complex models, and the inclusion of zebrafish assays were considered for these three tiers.

The current tiered testing construct may not provide enough information on neuroinflammation, highlighting the need for future modifications based on evolving science. The zebrafish model's potential to include metabolism was discussed, emphasising the importance of testing both parent compounds and major metabolites. The need for regulatory input on the tiered testing approach and its usefulness in different regulatory frameworks was highlighted. Finally, the importance of characterising uncertainty for decision points and integrating tier three data into a systematic WoE approach was discussed.

Overall, the breakout group and follow-up discussion provided a proposed tiered testing approach, its current state from the point of view of the information needed from each tier to progress to the next tier and perform chemical assessment. It also highlighted areas for future development and regulatory

consideration. Additional information can be found above in the paper provided by Ellen Fritsche (see Chapter 4).

Breakout group 3: Defined Approach/es (DAs) development for DNT testing, are we there yet?

The breakout group focused on DAs and their implementation. The group agreed that multiple DAs are needed depending on the regulatory use context. However, some similarities or shared elements between the various DAs are expected. The discussion revolved around the use of fixed information sources, standardised analysis approaches, and fixed interpretation procedures. Developing DAs for DNT requires several levels of guidance for each of these components. It was acknowledged that some information is already quite well developed such as the detailed description of information sources by the assay developers using an annotated toxicity test method template (ToxTemp) (Krebs et al., 2019). For data analysis, the preference would be either to keep assay developers recommended cut-off values or define and provide criteria, including guidance on pipeline methodologies e.g., normalisation approaches, computing response units, and curve fitting. The consensus was to provide transparent and detailed documentation on each of the components needed for a DA.

There is work that needs to be finalised or considered before DNT DAs can be implemented, but taking actions that progress towards that goal is advisable. Tasks that need to be finalised include standardised protocols for assays that have been modified (e.g., change in cell source) and assessing assay reproducibility by repeated testing of reference compounds and laboratory transferability. Considerations include the integration and interpretation of the DNT IVB data by establishing criteria to understand which assay endpoints to use for aggregation, and whether to assign equal or unequal weight to the various assays or key processes represented in the battery. Additional work should focus on specificity in terms of detecting negative versus positive controls to ensure the battery does not produce too many false positives or negatives. Considering the low number of confirmed human DNT compounds, this poses a challenge and might require more innovative approaches. One suggestion was to include in DAs the comparison of human exposure data with in vitro activity concentrations using in vitro to in vivo extrapolation (IVIVE) modelling to assess if there is concern for DNT effects. Suggested next steps from the group were to initially develop a DNT DA case study for screening and prioritisation to explore the various components identified in the discussion. Such exercise may suggest refinement and inclusion of additional components that can then be considered in subsequent DNT DAs.

Overall, the breakout group and follow-up discussion provided a comprehensive overview of the areas of work needed to progress with the development of DNT DAs, and areas for future advancements and regulatory consideration.

Next Steps and Action Items

Case Studies

- Continue to develop case studies for different types of decisions and classes of chemicals.
- Encourage submitting examples as formal IATA case studies in particular for problem formulations not covered to date

DNT IATA Framework

- Update the framework based on discussions and move towards finalisation

Defined Approach(es)

- Select 1 or 2 examples that could be developed as DA case-studies

- Extract pre-processing information from ToxCast for each assay and capture in ToxTemp forms for increased transparency.

Tiered testing and Complex Models

- Novel complex models are (becoming) available-examples of their readiness status and application to decisions are needed
- Formalisation of decision processes of when to use these models is needed
- Update and continue work on the draft of the tiered testing presented and based on the breakout group discussions.

Assay Transfer

- “Assay Performance” lists of chemicals need to be finalised and made available.
- Data for the compounds in these lists needs to be available to the community
- Need to identify and involve experts in assay transfer (ICCVAM, ECVAM OECD, etc) ASAP to provide external peer review and credibility to transfer activities, including protocols.
- Establish agreed assay performance standards for transfer of assays.
- Transfer vs me too assays have a different structure but still need to meet performance criteria.

Annex A. Programme of the OECD Workshop on Critical Innovations in pesticides safety testing and chemical risk assessment, for developmental neurotoxicity DNT

Day 1 – 28 October 2024

Theme: Leverage on available experience from applying DNT-In vitro Battery (IVB) data

9h00

Welcome by the OECD

Patience Browne and Anne Gourmelon, OECD Environment Directorate

9h15

Welcome by the CRP and introduction to the Programme

Lieve Herman, CPR Scientific Advisory Body

Session 1: Lessons learned from existing IATA case studies using DNT-IVB data

***Chairs*: Iris Mangas,** European Food Safety Authority (EFSA), Italy and **Tim Shafer**, United States Environmental Protection Agency (US EPA), United States

Build off previous OECD IATA Case Studies on DNT and the Initial Recommendations on Evaluation of Data from the DNT-IVB published in 2022 and 2023, respectively. These efforts identified the need to elaborate on what was learned so far and identify the focus of additional efforts to accelerate the uptake of DNT-IVB for regulatory purposes. The ultimate goal of the first theme is to take a step forward from those efforts and discuss specific solutions to specific issues that were raised from the case studies to address different regulatory problem formulations (e.g., IVIVE, AOPs, in vivo and in vitro uncertainties, applicability domain, molecular characterisation, etc.).

09h30

US Division of Translational Toxicology (DTT): IATA case study for DNT to prioritise a class of chemicals

Helena Hogberg, NICEATM, US Division of Translational Toxicology (DTT), NIEHS, United States

(presentation: 20min; technical Q&A: 5min)

09h55

Case studies from large European (Horizon 2020) projects to refine testing strategies

Marcel Leist, University of Konstanz, CAAT-Europe, Germany

(presentation: 20min; technical Q&A: 5min)

10h20

US EPA Case-studies: Prioritisation, Weight of Evidence and Waiving in vivo DNT test studies

Tim Shafer, United States Environmental Protection Agency (US EPA), United States

(presentation: 20min; technical Q&A: 5min)

Tea/Coffee Break (10h45-11h15)

11h15

EFSA's IATA Case Studies for hazard identification and characterisation lessons learnt

Iris Mangas, European Food Safety Authority (EFSA), Italy

(presentation: 20min; technical Q&A: 5min)

11h40

Industry perspective on leveraging the mechanistic understanding from the DNT-IVB to optimise the development of safe pesticides

Agnes Karmaus, Syngenta, United States

(presentation: 20min; technical Q&A: 5min)

12h05

Inclusion of the DNT-IVB in EU's Pesticides Risk Assessment – a European Member State Perspective

Verena Haudek-Prinz, Austrian Agency for Health and Food Safety, Austria

(presentation: 20min; technical Q&A: 5min)

Lunch break (12h30-13h30)

13h30

IATA for chemical classification: special considerations when using DNT-IVB data

Martin Paparella, University of Innsbruck, Austria

(presentation: 20min; technical Q&A: 5min)

13h55

DNT-IVB data application for screening chemicals: industry's perspective

Sue Marty, Dow Chemicals, United States

(presentation: 20min; technical Q&A: 5min)

Session 2: Exposure assessment and Quantitative In vitro to in vivo extrapolation (QIVIVE)

***Chairs*: Cecilia Tan,** United States Environmental Protection Agency (US EPA), United States, US and **Jochem Louisse,** European Food Safety Authority (EFSA), Italy

In risk assessment, tiered testing is a stepwise approach that combines existing information on both exposure and hazard with the goal of obtaining uncertainty levels acceptable to the regulatory need on both. Positive activity in one or more assays in the DNT-IVB should be coupled with existing exposure information and adequate uncertainty analysis to determine whether the data is acceptable for the regulatory need. The need to perform PBK modelling-facilitated QIVIVE of DNT-IVB data has been highlighted in the IATAs published with hazard characterisation or risk assessment problem formulations. Further work and a road map are needed to develop a tiered exposure modelling framework that will allow the use of the DNT-IVB in vitro bioactivities to derive a PoD (Point of Departure) for human health risk assessment.

14h20

Towards quantitative in vitro to in vivo extrapolation of DNT IVB data: principles and considerations for regulatory application

Jochem Louisse, European Food Safety Authority (EFSA), Italy

(presentation: 20min; technical Q&A: 5min)

14h45

Maternal exposure and development neurotoxicity effects in offsprings: tiered modelling approaches

Cecilia Tan, United States Environmental Protection Agency (US EPA), United States

(presentation: 20min; technical Q&A: 5min)

Tea/Coffee break (15h15-15h45)

15h45

Deltamethrin - PBK/IVIVE strategy to consider developmental neurotoxicity (DNT) in vitro data for human health risk assessment

Katrin Bothe and **Dennis Mueller,** Bayer, Germany

(presentation: 20min; technical Q&A: 5min)

16h10

QIVIVE in developmental neurotoxicity: The role of in vitro distribution kinetics

Nynke Kramer, Wageningen University, The Netherlands

(presentation: 20min; technical Q&A: 5min)

16h35

Panel Discussion of Sessions 1 and 2

Prioritisation of Future Efforts: Identification of common strengths and limitations of the approaches used among the various case studies. Aims to decide on the selection of strengths that can be standardised/reused and to identify current limitations (i.e., technical, lack of data, lack of specific guidance, etc.) that need development efforts. Within those limitations, identify those that must be solved to use the DNT-IVB data as PoD and those that would be nice to decrease the uncertainty when doing so and summarise future research or regulatory goals for each of them.

Moderator: **Patience Browne**, OECD Environment Directorate

17h30 END OF DAY 1

Day 2 – 29 October 2024

Theme: Regulatory implementation of the DNT-IVB

09h00

Break-out group discussion on case studies, guided by charge questions.

What can be a short-medium term plan for the use of the DNT-IVB for:

1. Use of DNT-IVB data as complementary information as part of the WoE in an IATA
2. Moving forward to an agreed tiered testing strategy
3. DNT Defined approach development

Breakout (BO) group	Topic	Moderators
BO group 1	*Use the DNT-IVB data as complementary information and part of the WoE in an IATA. What are the next steps for standardisation?*	Moderators: Iris Mangas & Jochem Louisse
BO group 2	*Moving forward to an agreed-tiered testing strategy. What is still missing?*	Moderators: Josh Harrill & Ellen Fritsche
BO group 3	*Defined approach/es development for DNT testing. Are we there yet?*	Moderators: Helena Hogberg & Agnes Karmaus

Tea/Coffee Break (during the BO session)

Theme: Tiered testing strategy for application of DNT-IVB

Session 3: Tiered testing, Additional Assays and Non-Mammalian Animal Models– What do they have to offer to the DNT-IVB?

***Chair*: Ellen Hessel**, RIVM, The Netherlands

The third session will focus on strategies for tiered testing, improvements to the current DNT-IVB, and non-mammalian models for DNT. It will be an opportunity to identify any knowledge gained from CROs' or researchers' experience with additional assays to cover neurodevelopmental processes not represented in the DNT-IVB and non-mammalian animal models. It will also highlight any areas of research that will inform revisions to Initial Recommendations.

11h30

The EFSA DNT-RAP project - Laboratory transferability and accessibility of the DNT-IVB test methods

Katharina Koch, IUF – Leibniz Research Institute for Environmental Medicine, DNTOX, Germany

(presentation: 25min; technical Q&A: 5min)

Lunch break (12h00-13h30)

13h30

Transfer of, data generation from, and comparison to existing data from the developmental neurotoxicity in vitro battery

Megan Culbreth, Human Foods Program, U.S. Food and Drug Administration (FDA), United States

(presentation: 25min; technical Q&A: 5min)

14h00

Tiered testing and considerations for integrating additional assays and models to the DNT-IVB

Ellen Fritsche, SCAHT - Swiss Centre for Applied Human Toxicology, Switzerland

(presentation: 25min; technical Q&A: 5min)

14h30

High-throughput Phenotypic Profiling with Cell Painting as a potential first-tier DNT Screen

Joshua Harrill, Center for Computational Toxicology and Exposure (CCTE), United States Environmental Protection Agency (US EPA), United States

(presentation: 25min; technical Q&A: 5min)

15h00

3D human stem cell-derived models for developmental neurotoxicity studies

Lena Smirnova, Center for Alternatives to Animal Testing (CAAT), Johns Hopkins Bloomberg School of Public Health, United States

(presentation: 25min; technical Q&A: 5min)

Tea/Coffee break (15h30-16h00)

16h00

The added value of non-mammalian animal models to fill the gaps in developmental neurotoxicity

Lee Ellis, National Research Council of Canada, Canada

(presentation: 25min; technical Q&A: 5min)

16h30

Panel Discussion of Session 3

Future directions: The ultimate goal of this discussion is to develop a roadmap through which improvements to the Initial Recommendations can be made. Specifically, i) identify the status of assay transfers and determine what additional efforts are needed, if any, ii) begin to define more clearly tiered testing approaches to DNT and how to implement them, and iii) understand the value of alternative species and how they fit into the current DNT-IVB.

Moderator: **Tim Shafer**, United States Environmental Protection Agency (US EPA), United States

17h30 END OF DAY 1

Day 3 – 30 October 2024

Session 3: Tiered testing, Additional Assays and Non-Mammalian Animal Models– What do they have to offer to the DNT-IVB? (Cont.)

09h00

Plenary feedback from breakout groups and discussion

Moderators from breakout groups: **Iris Mangas** European Food Safety Authority (EFSA), Italy**, Josh Harrill** United States Environmental Protection Agency (US EPA), United States **and Helena Hogberg** NICEATM, US Division of Translational Toxicology (DTT), NIEHS, United States

10h00

Developing new assays to predict glia-related KNDPs with transcriptomics data support

Oddvar Myhre, Norwegian Institute of Public Health, Norway

(presentation: 25min; technical Q&A: 5min)

10h30

Microglia for Studying Developmental Neurotoxicity

Kelly Carstens, United States Environmental Protection Agency (US EPA)

(presentation: 25min; technical Q&A: 5min)

Tea/Coffee Break (11h00-11h30)

11h30

Evaluation of neurotoxicity for pesticide-related compounds in human iPS cell-derived neurons using microelectrode array: Japanese experience

Yasunari Kanda, Division of Pharmacology, National Institute of Health Sciences, Japan

(presentation: 25min; technical Q&A: 5min)

Theme: Develop Action Plans to Implement Recommendations

12h00

Open Discussion-Workshop Results and Next Steps

Participants are invited to reflect on the presentations and discussions of the last two days and contribute to this agenda item to shape the recommendations, outcomes, and action plans for 1) Updating the Initial Recommendations to include transferability, 2) Efforts to develop tiered testing, and 3) Efforts to develop an IATA framework template and move gradually to defined approach(es) for DNT.

Moderators: **Iris Mangas,** European Food Safety Authority (EFSA), Italy, **Tim Shafer**, United States Environmental Protection Agency (US EPA), United States and **Helena Hogberg**, NICEATM, US Division of Translational Toxicology (DTT), NIEHS, United States, United States

12h45

Wrap-Up and Leaving Address by the CRP

Lieve Herman, CPR Scientific Advisory Body

13h00

Workshop closes

Annex B. Breakout (BO) group questions

BO GROUP 1: USE THE DNT-IVB DATA AS COMPLEMENTARY INFORMATION AND PART OF THE WOE IN AN IATA. WHAT ARE THE NEXT STEPS FOR STANDARDISATION?
Moderators: Iris Mangas & Jochem Louisse

- *Is currently the DNT IVB used in a standard way for DNT risk assessment?*
- *Which parts are standard and agreed among the stakeholders?*
- *Which further interpretative guidance/s are needed for a standard and optimised approach?*

Objective: Identify important next steps for acceptance and standardisation of DNT-IVB use in an IATA.

BO GROUP 2: MOVING FORWARD TO AN AGREED-TIERED TESTING STRATEGY. WHAT IS STILL MISSING?
Moderators: Ellen Fritsche &Josh Harrill

- *Assume that Tier 1 of a tiered testing paradigm is comprised of the DNT IVB. If so,*
  - *What information from Tier 1 is necessary for informing a decision to move towards more advanced tiers of testing (i.e. Tier 2 or Tier 3)?*
  - *What uncertainties should be considered when informing this decision?*
  - *Is a different preponderance (set) of information necessary for informing decision to move across tiers for different decision contexts?*
- *What information from prior knowledge or computational methods should be considered in a "Tier 0" prior to deciding to test a chemical in Tier 1?*
- *At what point should exposure and/or TK be considered in a tiered strategy?*
- *Where (at what tier) do alternative species models (e.g. zebrafish) and advanced in vitro models fit into a tiered strategy?*
- *Based on the current state of the science, what is more desirable?*
  - *A tiered testing toolbox (i.e. a collection of assays/information that can inform testing across different levels of assays/biological organisation)?*
  - *A tiered testing strategy (i.e. structured, but flexible within tiers)?*
  - *A tiered testing workflow (i.e. structured, but inflexible within tiers)?*

Objective: Determine consensus on the four topics (toolbox/strategy/workflow, tiers 0 &1, exposure, advanced in vitro models and alternative species).

BO GROUP 3: DEFINED APPROACH/ES (DAS) DEVELOPMENT FOR DNT TESTING, ARE WE THERE YET?

Hogberg & Agnes Karmaus

- *Is one DA sufficient, or are multiple DAs needed?*
- *What information do we have that can be leveraged to build a DA?*
- *What aspects needed for building a DA are missing and/or need significant additional work to develop?*

Objective: Define and prioritise milestones for the development of DNT DA(s).