Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

27-Jan-2017

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Annex 1

REPORT OF THE OECD/EFSA WORKSHOP ON DEVELOPMENTAL NEUROTOXICITY (DNT): THE USE OF NON-ANIMAL TEST METHODS FOR REGULATORY PURPOSES

Background document on integrated Testing Strategies for the identification and evaluation of chemical hazards associated with the developmental neurotoxicity (DNT), to facilitate discussions at the Joint EFSA/OECD Workshop on DNT

Series on Testing and Assessment
No. 261

This document was produced by the Test Guidelines Programme work plan project 4.110, agreed in April 2016, and led by the European Food Safety Authority

Please note the deadline for declassification is 17 January 2017

This document is only available in pdf format

JT03408272

Report on Integrated Testing Strategies for the identification and evaluation of chemical hazards associated with the developmental neurotoxicity (DNT), to facilitate discussions at the Joint EFSA/OECD Workshop on DNT

Ellen Fritsche

September 2016

OECD Environment, Health and Safety

INDEX

1. BACKGROUND

To date, there is only a small amount of data available for developmental neurotoxicity (DNT) e.g. in support of the authorisation of pesticides. This is mainly due to the fact that there is no *a priori* requirement for pesticides or other chemicals to be tested for DNT effects prior to their registration and use under the present regulation. While developmental toxicity testing is mandatory, DNT studies are only to be carried out when relevant observations are made in other studies (triggers: adult neurotoxicity or endocrine disruption) or when suggested appropriate on the basis of the mode of action of the active substance. Although systematic DNT testing is lacking, many chemicals have been shown to be neurotoxic in *in vitro* and *in vivo* assays and this, according to some authors, raises particular concern for the developing brain, inherently much more susceptible to toxic agents than adult brains. A recent review listed about 200 chemicals known to be neurotoxic in humans, and just 5 of these substances have been firmly documented as causes of developmental neurotoxicity. Given the potential vulnerability of the developing brain, it is likely that many of these substances are capable of causing also developmental neurotoxicity but that such effects have not been captured because of insufficient testing.

Therefore, EFSA has recently considered developmental neurotoxicity as a 'critical effect of particular significance', as highlighted in the Annex II of the Regulation No 1107/2009.

At the regulatory level, the current OECD guideline for DNT refers only to *in vivo* studies mainly performed in the rat: however, the experimental conditions of these tests are difficult to apply in a standardised manner, thus resulting in limited reproducibility of results. In addition, *in vivo* studies are unsuitable for screening large numbers of chemicals, due to the use of large number of animals and long duration of tests. Despite this, the use of *in vivo* studies based on animal models remains an important hurdle when data are to be extrapolated to the human case.

Reliable, fast and efficient screening and assessment tools are needed to improve the identification, and evaluation of chemicals with the potential to induce DNT. Ongoing international trends in DNT testing have nowadays shifted from the use of rodent studies to alternative methods with the aim to reduce the DNT *in vivo* testing (taking into account the 3Rs concept aiming at refining, reducing and replacing tests with vertebrates) with integrated testing strategies combining *in vivo* data sets with *in vitro* approaches (Fritsche, 2015).

In addition, the quickly growing field of 'Adverse Outcome Pathway' (AOP) development has started to allow chemical testing within 'Integrated Approaches for Testing and Assessment' (IATAs). Thus, it was also considered here if AOP-informed IATAs might be useful to support DNT in vitro testing.

2. SUMMARY OF DNT-AOPS SUBMITTED TO THE AOP-WIKI OR PUBLISHED OTHERWISE

As of Aril 13th 2016 only 6 AOPs had been submitted to the AOP-Wiki (Table 1). Information at that time available on the AOP-Wiki is summarized here and information is displayed.

Table 1: DNT AOP summary (as of April 13th 2016):

AOP Number	Name	MIE	AO	Status
13	Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	NMDAR	impairment of learning and memory abilities	EAGMST Approved
42	Xenobiotic Induced Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	TPO	neurodevelopmental outcomes	EAGMST Under Review
134	Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	NIS	neurodevelopmental outcomes	SAAOP AOP Under Development
54	Inhibition of Na+/I-symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	NIS	learning and memory deficits in children	EAGMST Under Development
8	Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	mulitple	neurodevelopmental outcomes	EAGMST Under Development

152	Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity			

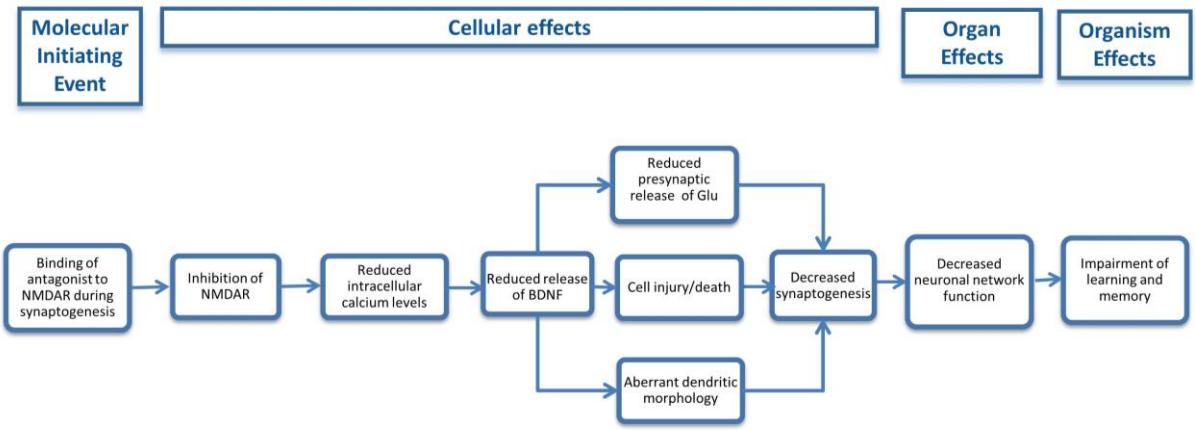
In the following paragraphs, each DNT-AOP will briefly be summarized (taken from https://aopwiki.org/wiki/index.php/Main_Page)

2.1 AOP13: CHRONIC BINDING OF ANTAGONIST TO N-METHYL-D-ASPARTATE RECEPTORS (NMDARS) DURING BRAIN DEVELOPMENT INDUCES IMPAIRMENT OF LEARNING AND MEMORY ABILITIES

Short name: Binding of antagonist to NMDARs impairs cognition

Abstract:
It is well documented and accepted that learning and memory processes rely on physiological functioning of the glutamate receptor N-methyl-D-aspartate (NMDAR). Both animal and human studies investigating NMDA itself, experiments with NMDAR antagonists and mutant mice lacking NMDAR subunits strongly support this statement (Rezvani, 2006). Activation of NMDARs results in long-term potentiation (LTP), which is related to increased synaptic strength, plasticity and memory formation in the hippocampus (Johnston et al., 2009). LTP induced by activation of NMDA receptors has been found to be elevated in the developing rodent brain compared to the mature brain, partially due to 'developmental switch' of the NMDAR 2A and 2B subunits (Johnston et al., 2009). Activation of the NMDAR also enhances brain derived neurotrophic factor (BDNF) release, which promotes neuronal survival, differentiation and synaptogenesis (Tyler et al., 2002, Johnston et al., 2009). Consequently, the blockage of NMDAR by chemical substances during synaptogenesis disrupts neuronal network formation resulting in the impairment of learning and memory processes (Toscano and Guilarte, 2005). This AOP is relevant to developmental neurotoxicity (DNT). The molecular initiating event (MIE) is described as the chronic binding of antagonist to NMDAR in neurons during synaptogenesis (development) in hippocampus (one of the critical brain structures for learning and memory formation). One of the chemicals that blocks NMDAR after chronic exposure is lead (Pb^{2+}), a well-known developmental neurotoxicant.

AOP overview:



AOP Evaluation:

Weight of Evidence:

Event	Description	Triggers	Weight of Evidence
NMDARs, Binding of antagonist	Directly Leads to	NMDARs, Inhibition	Strong
NMDARs, Inhibition	Directly Leads to	Calcium influx, Decreased	Moderate
Calcium influx, Decreased	Indirectly Leads to	Release of BDNF, Reduced	Weak
Release of BDNF, Reduced	Indirectly Leads to	Dendritic morphology, Aberrant	Weak
Release of BDNF, Reduced	Indirectly Leads to	Cell death, N/A	Weak
Release of BDNF, Reduced	Indirectly Leads to	Presynaptic release of glutamate, Reduced	Weak
Dendritic morphology, Aberrant	Indirectly Leads to	Synaptogenesis, Decreased	Weak
Cell death, N/A	Indirectly Leads to	Synaptogenesis, Decreased	Weak
Presynaptic release of glutamate, Reduced	Indirectly Leads to	Synaptogenesis, Decreased	Weak
Synaptogenesis, Decreased	Directly Leads to	Neuronal network function, Decreased	Weak
Neuronal network function, Decreased	Indirectly Leads to	Learning and memory, Impairment	Weak

Essentiality:

Molecular Initiating Event	Support for Essentiality

NMDARs, Binding of antagonist	Strong

Event	Support for Essentiality
NMDARs, Inhibition	Strong
Calcium influx, Decreased	Strong
Release of BDNF, Reduced	Strong
Dendritic morphology, Aberrant	Strong
Presynaptic release of glutamate, Reduced	Strong
Cell death, N/A	Strong
Synaptogenesis, Decreased	Strong
Neuronal network function, Decreased	Strong

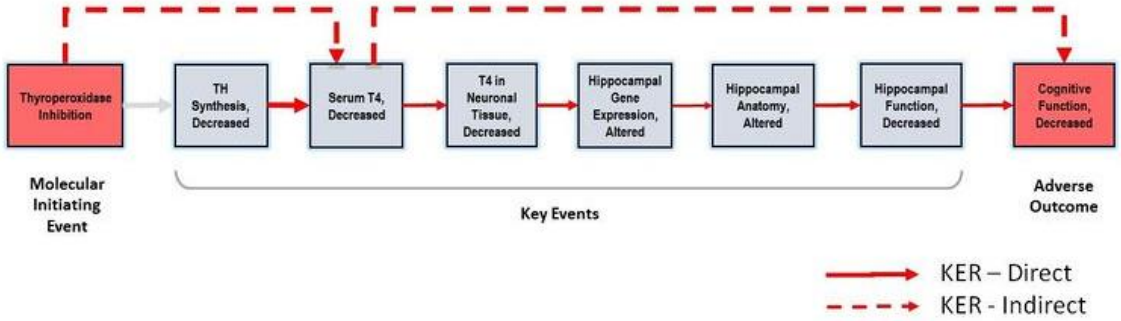
Adverse Outcome
Learning and memory, Impairment

2.2 AOP42: XENOBIOTIC INDUCED INHIBITION OF THYROPEROXIDASE AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS (INHIBITION OF THYROPEROXIDASE AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS)

Short name: TPO Inhibition and Altered Neurodevelopment

Abstract:
This AOP describes one adverse outcome that results from the inhibition of thyroperoxidase (TPO) during mammalian development. Chemical inhibition of TPO, the molecular-initiating event (MIE), results in decreased thyroid hormone (TH) synthesis, and subsequent reduction in circulating concentrations of THs. THs are essential for normal human brain development, both prenatally and postnatally, modulating neural proliferation and differentiation, synaptogenesis, and cell migration. Developmentally-exposed rat models to known TPO inhibitors, pharmacological agents methimazole (MMI) and 6-propyl-2-thiouracil, demonstrate impairment of neuronal migration and decreased myelination and oligodendrocyte development. Therefore, chemicals that interfere with TH synthesis have the potential to cause TH insufficiency that may result in adverse neurodevelopmental effects in offspring; this AOP focuses solely on the neurodevelopmental effects that may be mediated by TH insufficiency in the hippocampus. The hippocampus is critically involved in cognitive, emotional, and memory function. The adverse consequences of TH insufficiency depend both on severity and developmental timing, indicating that exposure to thyrotoxicants may produce different effects at different developmental windows of exposure. Herein we discuss the implications of developmental TPO inhibition for hippocampal anatomy, function, and ultimately cognitive function. This MOA was originally described and published by (Zoeller and Crofton, 2005).

AOP overview:



AOP Evaluation:

Weight of Evidence:

Event	Description	Triggers	Weight of Evidence	Quantitative Understanding
Thyroperoxidase, Inhibition	Directly Leads to	Thyroid hormone synthesis, Decreased	Strong	Weak
Thyroid hormone synthesis, Decreased	Directly Leads to	Thyroxin (T4) in serum, Decreased	Strong	Weak
Thyroxin (T4) in serum, Decreased	Directly Leads to	Thyroxin (T4) in neuronal tissue, Decreased	Moderate	Weak
Thyroxin (T4) in serum, Decreased	Indirectly Leads to	Cognitive Function, Decreased	Strong	Moderate
Thyroxin (T4) in neuronal tissue, Decreased	Directly Leads to	Hippocampal gene expression, Altered	Moderate	Weak
Hippocampal gene expression, Altered	Directly Leads to	Hippocampal anatomy, Altered	Moderate	Weak
Hippocampal anatomy, Altered	Directly Leads to	Hippocampal function, Decreased	Moderate	Weak
Hippocampal function, Decreased	Directly Leads to	Cognitive Function, Decreased	Moderate	Weak
Thyroperoxidase, Inhibition	Indirectly Leads to	Thyroxin (T4) in serum, Decreased	Strong	Weak

Essentiality:

Molecular Initiating Event	Support for Essentiality
Thyroperoxidase, Inhibition	Strong

Event	Support for Essentiality
Thyroid hormone synthesis, Decreased	Strong
Thyroxin (T4) in neuronal tissue, Decreased	Strong
Thyroxin (T4) in serum, Decreased	Strong
Hippocampal gene expression, Altered	Moderate
Hippocampal anatomy, Altered	Moderate
Hippocampal function, Decreased	Moderate

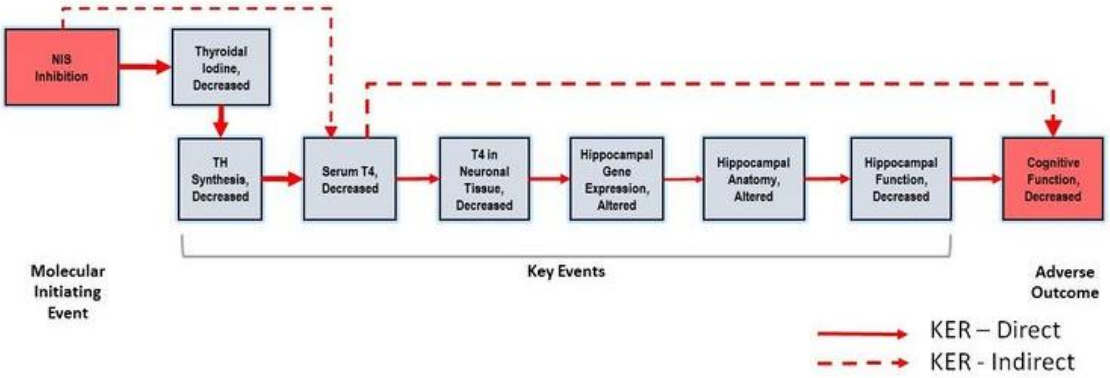
Adverse Outcome
Cognitive Function, Decreased

2.3 AOP134: SODIUM IODIDE SYMPORTER (NIS) INHIBITION AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS

Short name: NIS and Cognitive Dysfunction

Abstract:
This AOP describes one adverse outcome that results from the inhibition of the sodium iodine symporter (NIS) during mammalian development. Inhibition of NIS, the molecular-initiating event (MIE), results in decreased iodine uptake, decreased thyroidal iodine content, subsequent decreased synthesis of thyroid hormones (THs), and reduction in circulating levels of THs. THs are essential for normal human brain development, both prenatally and postnatally. Therefore, chemicals that interfere with TH synthesis have the potential to cause TH insufficiency that may result in adverse neurodevelopmental effects in offspring. This AOP includes changes in brain TH concentrations and subsequent impacts on hippocampal development that lead to declines in cognitive spatial behavior. The weight of evidence for this AOP is strong. And, while there are currently computational quantitative models that can predict serum TH levels from NIS inhibition, there is currently a lack of quantitative understanding of the degree of serum TH disruption that leads to the adverse outcome.

AOP overview:



AOP Evaluation:

Weight of Evidence:

Event	Description	Triggers	Weight of Evidence	Quantitative Understanding
Na+/I-symporter (NIS), Inhibition	Directly Leads to	Thyroidal iodide uptake, Decreased	Strong	Strong
Thyroidal iodide uptake,	Directly Leads to	Thyroid hormone synthesis, Decreased	Strong	Strong

Decreased				
Thyroid hormone synthesis, Decreased	Directly Leads to	Thyroxin (T4) in serum, Decreased	Strong	Strong
Thyroxin (T4) in serum, Decreased	Directly Leads to	Thyroxin (T4) in neuronal tissue, Decreased	Strong	Weak
Thyroxin (T4) in neuronal tissue, Decreased	Directly Leads to	Hippocampal gene expression, Altered	Moderate	Weak
Hippocampal gene expression, Altered	Directly Leads to	Hippocampal anatomy, Altered	Moderate	Weak
Hippocampal anatomy, Altered	Directly Leads to	Hippocampal function, Decreased	Moderate	Weak
Hippocampal function, Decreased	Directly Leads to	Cognitive Function, Decreased	Moderate	Weak
Thyroxin (T4) in serum, Decreased	Indirectly Leads to	Cognitive Function, Decreased	Strong	Weak

Essentiality:

Molecular Initiating Event	Support for Essentiality
Na+/I- symporter (NIS), Inhibition	Strong

Event	Support for Essentiality
Thyroidal iodide uptake, Decreased	Strong
Thyroid hormone synthesis, Decreased	Strong
Thyroxin (T4) in serum, Decreased	Strong
Thyroxin (T4) in neuronal tissue, Decreased	Strong
Hippocampal gene expression, Altered	Moderate
Hippocampal anatomy, Altered	Moderate
Hippocampal function, Decreased	Moderate

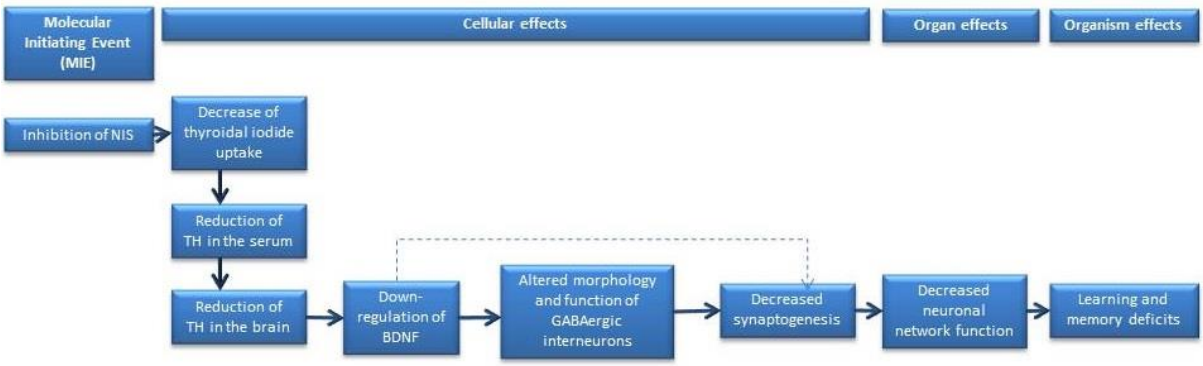
Adverse Outcome
Cognitive Function, Decreased

2.4 AOP54: INHIBITION OF NA+/I- SYMPORTER (NIS) DECREASES TH SYNTHESIS LEADING TO LEARNING AND MEMORY DEFICITS IN CHILDREN

Short name: NIS inhibition and DNT effects

Abstract:
The thyroid hormones (TH) are essential for brain development, maturation, and function as they regulate the early key developmental processes such as neurogenesis, cell migration, proliferation, myelination and neuronal and glial differentiation. Normal brain development and cognitive function in mammals relays on sufficient production of TH during the perinatal period. The function of Na^+/I^- symporter (NIS) is critical for the physiological production of TH levels in the serum, as it is a membrane bound glycoprotein that mediates the transport of iodide form the bloodstream into the thyroid cells, and this constitutes the initial step for TH synthesis. NIS is a well-studied target of chemicals, and its inhibition results in decreased hormone synthesis and secretion into blood leading to subsequent TH insufficiency in the brain with detrimental effects in neurocognitive function in children. The present AOP describes developmental neurotoxicity (DNT) effects induced by the decreased levels of TH in the blood and consequently in the brain, as a result of NIS inhibition. Many environmental chemicals have been reported to disrupt iodide uptake, but the studies that have been focused on NIS inhibition are mainly restricted to perchlorate and some small ionic or drug-like molecules. Perchlorate, which is the most potent inhibitor of NIS, has been associated with reduced TH production and also with cognitive deficits in animals and human.

AOP overview:



AOP Evaluation:

Weight of Evidence:

Event	Description	Triggers	Weight of Evidence	Quantitative Understanding
Thyroidal iodide uptake, Decreased	Directly Leads to	Thyroid hormone synthesis, Decreased	Strong	Strong

Na+/I-symporter (NIS), Inhibition	Directly Leads to	Thyroidal iodide uptake, Decreased	Strong	Strong
Thyroid hormone synthesis, Decreased	Directly Leads to	Thyroxin (T4) in serum, Decreased	Strong	Strong
Thyroxin (T4) in serum, Decreased	Directly Leads to	Thyroxin (T4) in neuronal tissue, Decreased	Strong	Weak
Thyroxin (T4) in neuronal tissue, Decreased	Directly Leads to	Release of BDNF, Reduced	Weak	Weak
GABAergic interneurons morphology and function , Altered	Directly Leads to	Synaptogenesis, Decreased	Strong	Weak
Release of BDNF, Reduced	Indirectly Leads to	GABAergic interneurons morphology and function , Altered	Moderate	Weak
Release of BDNF, Reduced	Indirectly Leads to	Synaptogenesis, Decreased	Moderate	Weak
Synaptogenesis, Decreased	Directly Leads to	Neuronal network function, Decreased	Weak	Weak
Neuronal network function, Decreased	Directly Leads to	Learning and memory, Impairment	Strong	Weak

Essentiality:

Molecular Initiating Event	Support for Essentiality
Na+/I- symporter (NIS), Inhibition	Strong

Event	Support for Essentiality

Thyroidal iodide uptake, Decreased	Strong
Thyroid hormone synthesis, Decreased	Strong
Thyroxin (T4) in serum, Decreased	Strong
Thyroxin (T4) in neuronal tissue, Decreased	Strong
Release of BDNF, Reduced	Strong
GABAergic interneurons morphology and function , Altered	Moderate
Synaptogenesis, Decreased	Strong
Neuronal network function, Decreased	Moderate

Adverse Outcome
Learning and memory, Impairment

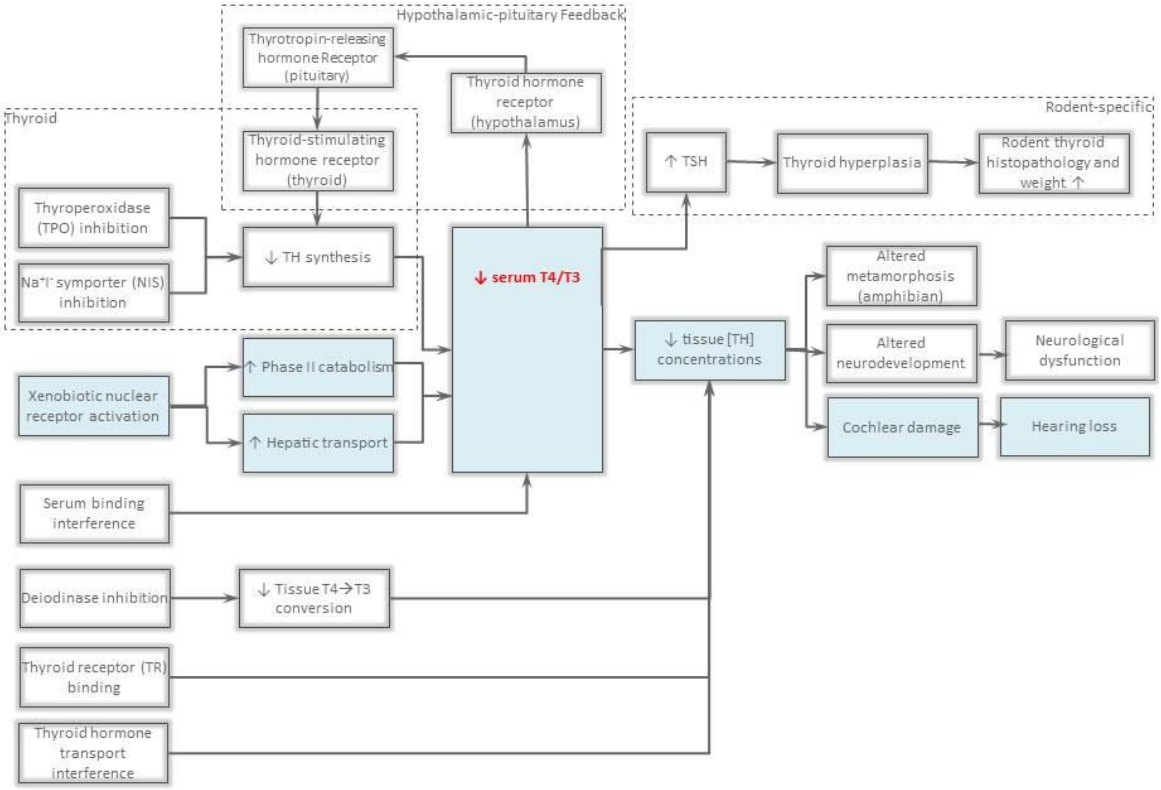
2.5 AOP8: UPREGULATION OF THYROID HORMONE CATABOLISM VIA ACTIVATION OF HEPATIC NUCLEAR RECEPTORS, AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS

Short name: Nuclear receptor induced TH Catabolism and Developmental Hearing Loss

Abstract:
Data from rodent studies demonstrate that thyroid hormone disruption during cochlear development culminates in ototoxicity. Developmental exposure of rats to polychlorinated biphenyls (PCBs) results in a low-frequency hearing loss in adult offspring (Crofton and Rice, 1999, Herr et al., 2016, Herr et al., 2001, Lasky et al., 2002). A body of work now supports the hypothesis that this ototoxicity results from PCB-induced hypothyroxinemia during a critical period of auditory development. Evidence for this hypothesis includes: a correlation between the severity of functional auditory impairment and the degree of thyroid hormone depletion (Crofton, 2004, Goldey et al., 1995a, Goldey and Crofton, 1998, Goldey et al., 1995b), a cross-fostering study demonstrating that the critical exposure period is postnatal (Crofton et al., 2000), and amelioration of the hearing loss following postnatal thyroxine replacement (Goldey and Crofton, 1998). Below an adverse outcome pathway is described for chemicals that activate xenobiotic nuclear receptors, including AhR, CAR, and PXR, leading to thyroid hormone disruption during cochlear development and resulting in permanent auditory loss.

This AOP is a revision and update of the original started on the Chemical Mode of Action wiki sponsored by WHO/IPCS. This MOA was described and published by (Crofton and Zoeller, 2005).

AOP overview:



AOP Evaluation:

Weight of Evidence:

Event	Description	Triggers	Weight of Evidence	Quantitative Understanding
Pregnane-X receptor, NR1I2, Activation	Directly Leads to	Upregulation of glucuronyltransferase activity, Induction	Strong	Weak
Constitutive androstane receptor, NR1I3, Activation	Directly Leads to	Upregulation of glucuronyltransferase activity, Induction	Moderate	Weak
Upregulation of glucuronyltransferase activity, Induction	Directly Leads to	Biliary excretion, Up Regulation	Moderate	
Upregulation of glucuronyltransferase activity, Induction	Indirectly Leads to	Serum thyroxine (T4), Decrease	Strong	Weak
Hepatic transporter gene expression, Up Regulation	Indirectly Leads to	Hepatic transport of parent T4, Increase	Moderate	
Hepatic transport of parent T4, Increase	Directly Leads to	Biliary excretion, Up Regulation	Moderate	

Biliary excretion TH glucuronide, Increase	Directly Leads to	Serum thyroxine (T4), Decrease	Moderate	Weak
Serum thyroxine (T4), Decrease	Directly Leads to	Tissue thyroid hormone concentration, Decrease	Moderate	Weak
Tissue thyroid hormone concentration, Decrease	Indirectly Leads to	TR-regulated cochlear proteins, Decrease	Weak	
TR-regulated cochlear proteins, Decrease	Directly Leads to	Structure of the cochlea, Damage	Weak	
Structure of the cochlea, Damage	Directly Leads to	Cochlear function, Loss	Moderate	
Pregnane-X receptor, NR1l2, Activation	Directly Leads to	Hepatic transporter gene expression, Up Regulation	Moderate	

Essentiality:

Molecular Initiating Event	Support for Essentiality
Pregnane-X receptor, NR1l2, Activation	Moderate

Event	Support for Essentiality
Upregulation of glucuronyltransferase activity, Induction	Strong
Biliary excretion TH glucuronide, Increase	Moderate
Thyroxin (T4) in serum, Decreased	Strong
Thyroxin (T4) in neuronal tissue, Decreased	Strong
Hippocampal gene expression, Altered	Moderate
Hippocampal anatomy, Altered	Moderate
Hippocampal function, Decreased	Moderate

Adverse Outcome
Cochlear function, Loss

2.6 AOP152: INTERFERENCE WITH THYROID SERUM BINDING PROTEIN TRANSTHYRETIN AND SUBSEQUENT ADVERSE HUMAN NEURODEVELOPMENTAL TOXICITY

Short name: Transthyretin interference

Abstract:
This AOP describes one adverse outcome (neurodevelopemental effects) that results from the interference with thyroid serum binding protein transthyretin (TTR). Binding of TTR by a xenobiotic (the MIE) during certain developmental windows may disrupt the normal neurodevelopment of certain mammals through a transient increase in free thyroxine (T4) levels, permitting increased tissue uptake of thyroid hormone (TH), followed by a decrease in both serum and neuronal tissue concentrations. The adverse consequences of TH insufficiency depend both on the severity and developmental timing, indicating that exposure to thyroid toxicants may produce different effects at different developmental windows of exposure. This AOP discusses the potential for developmental TTR interference to adversely impact hippocampal anatomy, function, and ultimately cognitive function.
Transthyretin is one of three serum binding proteins found in man that collectively act to transport thyroid hormone (TH) and thus maintain normal homeostasis via modulation of the hypothalamic/pituitary/thyroid axis. In addition to TTR, albumin and thyroxine-binding globulin (TBG) also serve to transport TH in serum and the relative contribution of each binding protein differs across species (although TTR itself is highly conserved). In man, TBG has the greatest affinity for thyroxine (T4), followed by TTR and ALB shows the lowest affinity for T4 while prevalence in serum is the opposite, with ALB being most prevalent (42 g/L in serum), followed by TTR (0.25 g/L) and TBG is the least prevalent in serum (0.015 g/L) (Mendel et al 1989, Richardson et al 2015). In rat, TTR is the major serum transport protein (as rats lack TBG); however, in man and primates, only a small fraction of T4 is bound to TTR or albumin.

AOP overview:
not available

AOP Evaluation:

Weight of Evidence:
not available

Essentiality:

Molecular Initiating Event	Support for Essentiality
Transthyretin in serum, Binding	Strong

Key Events

Event	Support for Essentiality

Serum thyroxine (T4) from transthyretin, Displacement	
Free serum thyroxine (T4), Increased	
Uptake of thyroxine into tissue, Increased	
Clearance of thyroxine from tissues, Increased	
Thyroxin (T4) in serum, Decreased	
Thyroxin (T4) in neuronal tissue, Decreased	
Hippocampal gene expression, Altered	
Hippocampal anatomy, Altered	
Hippocampal function, Decreased	

Adverse Outcome
Cognitive Function, Decreased

2.7 ADDITIONAL PUTATIVE AOPS PUBLISHED IN THE SCIENTIFIC LITERATURE

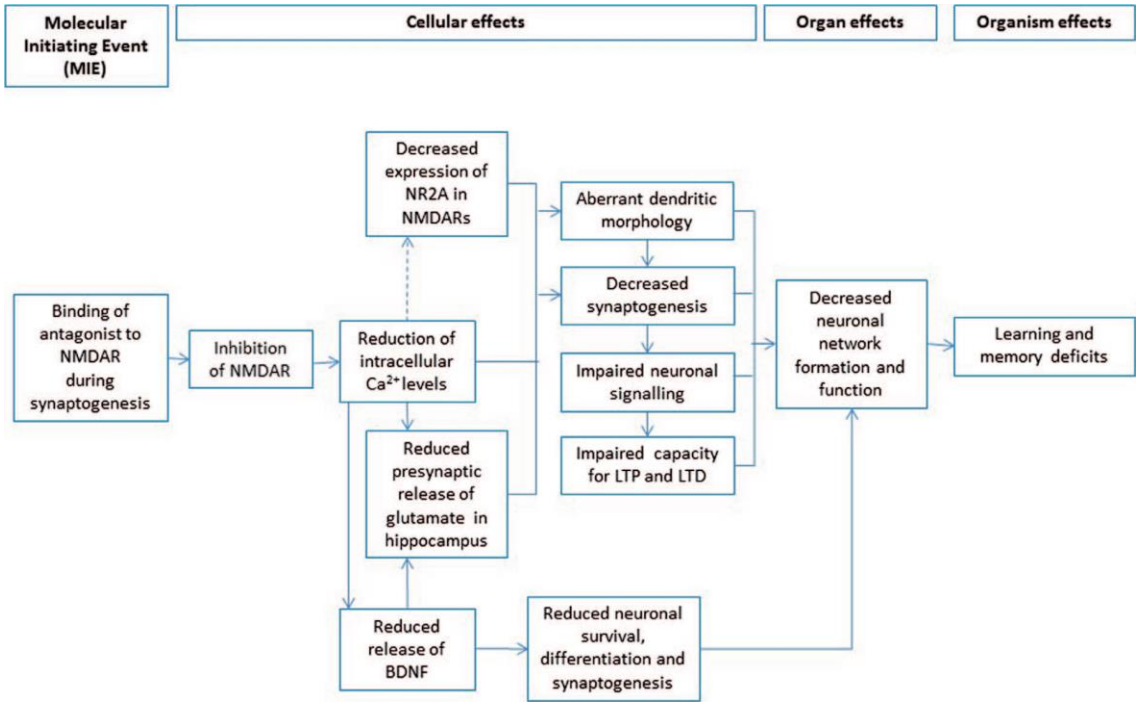
In addition to AOPs published on the AOP Wiki, putative AOPs for DNT were published in a review article by (Bal-Price et al., 2015b) and in the report (Bal-Price et al., 2016). These articles summarized AOPs for neurotoxicity, amongst them four concerned with DNT. These four will be summarized here briefly:

AOP I: Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities (Bal-Price et al., 2015b)

Summary:

Binding of an antagonist to NMDAR in hippocampus during synaptogenesis leads to inhibition of receptor activity and to a delay in the ontogeny of the NR2A subunit, contributing to decreased calcium influx into neurons and decreased glutamate release, causing a concomitant reduction of BDNF release. The resulting cellular key events eventually lead to impaired capacity for processes underlying learning and memory like long-term potentiation (LTP) and long-term depression (LTD).

AOP overview:

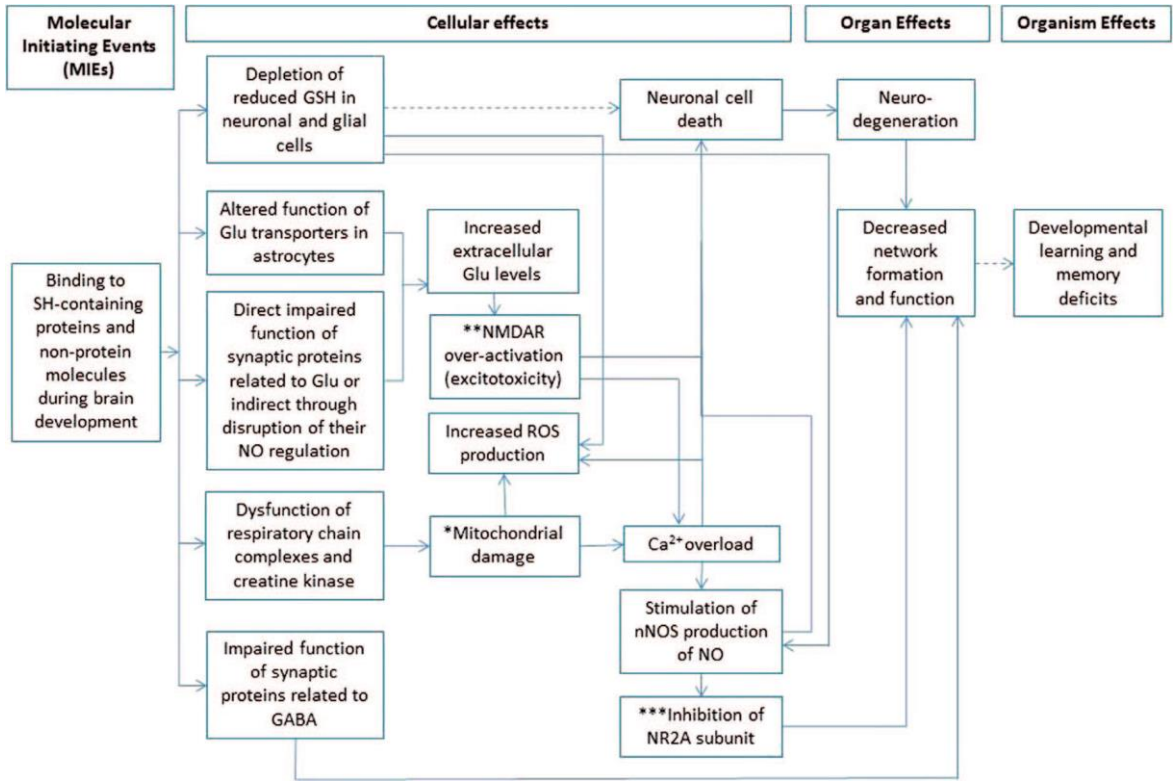


AOP VI: Impairment of learning and memory induced by binding of electrophilic chemicals to the SH(thiol)-group of proteins and non-protein molecules in neuronal and glial cells during development (Bal-Price et al., 2015b)

Summary:

By binding of a compound to the SH(thiol)- groups of proteins and non-protein molecules (Molecular Initiation Event) during brain development and the subsequent functional modification of their function leads to several distinct cellular key events that depend on the function and location of these proteins in the specific brain cell type and the brain structure. Mainly proteins and non-protein molecules associated with mitochondria, antioxidant defense, and glutamate storage, release and uptake are targeted. This binding leads to the depletion of reduced glutathione, increased of extracellular Glu levels inducing over activation of NMDAR, possible neuronal/glial dysfunction of respiratory chain complexes, triggering oxidative and nitrosative stress causing neuronal cell death. The induced neurodegeneration contributes to the decreased neuronal network formation and function responsible for the deficits in developmental learning and memory processes (AO).

AOP overview:

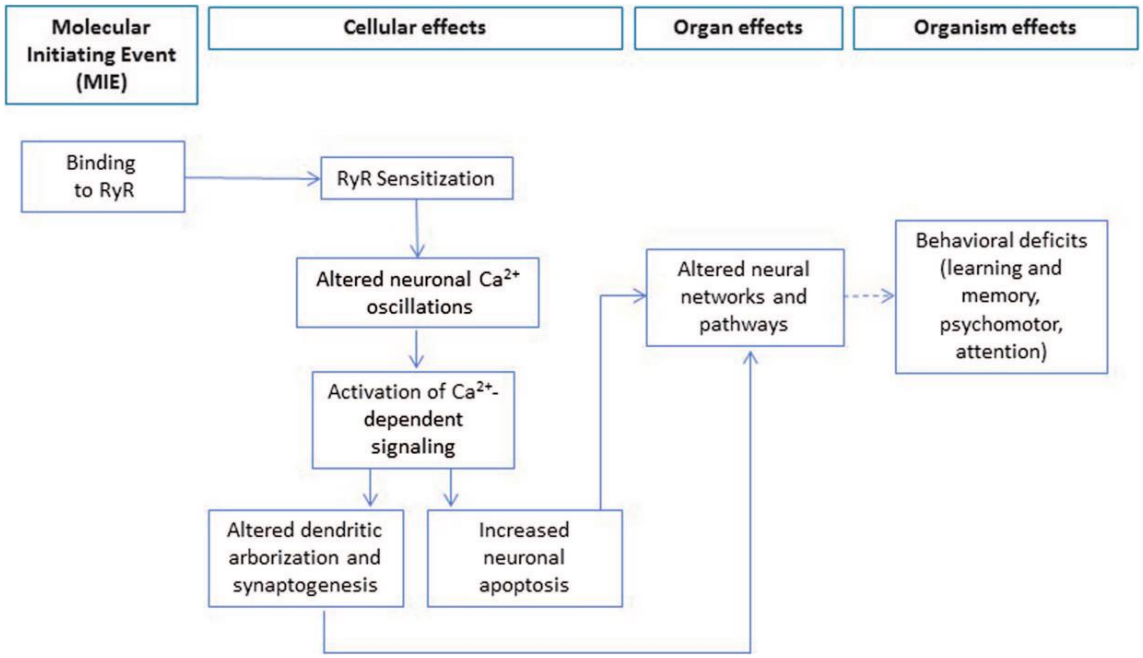


AOP IX: The interaction of non-dioxin-like PCBs with ryanodine receptors (RyRs) causes their sensitization affecting neuronal connectivity that results in behavioral deficits (developmental neurotoxicity; (Bal-Price et al., 2015b)

Summary:

NDL (non-dioxin-like) PCBs sensitize ryanodine receptor (RyR) activity and alter Ca^{2+} -dependent signalling mechanisms that link neuronal activity to dendritic growth and plasticity and to neuronal apoptosis. These cellular effects alter normal patterns of neuronal connectivity in the brain and contribute to behavioral and psychomotor deficits observed at the organismal level (adverse outcome).

AOP overview:

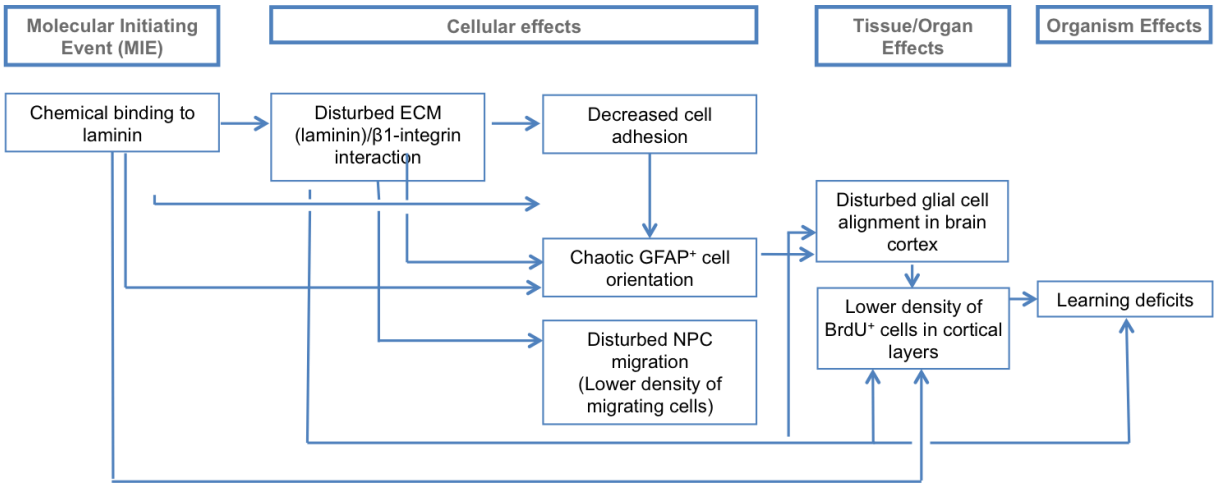


AOP: Disrupted laminin-β1-integrin interaction leading to developmental neurotoxicity (Bal-Price et al., 2016)

Summary:

This putative AOP is composed of the following KE: The MIE describes the binding of compound to laminin causing interference of the laminin-β1-integrin binding. This causes disturbed adhesion followed by chaotic cell orientation and altered cell migration. Altered migration leads to decreased cell density and impairment of learning based on studies with conditional β1-integrin deficient transgenic animals.

AOP overview:



2.8 SUMMARY OF AOPs AND THEIR CELLULAR KEY EVENTS (KEs)

The DNT-AOPs submitted to the AOP-Wiki are rather limited (Table 1). MIEs addressed in the AOPs currently available on the AOP Wiki are leading to either KE altering TH homeostasis or modulating NMDA receptors. In addition, four putative DNT AOPs have been published that add data. Targets for MIEs within these AOPs are NMDAR, SH-groups of proteins, RyR or the extracellular matrix (ECM) protein laminin. As MoA of DNT compounds reach far beyond interference of compounds with these receptors/pathways, at this point the development of an only on AOP-based testing strategy is not feasible. Development of AOPs for DNT is highly warranted, however, considering the multiple neurodevelopmental targets that might possibly serve as sites of molecular initiation and the time it takes for developing AOPs that are in accordance with the OECD guidelines for AOP development, such an AOP-informed testing strategy will have to be envisioned for the future. In addition to cellular KE identified by AOPs (Table 2), that are helpful in setting up assays for a DNT testing strategy, one can make use of data addressing essential neurodevelopmental processes. This approach has been promoted by the series of TestSmart John's Hopkins DNT1-4 meetings as well as by the ISTNET meeting (Bal-Price et al., 2015a).

Table 2: Cellular KEs identified within DNT AOPs published within the AOP Wiki and in Bal-Price et al. (2015,2016):

No	Timing of KE	KE	AOP number
1	Early	Intracellular calcium levels	Wiki 13; (Bal-Price et al., 2015b), AOP I & IX
2	Early	BDNF release	Wiki 13
3	Early	T4 decrease in hippocampus	Wiki 42,134
4	Early	TH decrease in brain	Wiki 54,8,152
5	Early	Hippocampal gene expression	Wiki 42,134,152
6	Early	Inhibition of NMDAR	(Bal-Price et al., 2015b), AOP I
7	Early	Depletion of reduced glutathione in neuronal and astrocyte cells	(Bal-Price et al., 2015b), AOP VI
8	Early	Altered function of Glu transporters in astrocytes	(Bal-Price et al., 2015b), AOP VI
9	Early	Direct impaired function of synaptic proteins related to Glu or indirect through disruption of their NO regulation	(Bal-Price et al., 2015b), AOP VI
10	Early	Dysfunction of respiratory chain	(Bal-Price et al.,

		complexes and creatin kinase	2015b), AOP VI
11	Early	RyR sensitization	(Bal-Price et al., 2015b), AOP IX
12	Early	Disturbance in NPC adhesion and migration	(Bal-Price et al., 2016)
13	Intermediate	Altered function of GABAergic neurons	Wiki 54; (Bal-Price et al., 2015b), AOP VI
14	Intermediate	Reduced release of BDNF	(Bal-Price et al., 2015b), AOP I
15	Intermediate	Reduced presynaptic glutamate release in hippocampus	(Bal-Price et al., 2015b), AOP I
16	Intermediate	Increased extracellular Glu levels	(Bal-Price et al., 2015b), AOP VI
17	Intermediate	NMDAR over-activation	(Bal-Price et al., 2015b), AOP VI
18	Intermediate	Increased ROS production	(Bal-Price et al., 2015b), AOP VI
19	Intermediate	Mitochondrial damage	(Bal-Price et al., 2015b), AOP VI
20	Intermediate	Stimulation of nNOC with NO production	(Bal-Price et al., 2015b), AOP VI
21	Intermediate	Inhibition of NR2A subunit	(Bal-Price et al., 2015b), AOP I & VI
22	Intermediate	Altered dendritic arborization	(Bal-Price et al., 2015b), AOP IX
23	Intermediate	Altered alignment of GFAP+ cells	(Bal-Price et al., 2016)
24	Late	Neuronal cell death	(Bal-Price et al., 2015b), AOP VI
25	Late	Increased neuronal apoptosis	(Bal-Price et al., 2015b), AOP IX
26	Late	Altered Synaptogenesis	Wiki 13,54; (Bal-Price et al., 2015b), AOP I, IX
27	Late	Altered Neuronal network formation	Wiki 13,54
28	Late	Reduced neuronal survival, differentiation and synaptogenesis	(Bal-Price et al., 2015b), AOP I
29	Late	Impaired neuronal differentiation	Bal-Price et al. (2016)

2.9 AOP-BASED FRAMEWORK: AN ATTEMPTED CASE STUDY FOR USAGE OF AOPS FOR EVALUATING METAL MIXTURE EFFECTS ON DNT

Independently of DNT-AOPs within the AOP-Wiki, an AOP-based DNT framework has recently been published not for single initiating events/chain of key events, but for application of the AOP-concept to mixtures of metals (von Stackelberg et al., 2015): 'Exposure to Mixtures of Metals and Neurodevelopmental Outcomes: A Multidisciplinary Review Using an Adverse Outcome Pathway Framework'). Specifically, the authors propose to intersect domains of evidence including exposure, mode of action, disease and genetic susceptibility and putting it in an AOP framework (Fig. 1). The authors performed a systematic literature review on neurodevelopmental effects of arsenic, lead, manganese and cadmium in single or combined exposure scenarios in vitro, in vivo and in epidemiological studies. Although the authors successfully compiled a large amount of studies on adverse neurodevelopmental effects of metals, this work is far from being an AOP according to the OECD guidelines. Especially information on MIEs and key event relationships are missing, it is rather a collection of modes of action. I.e., specifically the author's identification of reduced BDNF could have formed a common KE with AOP 13 published within the AOP Wiki. Here, altered BDNF expression is causally linked to aberrant dendritic morphology, followed by reduced presynaptic release of glutamate, increased cell death with reduced synaptogenesis and ultimately impaired neuronal network function. Such a chain of events was not elaborated in this publication.

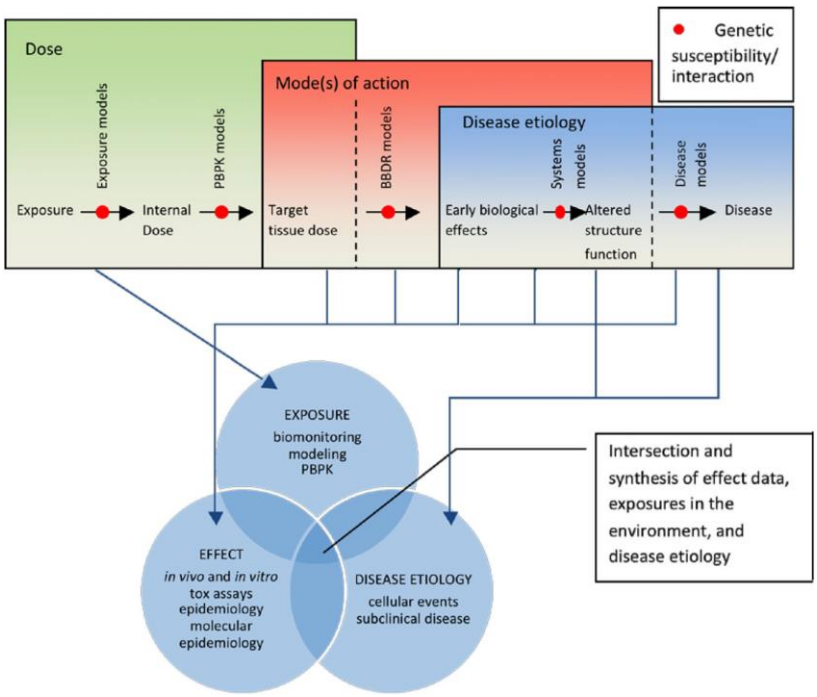


Figure 1: Schematic showing the intersection of domains of evidence. Notes: BBDR, biologically-based dose response; PBPK, physiologically-based pharmacokinetic (von Stackelberg et al., 2015).

In any case the authors tried to identify KEs relevant for DNT (Fig. 2) and discussed these in the context of putative DNT AOPs (Bal-Price et al., 2015b). These identified KE, although not really placed in an AOP framework, are still helpful for setting up an in vitro testing strategy for identification of DNT compounds as they re-iterate the importance of some modes of action like increased calcium release, ROS-induced apoptosis, impairment in synapse formation and altered neurotransmitter releases for cognition and learning.

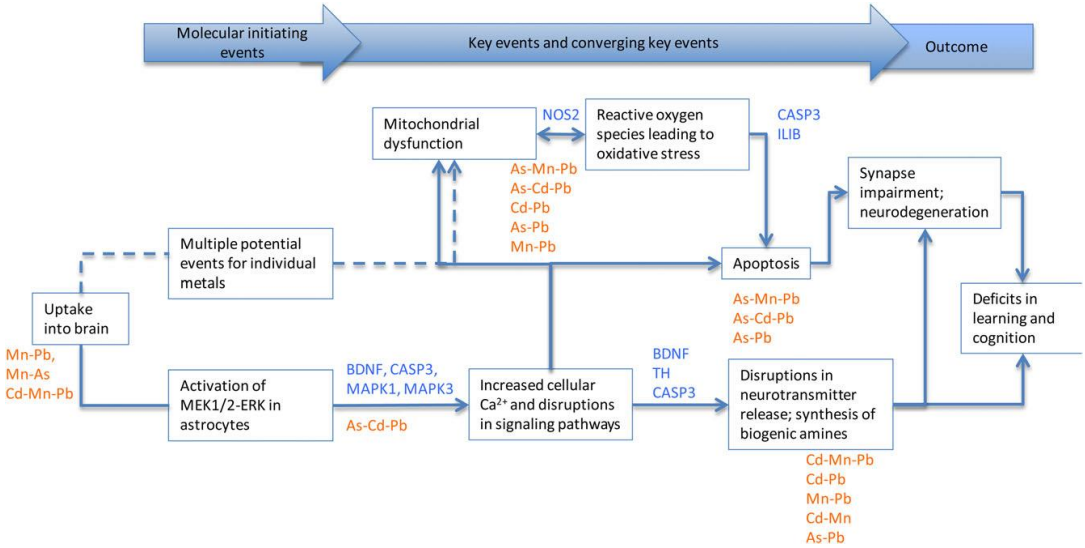


Figure 2: Proposed pathways of potential neurotoxicity of As–Cd–Mn–Pb (von Schackelberg et al., 2015).

3. EVALUATION OF NEURODEVELOPMENTAL SIGNALLING PATHWAYS

Because the number of available AOPs is not sufficient for proposing an AOP-informed in vitro testing strategy, here pathways of toxicity in models representing DNT are summarized. For one, the literature was screened for the published modes-of-action of the 12 known human DNT compounds (Grandjean and Landrigan, 2014), which have a known adverse outcome for behaviour/cognition in humans. These data is summarized in Table 3. As a second strategy, knowledge on pathways contributing to normal brain development was collected and is summarized here in Table 8. As this was not a systematic review, these data might not be comprehensive.
It is to note that proposed modes-of-action on DNT summarized here are not restricted to pesticides and their metabolites. The reason is that over all there is very few data

available that address DNT in humans. Thus, DNT information on all available compounds is displayed here.

3.1 SIGNALLING PATHWAYS ALTERED BY HUMAN DNT COMPOUNDS

In the following paragraphs, pathways contributing to DNT for the 12 known human DNT compounds identified by (Grandjean and Landrigan, 2014) were summarized. Therefore, PubMed searches were performed containing the chemical name combined with the Bolean operator AND brain development OR neurodevelopment OR developmental neurotoxicity in combination with AND signalling OR pathway. Retrieved results were analysed for alterations of pathways/disturbance of neurodevelopmental processes involved in DNT of compounds either in vivo or in vitro.

Methylmercury:
Methylmercury is so far the most extensively studied DNT compound. It acts through multiple cellular targets because of its ability to bind to SH-groups of proteins or peptides (Putative AOP VI in (Bal-Price et al., 2015b)). By this mechanism it induces oxidative stress, alters NO signalling, and interferes with mitochondrial and synaptic functions, leading to disturbed neurotransmission. Developmental exposure to methylmercury also disturbs human neural cell migration (Choi, 1986, Choi et al., 1978), which might also be the consequence of SH-group interference.

Arsenic:
Developmental exposure towards arsenic induces ROS formation and reduces NPC proliferation in rats in vivo (Chattopadhyay et al., 2002). Arsenic-induced ROS formation was also made responsible for mitochondrial dysfunction, caspase activation (Flora et al., 2009) and astrocyte cell death (Rai et al., 2010). In addition, Arsenic produces axon and fibre damage possibly via reduction of NO signalling (Rios et al., 2009). Also epigenetic alterations (reduction in acetylation and methylation) might contribute to DNT effects of arsenic (Cronican et al., 2013, Martinez et al., 2011, Zarazua et al., 2010).

Polychlorinated Biphenyls (PCBs):
PCBs activate GABAA receptors as a potential mode-of-action of their developmental toxicity ((Fernandes et al., 2010a, Fernandes et al., 2010b); common KE with AOP54 (AOP-Wiki)). In addition, they interfere with ryanodine receptors (RyR) leading to increased dendritic growth (Putative AOP IX in (Bal-Price et al., 2015b)). They also act as thyroid hormone (TH) disruptors in the developing brain, possibly through multiple mechanisms (AOP42,134,54,8,152 (AOP-Wiki)). That THs are crucial for brain development has long been established (Cranefield, 1962).

Toluene:
Toluene alters the expression of specific NMDA receptor subunits and the related signalling pathway components CaMKIV and CREB1 as well as the apoptosis-related

gene Bax and activates caspase-3 (Win-Shwe et al., 2010). It also alters gene expression related to synaptogenesis (Hester et al., 2011). A putative AOP for neurotoxicity of NMDA-receptor stimulation was published earlier, however, here the focus was not on the developmental adverse outcome (Putative AOP II, (Bal-Price et al., 2015b)).

Lead:
Lead can bind to NMDA-receptors during synaptogenesis. Thereby, lead inhibits the NMDA-receptor, causing inhibition of NMDA-dependent calcium currents with aberrant dendritic morphology and decreased synaptogenesis (Putative AOP I in (Bal-Price et al., 2015b)). Lead also dysregulates BDNF-Trk signalling causing alterations in synaptogenesis as a mechanism of DNT action (Stansfield et al., 2012).

Ethanol:
Ethanol alters developmental expression of BDNF and TrkB receptors (Light et al., 2001, Moore et al., 2004). Moreover, it alters genes involved in calcium signalling (Mandal et al., 2015). Developmental neurotoxicity of ethanol is also discussed to be mediated through interference with acetylcholine muscarinic receptor-stimulated inositol metabolism (Costa, 1994). In addition, it interferes with Phospholipase D activity with reduced generation of phosphatidic acid due to the generation of phosphatidylethanol. Phospholipase D is thought to be involved in cellular proliferation, exo- and endocytosis and neurite formation of different brain cell types due to its expression in neurons, astrocytes and oligodendrocytes (Klein, 2005).

Valproic Acid:
Valproic acid acts on NPC during brain development in a stage-specific manner. During embryonic development valproic acid interferes with wnt-catenin signalling (Foti et al., 2013) and reduces apoptosis (Go et al., 2012). During the fetal stage it induces apoptosis (Yochum et al., 2010, Go et al., 2012), decreases proliferation due to HDAC inhibition (Baumann et al. in preparation) and decreases neuronal differentiation (Foti et al., 2013). Valproic acid causes transcriptome changes (1000 – 4000 genes; (Krug et al., 2013b)) and specifically dysregulates for example genes coding for BDNF (Fukuchi et al., 2009, Almeida et al., 2014), GABAA-receptors, GAD65,67 (Fukuchi et al., 2009) and neuroligin3 (Kolozsi et al., 2009).
In addition, VPA as well as other HDAC inhibitors inhibit neural crest cell (NCC) migration (Dreser et al., 2015, Zimmer et al., 2014) and thus interfere with the development of a large number of cell types of the body including neurons and glial cells of the peripheral nervous system in addition to cranial bone and cartilage (Le Douarin et al., 2008). VPA also inhibits the differentiationfrom hESC to neuroepithelial precursors in vitro (Waldmann et al., 2014).

Manganese:
Manganese induces cell death via formation of oxidative stress (Dukhande et al., 2006) due to mitochondria-induced apoptosis (Tamm et al., 2008). Moreover, it dysregulates autophagy (rev in (Chen et al., 2016)).

Fluoride:
no mechanistic DNT data available

Tetrachloroethylene:
no mechanistic DNT data available

Pesticides:
DDT/DDE
DDE activates RXR and thereby promotes caspase-3-dependent NPC apoptosis (Wnuk et al., 2016). Moreover, AhR activation and inhibition of GPR30 are involved in NPC apoptosis (Kajta et al., 2014).

Organophosphates
Chlorpyrifos causes neuroinflammation with activation of NFkB in the substantia nigra of juvenile rats accompanied by a loss of dopaminergic neurons (Zhang et al., 2015).
Chlorpyrifos-oxon directly inhibits neurite outgrowth due to its reactive moiety by directly interfering with proteins including cytoskeleton proteins or Acetylcholinesterase (AChE, morphogenetic function; Rev. in (Flaskos, 2012)).
The OP insecticides and their oxons (chlorpyrifos (CPF), diazinon (DZ) and parathion (P), their oxygen analogs chlorpyrifos oxon (CPO), diazoxon (DZO) and paraoxon (PO), and their metabolites 3,5,6-trichloro-2-pyridinol (TCP), 2-isopropyl-6-methyl-4-pyrimidol (IMP) and para-nitrophenol (PNP)) affect astroglial cell proliferation. The transition from the G(0)/G(1) to the S/G(2) phase of the cell cycle may be particularly sensitive to the action of these compounds (Guizzetti et al., 2005).
The organophorphorous pesticide chlorpyrifos (CPF) induces apoptosis via an NFkB-dependent mechanism in hNPCs (Lee et al., 2014a). Moreover, CPF causes cholinergic hyperstimulation, oxidative stress, and interference with adenylate cyclase signalling in PC12 cells (Slotkin et al., 2007).
CPF or its metabolites CPF-oxon (CPFO) and TCP perturb neuronal morphogenesis of primary cultures of embryonic rat sympathetic neurons derived from superior cervical ganglia (SCG). Axon outgrowth was significantly inhibited by CPF or CPFO, but not TCP, at concentrations ≤0.001 μM or ≤0.001 nM, respectively. In contrast, all three compounds enhanced BMP-induced dendritic growth. Acetylcholinesterase was inhibited only by the highest concentrations of CPF (≥1 μM) and CPFO (≥1 nM); TCP had no effect on this parameter suggesting that the perturbed neuronal morphogenesis is independent of Acetylcholinesterase inhibition (Howard et al., 2005).
CPF and CPFO inhibited axonal growth in AChE+/+ DRG neurons, they had no effect on axonal growth in AChE−/− DRG neurons. However, transfection of AChE−/− DRG neurons with cDNA encoding full-length AChE restored the wildtype response to the axon inhibitory effects of OPs. These data indicate that inhibition of axonal growth by OPs requires AChE, but the mechanism involves inhibition of the morphogenic rather than enzymatic activity of AChE (Yang et al., 2008).

Exposure to CPF or CPFO (resulted in a concentration-dependent increase in mitochondrial length, a decrease in mitochondrial number (indicative of increased fusion events), and a decrease in their movement in axons of primary cortical neurons (Middlemore-Risher et al., 2011).
CPF, CPFO and TCP inhibit NGF-induced neurite outgrowth (branches per cell, fragments per cell, total neurite outgrowth per cell) in PC12 cells. While CPFO inhibits neurite outgrowth at concentrations inhibiting Cholinesterase (ChE), CPF and TCP inhibit neurite outgrowth without inhibiting ChE (Das and Barone, 1999).
CPF and CPFO altered neurodevelopmental biomarkers on the gene expression level in differentiating mouse D3 cells and thus a prediction model classified these OP as weak DNT compounds. Biomarkers are gene products for nestin, patatin-like phos- pholipase domain containing 6, and neurofilament medium polypeptide (Estevan et al., 2014).
DZO, a major metabolite of the insecticide DZ, disrupts the neuronal microtubule network formation of N2a cells by specifically reducing the betaIII-tubulin and the microtubule-associated protein 1B (MAP 1B), which are important for axonogenesis, while total beta tubulin as well as neurofilaments were unchanged (Sachana et al., 2014b).
DZO reduces axonal outgrowth of N2a cell and increased the expression of phosphorylated neurofilament heavy chain (NFH), while there was no significant change in total NFH. It also reduced the expression of the axon growth-associated protein GAP-43 at concentrations where Acetylcholinesterase was unaffected (Sidiropoulou et al., 2009).

Pyrethroids
Cypermethrin, a pesticide of the pyrethroid class causes astrocyte toxicity. An increased intracellular calcium release is found with activation of the MAP kinases pathways, increase in MMP2 expression and activation of the Reelin-Dab pathway with altered astrocyte migration (Maurya et al., 2014).
Bifenthrin belongs to the pyrethroid insecticides and inhibits neurite outgrowth of PC12 cells (Tran et al., 2006).

Organochlorines
Dieldrin alters gene expression of GABA(A) receptor subunits, which could produce GABA(A) receptors with altered functional properties (Liu et al., 1997).

Phenylpyrazoles
Fipronil (FIP) is an N-phenylpyrazole insecticide that reduces neurodevelopmental phosphorylation of the MAP-kinases ERK1/2. Moreover, FIP inhibits outgrowth of axon-like processes in differentiating mouse N2a (Sidiropoulou et al., 2011) cells as well as in PC12 cells (Lassiter et al., 2009).

Table 3: Pathways contributing to DNT potential of compounds with known human/animal/in vitro DNT adverse outcomes

Pathway	Function	Model Compound	Literature
SH-group binding by electrophils	Ubiquitous effects including reduced synaptic functions; possibly inhibition of cell migration	MeHgCl, acrylamide, acrolein	Putative AOP in (Bal-Price et al., 2015b, Moors et al., 2007)
ROS formation	Proliferation reduced	Arsenic	(Chattopadhyay et al., 2002)
	Mitochondrial function reduced, Caspase-3 activity induced	Arsenic	(Flora et al., 2009)
	JNK-ERK activation, Astrocyte cell death		(Rai et al., 2010)
	Apoptosis	Valproic Acid	(Go et al., 2012), Baumann et al. in preparation
	Cell death via mitochondria-induced apoptosis	Manganese	(Dukhande et al., 2006, Tamm et al., 2008)
Ca^{2+} release, ROS formation, JNK and P38 activation	(multiple) Inhibition of astrocyte proliferation, possibly glia migration	Cypermethrin	(Guizzetti et al., 2005, Maurya et al., 2014)
Inhibition of ERK1/2 phosphorylation	Inhibition of neuritogenesis	Fipronil	(Lassiter et al., 2009, Sidiropoulou et al., 2011)
Reduced nNOS function	Axon and fibre damage	Arsenic	(Rios et al., 2009)
Histone Hypoacetylation	Reduction in GFAP	Arsenic	(Cronican et al., 2013)
Reduced DNA methylation		Arsenic	(Martinez et al., 2011)
	Dimethylated	Arsenic	(Zarazua et al.,

	myelin		2010)
Activation of GABAA-receptors **Alteration of GABAA-receptor expression**		NDL-PCB Dieldrin	(Fernandes et al., 2010b) (Liu et al., 1997)
Ryanodine Receptor Sensitization	Increased dendritic growth	NDL-PCB	Putative AOP in (Bal-Price et al., 2015b)
TH disruption	Reduction in gene expression, e.g. BDNF	PCB, PTU	(Gilbert et al., 2016)
	Alterations in GABAergic neurons	PTU	(Gilbert et al., 2007)
	Altered learning and memory	PTU	(Westerholz et al., 2010)
	Reduced oligodendrocyte maturation	PBDE	(Dach et al. in revision)
	THRβ function	Carbaryl	(Sun et al., 2008)
NMDA-receptor activation	apoptosis	Toluene	(Win-Shwe et al., 2010); (Bal-Price et al., 2015b)
	mRNA expression synaptogenesis	Toluene	(Hester et al., 2011)
NMDA-receptor inhibition	Inhibition of synaptogenesis	Lead	Putative AOP in (Bal-Price et al., 2015b)
Inhibition of BDNF-Trk signalling		Ethanol, Lead	(Light et al., 2001, Moore et al., 2004, Stansfield et al., 2012)
Interference with calcium signalling			(Mandal et al., 2015)
Inositol metabolism		Ethanol	(Costa, 1994)
Interference with		Ethanol	(Klein, 2005)

Phospholipase D activity with reduced generation of phosphatidic acid			
HDAC inhibition	Alteration of Proliferation	Valproic Acid	(Foti et al., 2013)
	Alteration of neuronal differentiation	Valproic Acid	(Foti et al., 2013)
	Alteration of neural crest cell (NCC) migration	Valproic Acid	(Zimmer et al., 2012)
	Reduction of hESC differentiation of NEP		(Waldmann et al., 2014)
Dysregulation of autophagy		Manganese	Rev. in (Chen et al., 2016)
RXR activation	NPC Apoptosis	DDE	(Wnuk et al., 2016)
AhR activation	NPC Apoptosis	DDE	(Kajta et al., 2014)
GPR30 inhibition	NPC Apoptosis	DDE	(Kajta et al., 2014)
NFkB activation	Dopaminergic cell death	Chlorpyrifos	(Zhang et al., 2015)
	Apoptosis		(Lee et al., 2014a)
Direct interaction with cytoskeleton	Impaired neurite outgrowth	Chlorpyrifos-oxon	Rev. in (Flaskos, 2012)
		Chemotherapeutic alkaloids like Colchicine, Vinchristine, Nocodazole	(Krug et al., 2013a) Rev. in (Flaskos, 2012)
	Impaired glia differentiation	Chlorpyrifos-oxon	
	Impaired neuritogenesis by microtubule disruption	Diazinon (Diazoxon)	(Sachana et al., 2014a)
Binding to AChE	Inhibition of	Chlorpyrifos-oxon	Rev. in

	morphogenetic function of AChE		(Flaskos, 2012)
Intracellular calcium release	Astrocyte toxicity	Cypermethrin	(Maurya et al., 2014)
Activation of MAP kinase and Reelin-Dab pathways	Inhibition of astrocyte migration	Cypermethrin	(Maurya et al., 2014)
Interference with PIP metabolism	Alteration of synapse formation	Ethanol	(Tong and Sun, 1996)
Reduction of GAP43 protein			
	Inhibition of neurite outgrowth	Befenthrin	(Tran et al., 2006
(possibly disturbed cAMP-CREB signaling)	Perturbation of neuronal morphogenesis (axons and dendrites)	Chlorpyrifos	(Howard et al., 2005)
cAMP-CREB signaling	Memory	Organophosphates	(Adigun et al., 2010, Verma et al., 2009)
	Altered mitogenesis and mitochondrial movement	Chlorpyrifos	(Middlemore-Risher et al., 2011)

3.2 PATHWAYS CONTRIBUTING TO NEURODEVELOPMENTAL PROCESSES

The following pathways, which are collectively displayed in table 8, are pathways known to play a role in a variety of also non-neuronal related diseases. However, besides the role of miRNAs for brain development where data of essentiality is generated from knockout animal models, for all pathways indicated below there is evidence from human studies that their deregulation is involved in syndromes related to brain disorders.

3.2.1 THE CONVERGING SIGNALLING CASCADE OF BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF), EXTRACELLULAR SIGNALLING-RELATED KINASE (ERK) AND CAMP RESPONSIVE ELEMENT BINDING PROTEIN (CREB):

Learning about motivationally important stimuli involves plasticity in the amygdala, a temporal lobe structure. Amygdala-dependent learning involves a growing number of plasticity-related signalling pathways also implicated in brain development, suggesting that learning-related signalling in juveniles may simultaneously influence development. Rodent research has identified maturation of amygdala neurons throughout infancy and into adolescence. The recent review of Ehrlich and Josselyn (2016) summarizes comprehensively the converging BDNF–ERK– CREB signalling cascade in brain development and amygdala-dependent learning, especially fear-conditioning (Fig. 3). BDNF is constantly secreted from neurons as a survival factor. Moreover, it is a signal for activity-dependent synaptic modulation. It can act in an autocrine as well as paracrine manner. BDNF dimers bind the extracellular domain of the tropomyosin-related kinase B (TrkB) receptor, causing receptor dimerization and autophosphorylation. Subsequent binding of intracellular adaptor proteins leads to the activation of three major signalling cascades: ERK, phosphatidylinositol 3-kinase (PI3K) and phospholipase C-□ (PLC□). Extracellular signalling-related kinases are a family of effectors for a plasticity-related intracellular signal- ing cascade, activated not only by BDNF but also by neurotransmitter-dependent calcium signalling. CREB acts as an effector of multiple signalling cascades to transduce signals from synapses to the nucleus, regulating transcription of plasticity-related genes. It also serves as a point of convergence for the three major pathways activated by BDNF, and CREB is activated by a variety of extracellular signals including hormones, growth factors and synaptic activity as well as by activity-dependent calcium influx. In addition, CREB is a phosphorylation target of Akt (also known as protein kinase B), which is activated by BDNF and TrkB receptors via the PI3K pathway. Specific phosphorylation of CREB allows it to interact with transcriptional co-activators to promote transcription of genes enabling structural and functional plasticity of neurons. It is suggested that BDNF, ERK and CREB work in concert to adapt neuronal gene expression and function to developmental and environmental demands, i.e. for neuronal plasticity.

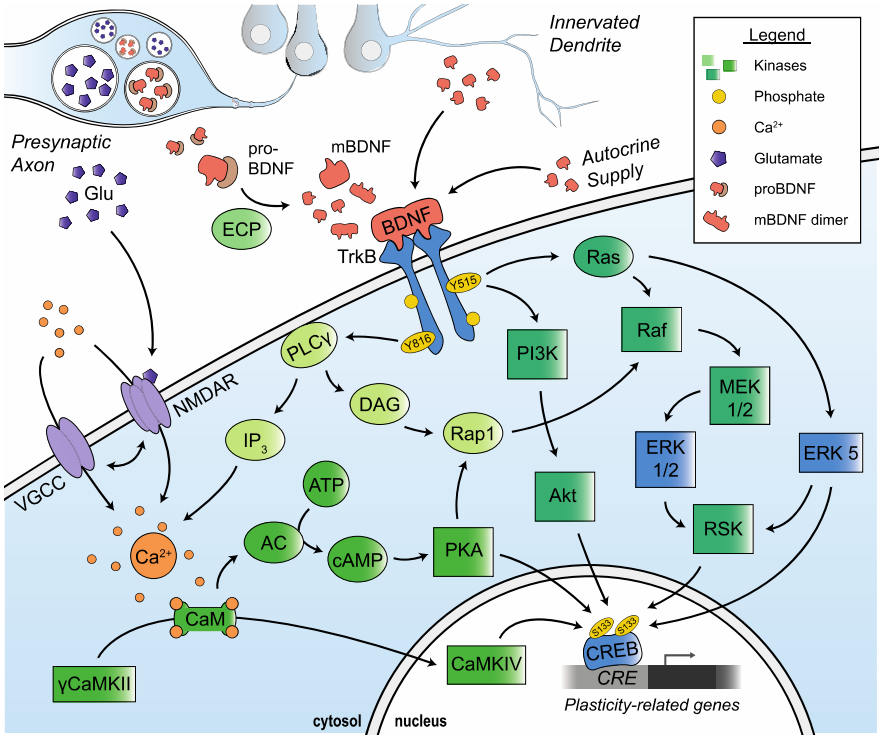


Figure 3: Intracellular signalling pathways linking BDNF, ERK, and CREB. (Ehrlich and Josselyn, 2016).

Given the myriad contributions of BDNF, ERK and CREB to neural development, dysregulation of their activity during development may influence brain function later in life. BDNF – ERK – CREB signalling influences a variety of structural and functional outcomes during brain development. These include cell density (neuronal survival), neuron morphology, synaptic connectivity (e.g. potentiates transmitter release from developing axons, promotes dendritogenesis), neuron excitability, GABAergic neurotransmission (Fig. 4) and promoting proliferation and differentiation of oligodendrocytes. Hence, interference with these signalling pathways during brain development is predicted to also affect amygdala-dependent motivation-associated learning later in life. It is suggested that the survival signals of the BDNF – ERK – CREB pathway might cause enhanced amygdala volume and neuronal density. Increased volume of the amygdala may result in greater amygdala activation and downstream signalling, potentially yielding exaggerated motivational learning (Fig. 4). In support of this notion, stress exposure in juveniles, which likely stimulates BDNF–ERK–CREB signalling, results in greater amygdala volume and emotional dysfunction in humans and non-human primates.

Intracellular signalling pathways may provide a direct interface between genetic and environmental risks for psychiatric illness. Due to the multitudes of its function, this signalling pathway poses a point of vulnerability to insult for the developing brain. This is supported by the observation that aberrant expression of BDNF, ERK and CREB has been linked to numerous psychiatric disorders, including autism spectrum disorders, mood disorders and schizophrenia.

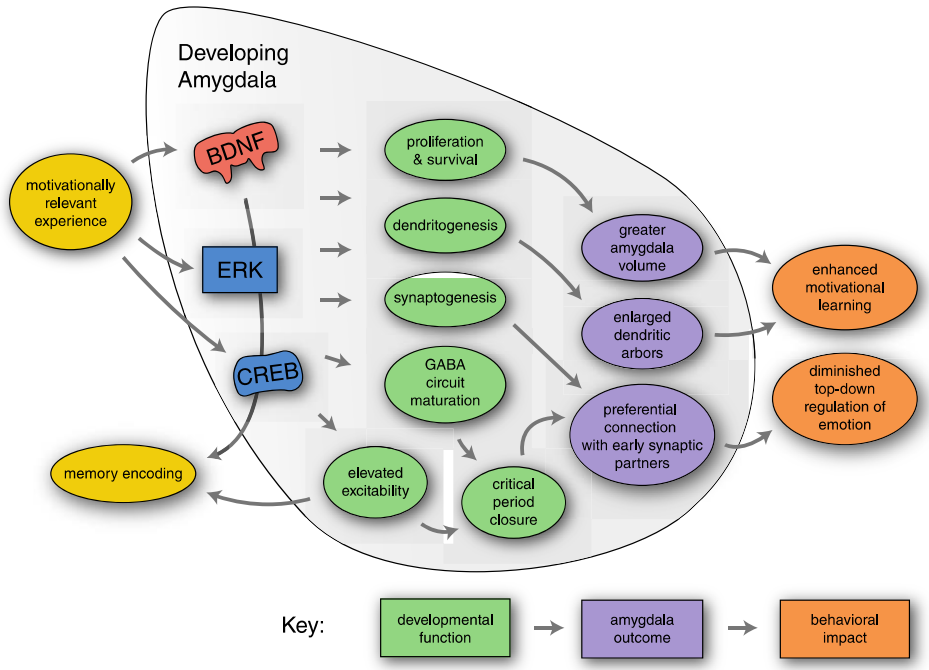


Figure 4: Predicted effects of learning-induced BDNF–ERK–CREB signalling on amygdala development (Ehrlich and Josselyn, 2016).

Contribution of the BDNF–ERK–CREB signalling pathway for neurodevelopmental processes (green boxes in Fig. 4) as well as for amygdala-dependent motivational learning are well established. In contrast, the effects of developmental alteration of this pathway for amygdala-related brain function later in life is not entirely supported by causality. However, indications for developmental functions of BDNF–ERK–CREB signalling are sufficient for regarding these pathways as neurodevelopmentally relevant. All references of primary literature for this chapter can be found in Ehrlich and Josselyn (2016).

3.2.2 NOTCH SIGNALLING IN CELL FATE DETERMINATION

The notch signalling pathway is well known as an important signalling mechanism for communication between neighboring cells. The pathway is crucial for NSC maintenance and differentiation. Notch signalling is essential for a process called 'lateral inhibition', which warrants that cells differentiate into distinct cell types from an initially uniform cell population (Fig. 5). In the germinal zone of developing mammalian brains, NSCs initially undergo proliferation only, and then subsets of cells start neuronal differentiation, while others remain as NSCs. During this process, pro-neural genes, such as Mash1/Ascl1 and Neurogenins (Ngn1, Ngn2), are expressed by a subset of NSCs thereby inducing the neuronal differentiation. In those cells undergoing neuronal differentiation, notch ligands such as Delta-like1 (Dll1) are up-regulated, which in turn activate notch signalling in neighboring cells. As a result of notch activation, neuronal

differentiation is inhibited in neighboring cells and they remain as NSCs (Rev. in (Imayoshi et al., 2013)).

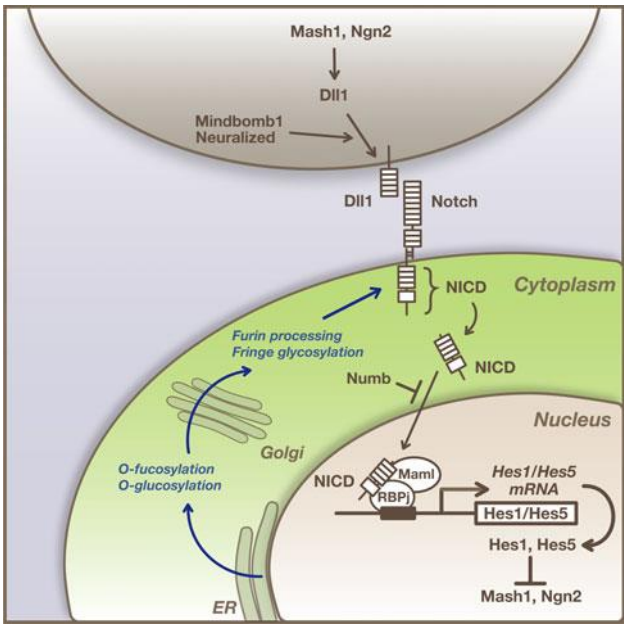


Figure 5: Notch signaling pathway. The proneural genes Mash1 and Ngn2 induce expression of notch ligands such as Dll1, which activate notch signaling in neighboring cells. Upon activation, the notch intracellular domain (NICD) is released from the transmembrane region and transferred into the nucleus, where NICD forms a complex with RBPj and induces Hes1 and Hes5 expression. Hes1 and Hes5 repress proneural gene expression. During maturation and trafficking to the cell surface, notch receptors undergo furin processing and glycosylation, which can impact their responsiveness to their ligands (Imayoshi et al., 2013).

3.2.3 CONSEQUENCES OF STIMULATION OF AKT-RELATED SIGNALLING FOR BRAIN DEVELOPMENT

Disorders of brain overgrowth are significant causes of intractable epilepsy, intellectual disability, autism, and other complex neurological problems like the rare disorders focal cortical dysplasias (FCDs), hemimegalencephaly (HMEG), dysplastic megalencephaly (DMEG), tuberous sclerosis, thanatophoric dysplasia and Apert syndrome. Recent genetic studies indicate that diverse forms of brain overgrowth are caused by *de novo* mutations that stimulate activity in the receptor tyrosine kinase (RTK)–phosphatidylinositol-3- kinase (PI3K)–AKT signalling pathway, a key mediator of growth factor, i.e. fibroblast growth factor (FGF), signalling in the developing brain. RTK-PI3K-AKT signalling is a key mediator of cell proliferation and growth in many biological systems (Fig. 6). RTKs are activated by growth factors that, in the developing brain, include insulin-like growth factor 2 (IGF2), FGF2, and others. All four FGF receptors, and the IGF I receptor (IGF1R), are RTKs that activate PI3K-AKT. Not

surprisingly, IGF1R and FGFRs also activate other signalling pathways, notably mitogen-activated protein kinase (MAPK) signalling mediated by the Ras-Raf-MEK-ERK cascade, so RTK-PI3K-AKT signalling is not purely linear. Indeed, divergent and convergent signalling occurs at all levels of the RTK-PI3K-AKT pathway, influencing the different phenotypes generated by specific gene mutations in the above-mentioned neurodevelopmental disorders.

Ligand-stimulated RTKs in turn activate PI3K, a lipid kinase that phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), a potent second messenger. Pten, a negative modulator of RTK-PI3K-AKT signalling, converts PIP3 back to PIP2. PIP3 binds and activates AKT (also known as protein kinase B), a protein kinase with more than 100 substrate proteins. Accordingly, AKT activation has pleiotropic effects that are often context-specific. In brain development, as in cancer, AKT activation generally leads to increased proliferation, decreased apoptosis, and increased cell growth, although the specific effects differ among neural progenitors, neurons, and glia. Some processes, such as proliferation and cell growth, have been related to specific pathways downstream of AKT, some of the most important of which are shown in Fig. 6.

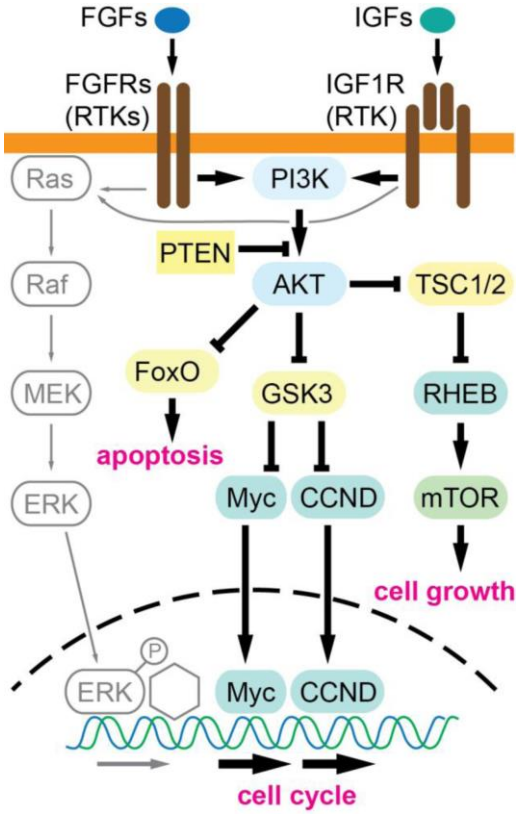


Figure 6: The RTK-PI3K-AKT signalling pathway. Growth factors bind RTKs such as FGFRs and IGF1R, leading to activation of PI3K, then AKT, then multiple divergent downstream pathways. RTKs can also activate the parallel Ras-Raf-MEK-ERK cascade, illustrated for comparison (Hevner, 2015).

The best-known pathway downstream of AKT is mTOR (mammalian target of rapamycin), a pathway that positively regulates cell growth. The mTOR protein is part of two different complexes, mTORC1 and mTORC2. Cell growth is promoted by AKT activation of mTORC1. The pathway from AKT to mTOR is controlled by hamartin (TSC1) and tuberin (TSC2) negatively regulating mTOR signalling (Fig. 7). Thus, the mTOR pathway is highly activated in tuberous sclerosis, representing a major branch downstream of RTK-PI3K-AKT signalling. Other major pathways influenced by AKT include the GSK3 (glycogen synthase kinase 3) pathway, which promotes cell proliferation via Myc and Cyclin-D (CCND). More detailed information in the role of mTor in brain development can be found below. Also FoxO (forkhead box O) is linked to the AKT pathway: FoxO activation promotes apoptosis and is negatively regulated by AKT. The two brain overgrowth syndromes, DMEG and HMEG, have been associated with genes all along this pathway, indicating that genetic heterogeneity probably explains much of the gross and histopathologic heterogeneity in these disorders.

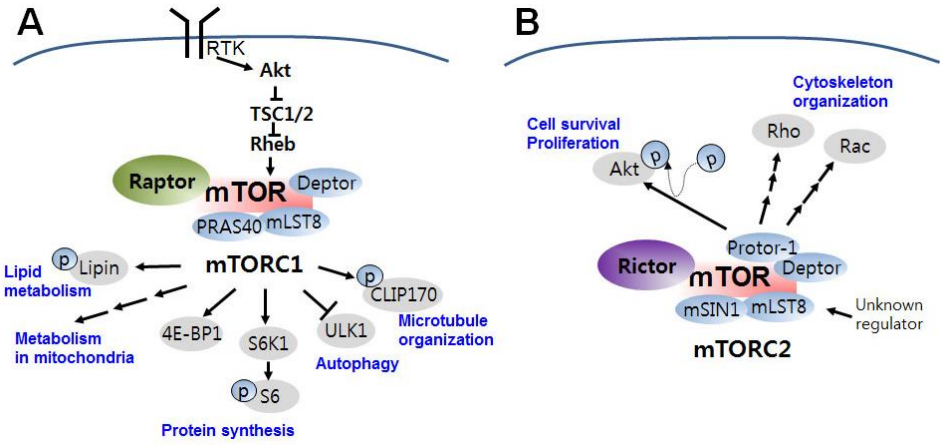


Figure 7: Involvement of mTORC1 (A) and mTORC2 (B) complexes and their downstream signalling targets in multiple cellular functions (Lee, 2015).

Clinical manifestations and structural pathologies with the genetic bases of these disorders targeting RTK-PI3K-AKT pathways are summarized in Table 5:

Table 5: Mutations in genes belonging to the RTK-PI3K-AKT pathways causing clinical syndromes in humans

Mutation	Clinical feature
FGFR3 activating mutations	Thanatophoric dysplasia
FGFR2 activating mutations	Apert syndrome
IGFR activating	Brain overgrowth
IGFR inhibiting	Microcephaly
PIK3CA or PIK3R2 activating mutations (encoding PI3K subunits)	Severe brain overgrowth (HMEG or DMEG)
Mosaic PIK3CA mutation	FCD type IIa (a disorder with many features of HMEG and DMEG)*
GSK3-dependent CCND2 (cyclin-D2) mutation	Megalencephaly syndrome
Mosaic mTOR mutation	HMEG
TSC1 & TSC2 (upsteam of mTOR) loss-of-function mutations	Tuberous sclerosis
PTEN & TSC mutations	Association with some autism cases

FCD - focal cortical dysplasia, HMEG - hemimegalencephaly, DMEG - dysplastic megalencephaly

* FCD type II, HMEG, and DMEG seem to form a spectrum differing mainly in the extent of cortex containing mutant cells

All references concerning this paragraph are found in Hevner et al. 2015.

3.2.4 ROLES OF mTOR SIGNALLING IN BRAIN DEVELOPMENT

mTOR signalling is transported by two functionally and structurally distinct types of mTOR complexes. Type I mTOR complex (mTORC1) is composed of mTOR, raptor, mLST8, PRAS40 and DEPTOR. mTORC1 and plays a role in cell proliferation, growth

through the regulation of RNA translation, nutrient metabolism and autophagy (Fig. 7A). mTORC1 signalling pathway is controlled by the signals from receptor tyrosine kinase-RAS in the brain. Type 2 mTOR complex (mTORC2) is composed of rictor, mSIN1, Protor-1, mLST8 and DEPTOR. mTORC2 modulates cell survival and proliferation through the activation of AKT/PKB by direct interaction through phosphorylation of AKT/PKB at position Ser473. However, the upstream signalling molecule leading to mTORC2 activation has not been well identified so far (Fig. 7B).
mTOR signalling is crucial for different processes of brain development including maintenance of NPC stemness, migration and neurogenesis (Fig. 8).

- mTOR and regulation of stemness in brain

mTOR- GSK3 signalling pathway activation is essential for the maintenance of NPC homeostasis showing that the inactivation of mTOR in nestin-positive NSCs results in smaller brain size and abnormalities in NSC self-renewal and proliferation.

- Function of mTOR signalling for neurogenesis/migration

Neuronal differentiation is controlled by fine tuning the processes of both spatial and temporal patterning of neurons thereby ensuring normal brain development. Defects in neuronal differentiation result in abnormal neuronal network formation in the brain causing in impairment of cognition, movement and perception. mTORC1 hyperactivation in subventricular NPC by ectopic expression of constitutively active Rheb, an upstream positive regulator of mTORC1, causes tuberous sclerosis-like lesions, i.e. ectopic and premature neuronal differentiation and integration, micronodule formation, and hypertrophic neuronal morphogenesis. Ectopic neural cells are found due to disturbed cell migration (Lafourcade et al., 2013).

- mTOR signalling in gliogenesis

Glial cell functions are critical for maintaining homeostasis of neurons by ensuring energy metabolite supply, clearance of extracellular glutamate and potassium, myelination, modulation of neuronal activity and synaptic plasticity. In the process of astrocyte differentiation, mTORC1 signalling pathway has a crucial function. Raptor deficiency in NSCs results in reduced NSC growth and inhibited astrocyte differentiation via downregulation of the mTOR downstream signalling pathway STAT3.

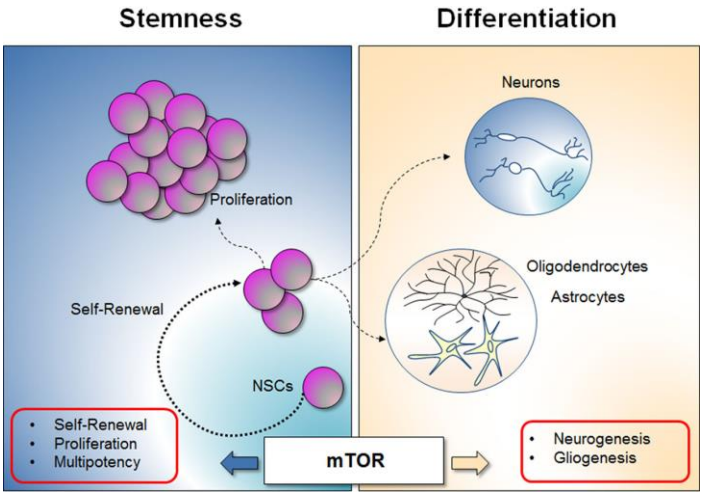


Figure 8: Functions of mTOR in NSCs. The activity of mTOR complexes is one of the key regulation factors for both the maintenance of NSC stemness and the process of neuronal and glial differentiation (Lee, 2015).

As mTOR signalling is crucial for a variety of neurodevelopmental processes, genetic variations in mTOR-related pathways have been associated with neurodevelopmental disorders in children. These are summarized in Fig. 9.

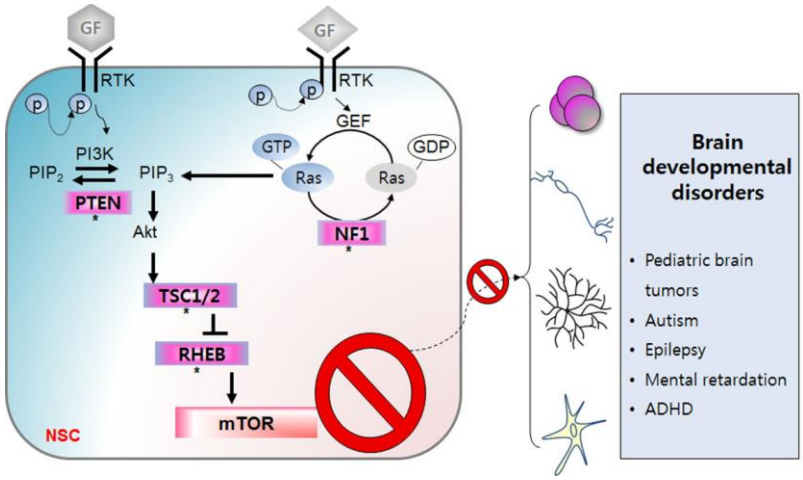


Figure 9: Clinical implication of mTOR upstream regulators in pediatric brain tumors and various brain developmental disorders. Receptor tyrosine kinase (RTK) signals induced by growth factors (GFs; e.g., EGF and PDGF) lead to the activation of mTOR through the modulation of upstream molecules including RAS, PTEN, AKT, RHEB and TSC1/2 in NSCs. The mTOR signal is involved in multiple NSC functions, such as NSC proliferation and differentiation into neurons and glial cells. The abnormalities in mTOR activity caused by mutations in PTEN, TSC1/2, RHEB and NF1 (neurofibromin) (*) are frequently observed in the patients with pediatric brain tumors (gliomas) and neurological disorders (autism, epilepsy, mental retardation and ADHD) (Lee, 2015).

All references belonging to this chapter can be found in Lee (2015).

3.2.5 ROLES OF PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C γ1 (PLCγ1) IN BRAIN DEVELOPMENT

During neural development, RTK-mediated PLCγ1 activation contributes to the neurodevelopmental processes neurite outgrowth, neuronal migration, axon guidance, synapse formation and maturation. For these contributions, different upstream molecules are activated (see Fig. 10 and 11). Cell-specific effects of upstream molecule-PLCγ1 interaction are regulated via cell type-specific expression of these molecules.

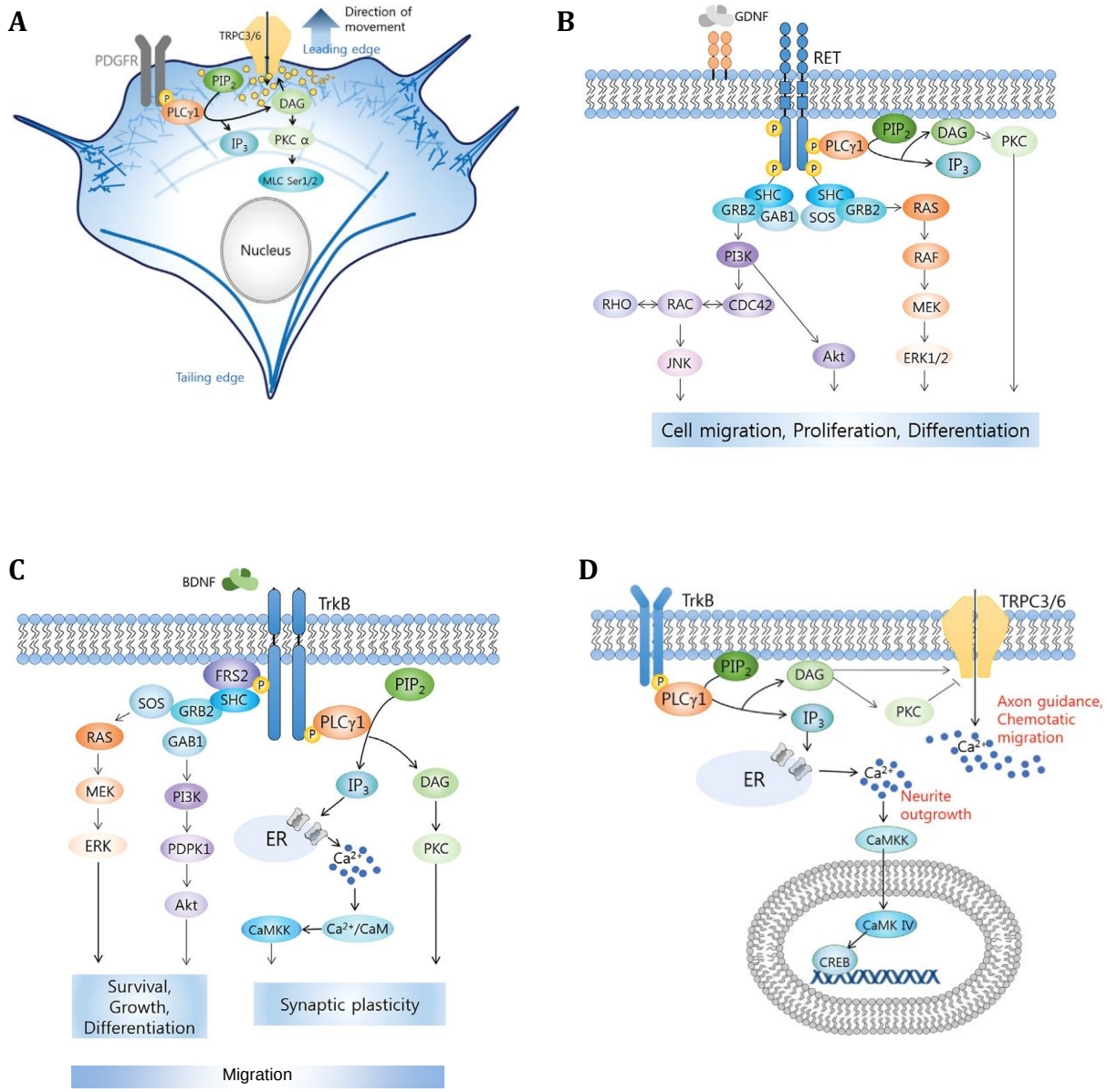


Figure 10: Distinct contributions of PLCγ1 to neurodevelopmental processes. A PDGFR-dependent migration; B RET & JNK-dependent migration, RET & AKT-dependent proliferation, RET & ERK-dependent differentiation; C Phosphorylation and recruitment of adaptor proteins to TrkB leads to activation of the Ras- MAPK signalling pathway. It induces neuronal differentiation and proliferation through MAPK/ERK kinase. TrkB recruitment and activation of PLCγ1 results in the generation of IP3 and DAG. DAG stimulates protein kinase C (PKC) and TRP channels contributing to synaptic plasticity; D BDNF/TrkB-mediated signalling activates PLCγ1. DAG directly activates TRP3/6

channels. Localized Ca^{2+} signalling in the growth cone can provide the intracellular directional cue for extension and induce growth cone turning. To induce the axon growth cone turning response, transient Ca^{2+} influx via TRPC3 and TRPC6 channels is necessary (Kang et al., 2016).

- Neurite outgrowth:

PLCγ1 mediates regulation of intracellular signalling in response to activation of FGF receptor (FGFR). Especially, the orchestration of both calcium and Protein kinase C (PKC) signalling is crucial in the FGFR activation through PLCγ1 during the regulation of neurite outgrowth (Fig. 11). The calcium signalling influences on several other kinases and members of the Rho family of small GTPases, such as Rho A, Rac1, or Cdc42 which eventually polymerize or depolymerize the actin filaments.

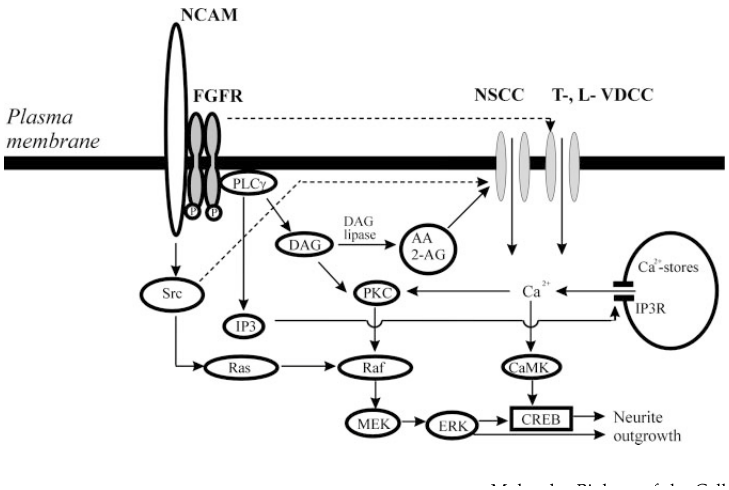


Figure 11: Specific roles for NCAM/FGFR/PLCγ1 in neurite outgrowth. Concerted actions of Ca^{2+} release, PKC and MAPK activation are required for neurite outgrowth. Signalling pathways activated NCAM activation. CaMK, calcium-calmodulin kinase; CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; Src, Src-family kinases. (Kiryushko et al., 2006).

- Neuronal migration:

The local concentration of diacylglycerol (DAG) is critical for generating the directional movement in mesenchymal chemotaxis. Platelet-derived growth factor receptor (PDGFR)-mediated activation of PLCγ1 produces an intracellular gradient of DAG (Fig. 10A). Subsequently, the asymmetric distribution of DAG activates PKC, which in turn inactivates Myosin IIA through a Ser1/2 phosphorylation of the regulatory light chain (RLC; also known as MLC-20). Inactivation of MyoIIA at the leading edge of the migratory cell generates the asymmetric moving force in directional migration. These data indicates a mechanism of PLCγ1 signalling that is involved in directional migration.

GDNF-RET signalling regulates neocortical neuronal progenitor migration through the PLCγ1 binding domain Tyr1015. GDNF signalling stimulates cytosolic Ca^{2+} release through the PLCγ1 binding on Tyr1015 of RET. It induces the increment of cytosolic Ca^{2+} level and subsequently phosphorylates to ERK1/2 and calcium/calmodulin-dependent protein kinase II (CaMKII; Fig. 10B).

BDNF-mediated PLCγ1 activation is important for cortical neuronal migration. BDNF/TrkB regulates the Ras/mitogen activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K) pathway. These signalling pathways are activated by SHC/FRS-2 binding at Y515 site of receptor TrkB. In addition, phosphorylation of the Y816 site in TrkB activates a CaMKII pathway through PLCγ1. During cortical development, mutation in both the Shc and PLCγ1 docking site of TrkB caused radial migration defect and consequent delayed cortical development, whereas each of single mutation in the docking sites of TrkB did not lead delayed cortical development. This suggests that Ras/ERK, a downstream signalling of PLCγ1, and PI3K/Akt signalling serve as complementary to the TrkB- mediated neuronal migration (Fig. 10C).

- Axon guidance:

Asymmetric Ca^{2+} gradients provide an instructive signal to the growth cone. Asymmetric Ca^{2+} gradients are essential to induce neuronal axon guidance as well as directional cell migration. Interestingly, the treatment with blockers to IP3 receptor restricted axon outgrowth, while the blocking Ca^{2+} release from the stores had no effect on guidance of axons. In contrast, chemically blocking Ca^{2+} entry through TRP channels not only restricted axon outgrowth but also severely misrouted axons. Hence, calcium signalling by store release or by Ca^{2+} entry through TRPC3/6 channels respectively mediates neurite outgrowth or both axon outgrowth and guidance respectively (Fig. 10D).

- Synapse formation and maturation:

PLCγ1 regulates functional and structural synaptic plasticity through BDNF/TrkB and ephrin-A1/EphA4 signalling, respectively.

During brain development, defective PLCγ1 signalling is associated with a variety of neurological diseases. Based on genomic fidelity and copy number variations, PLC□1 has been related to the following neurodevelopmental disorders as a contributing genetic factor (Table 7):

Table 7: Mutations in PLCγ1 causing clinical syndromes in humans

Mutation	Clinical feature
PLCγ1	Autism spectrum disorders Hippocampal atrophy Attention deficit hyperactivity disorder

All references concerning these pathways can be found within (Kang et al., 2016).

3.2.6 PHOSPHOINOSITIDES (PIPS) METABOLISM IN BRAIN DEVELOPMENT

Despite phosphoinositides (PIPs) are minor components of synaptic membranes, their exceptional high rate of metabolic turnover and their compartmentalization make them key players in postsynaptic excitability. The presence at dendritic spines of the enzymes that interconvert different PIPs supports a relevant role for these lipids in the dynamics of these structures (Fig. 12). Continuous synthesis and availability of phosphatidylinositol (3,4,5) triphosphate (PIP3) at the postsynaptic terminal is necessary for sustaining synaptic function. Appropriate levels and clustering of phosphatidylinositol(4,5) diphosphate (PIP2) at the postsynaptic membrane, which are modulated by the activities of Phospholipase γ (PLCγ) and PIP5K, are important for synaptic plasticity, both Long Term Potentiation (LTP) and Long Term Depression (LTD). The class I phosphatydilinsositol-3-kinase (PI3K) constitutively localizes at synapses by means of a direct interaction between its p85 subunit and the AMPARc. By converting PIP2 into PIP3 this kinase ensures the delivery of new AMPARc into spines in response to NMDARc activation and the maintenance of AMPARc clustering at the postsynaptic membrane (Fig. 12; Rev. in (Dotti et al., 2014)).

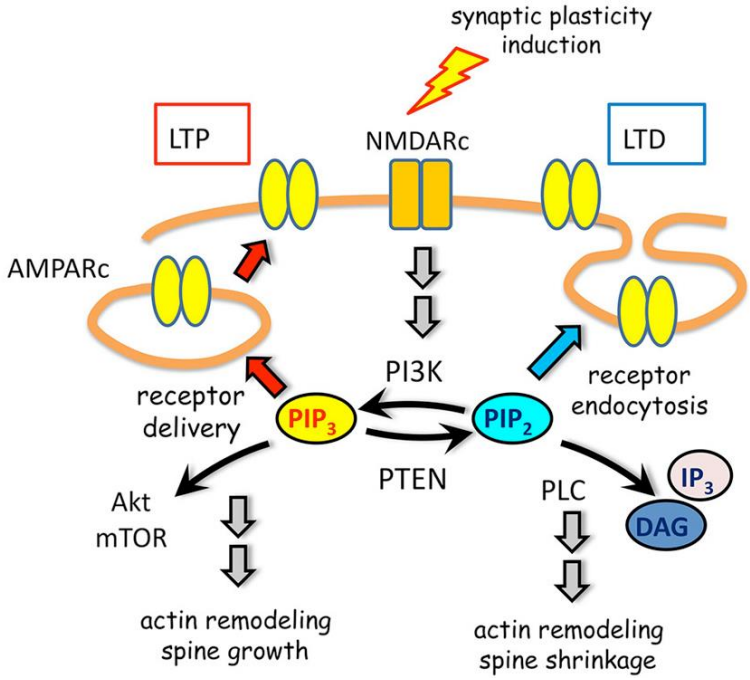


Figure 12: PIP metabolism in spine plasticity. Activation of NMDARc modulate AMPARc trafficking through spatially and timely controlled activity of PIPs and their metabolic enzymes. On one hand, PI3K association with AMPARc is required for receptor cell surface delivery during LTP. On the other hand, PTEN activity leading to PIP3 downregulation promotes migration of AMPARc from the postsynaptic density to the perisynaptic membrane. This depresses AMPARc synaptic responses by promoting receptor endocytosis during LTD. Moreover, signalling pathways initiated by PIP3 or PIP2, in which Akt/mTOR, DAG and IP3 are involved, contribute to actin remodeling and spine changes in size (Dotti et al., 2014).

Lipids participate in dendritic spine physiology as they play relevant roles in neurotransmission, through the control of spine architecture and by modulating neurotransmitter receptor function. As key components of postsynaptic membranes, lipids affect synaptic plasticity by shaping the membrane and modulating the levels, compartmentalization, interactions, trafficking and signalling properties of many proteins that are essential for synaptic function. By these means lipids regulate glutamate receptor function and actin cytoskeleton dynamics, which are instrumental features for postsynaptic plasticity (Rev. in Dotti et al. 2014).

Based on the important role of PIPs in the neurodevelopmental process of synaptogenesis as well as LTP and LTD, it is not surprising that alterations in PIP metabolism in humans contributes to neurodevelopmental syndromes. One syndrome that amongst others has the feature of mental retardation is the Lowe Syndrome (also known as Oculocerebrorenal Syndrome of Lowe; Fig. 13). It is caused by mutations in an inos- itol-5-phosphatase, which was named OCRL after the initials of the name of the syndrome. In addition, altered PIP metabolism is seen in Down Syndrome and other

psychiatric diseases like schizophrenia or bipolar disorder (Rev. in McCrea and De Camilli, 2009).

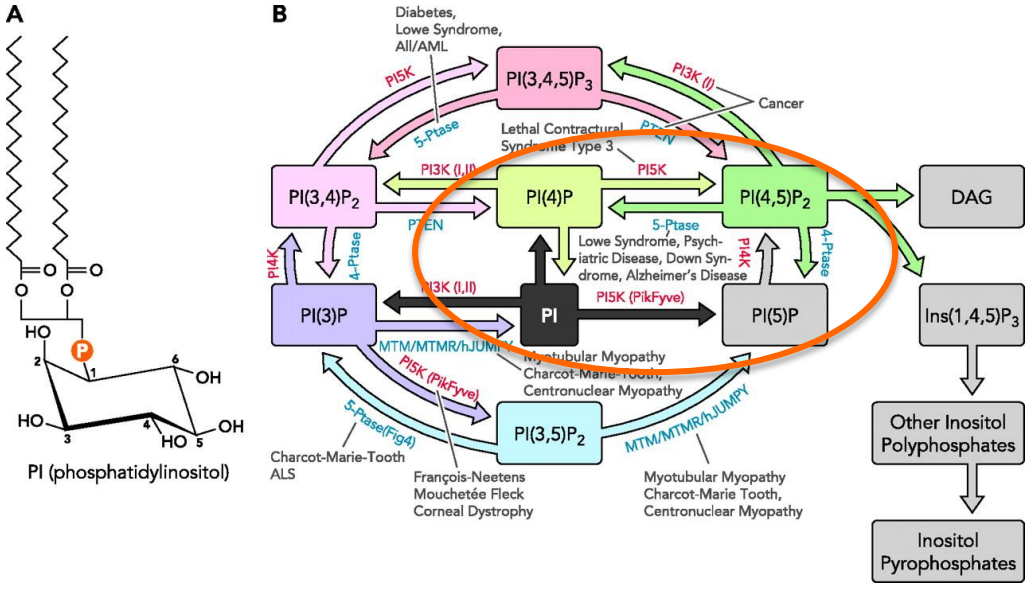


Figure 13: Phosphoinositide metabolism and associated disease.
A: chemical structure of phosphatidylinositol with numbered positions of the inositol ring indicated. B: depiction of the main pathways of phosphoinositide synthesis and degradation. Diseases associated with the kinases and phosphatases that regulate this interconversion are indicated (McCrea and De Camilli, 2009).

In addition, there are neurotoxicological indications that disturbance of PIP metabolism by exogenous noxae, like ethanol, during development might cause DNT (Tong and Sun, 1996).

3.2.7 THYROID HORMONES (TH) AS TROPHIC FACTORS DURING BRAIN DEVELOPMENT

Clinical studies link inadequate TH levels during gestation with compromised neurodevelopment that manifests as moderate to severe mental retardation, ataxia and sensory deficits (de Escobar et al., 2004, Patel et al., 2011, Williams, 2008). One clinical example for the essentiality of TH for brain development in humans is the Allan-Herndon-Dudley-Syndrome (AHDS). These patients possess a mutated TH transporter (MCT-8) gene leaing to a non-functional MCT-8 and thus a lack of TH uptake across the blood-brain-barrier into the brain. This brain hypothyroidism causes severe health problems in the AHDS children with severe mental retardation (rev. in Bernal et al., 2015). Even mild reductions in maternal TH levels during early pregnancy are associated with decreased IQ in offspring (Gyamfi et al., 2009, LaFranchi and Austin, 2007). Experimental studies in rodents confirm that thyroxine (T4) and the more active 3,3′,5-triiodothyronine (T3), are essential for normal neurodevelopment (de Escobar et al., 2004, Patel et al., 2011, Williams, 2008, Haddow et al., 1999, Schalock et al., 1977, Zoeller and Crofton, 2005), and can influence numerous neurodevelopmental processes including proliferation of neural precursor cells (NPC) and glia, neuronal migration, axonal and dendritic morphogenesis, synaptogenesis and myelination (Patel et al., 2011). Such studies establish TH signaling as a critical factor in regulating human brain development.

3.2.8 PROSTAGLANDIN E_2 IN BRAIN DEVELOPMENT

Lipid mediators are important signalling molecules. One of particular importance in brain development is Prostaglandin E_2 (PGE_2). PGE_2 is a bioactive fatty acid that is derived from arachidonic acid, a major structural component of plasma membrane phospholipids, through enzymatic metabolism of cyclooxygenases −1 and −2 (COX-1,-2) and different prostaglandin synthases. Diverse actions of PGE_2 are mediated through activation of 4 different G-protein coupled E-prostanoid receptors (EP1 through 4). Different PGE_2 functions are amplified by the variety of different kinase- mediated signalling cascades that are activated through its EP receptors, such as the protein kinase A (PKA), phosphatidylinositide 3-kinases (PI-3K), and protein kinase C (PKC) pathways. EP receptor expression peaks during the time of neurogenesis in mouse brain implying a role of PGE_2 in brain development. Specifically, PGE_2 plays a regulatory role in membrane excitability and synaptic transmission, thus synaptic plasticity in neurons, increases dendritic length and complexity of Purkinje neurons, and can alter neuronal firing activity in the developing brain. PGE_2 can also be involved in neuronal apoptosis and neuronal differentiation. Moreover, inhibition of COX-2 suppresses neurogenesis and proliferation of NPC.

Abnormal PGE_2 signalling may contribute to the pathology of neurodevelopmental disorders such as Autism Spectrum Disorders (ASD) as abnormal levels of PGE_2 and other fatty acid metabolites have been identified as potential biomarkers for ASD. Moreover, maternal exposure towards the drug misoprostol (prostaglandin E analogue), was associated with the development of autistic-like symptoms in children. Mechanisms of PGE_2 controlling neurodevelopmental processes might involve interaction with wnt singnalling, one important player in embryogenesis. Wnt controls a variety of processes like neural stem cell self-renewal, expansion, asymmetric cell division, maturation and differentiation (rev. in (Bengoa-Vergniory and Kypta, 2015)). Wong et al. (2014) recently showed a crosstalk between wnt and PGE_2 singnalling (Fig. 14) affecting neural proliferation and migration as well as gene expression. These features were also found altered in patients with ASD.

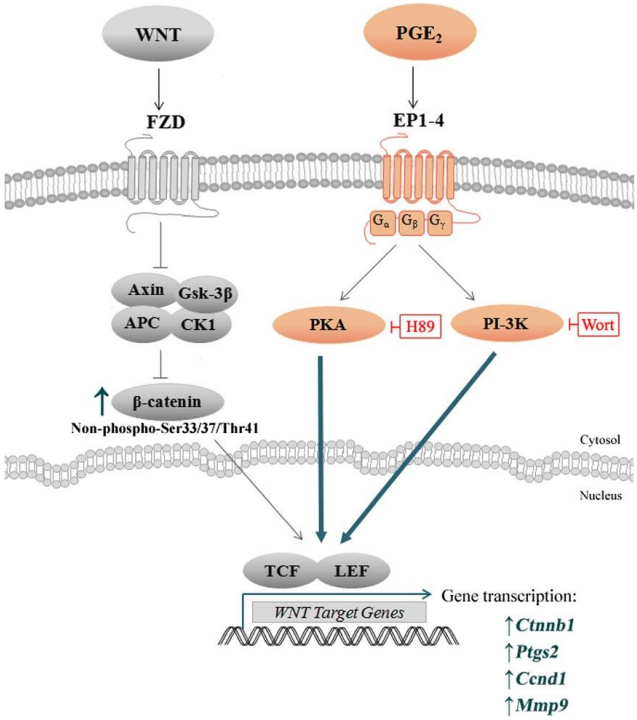


Figure 14: A proposed model for PGE_2-Wnt interactions in Wnt-induced NE-4C cells. From the compilation of our results (bolded) and other studies, a schematic model is drawn of the mechanism by which PGE2 might interact with the canonical Wnt pathway (Wong et al., 2014).

3.2.9 ROLE OF miRNAS IN BRAIN DEVELOPMENT

MicroRNAs (miRNAs) are single-stranded, endogenously expressed non-coding RNAs of 22–24 nucleotides acting as regulators of gene expression via the posttranscriptional

level (Bartel, 2004). Knowledge on their contribution to brain development has been gained through knockout (KO) studies in mice by either creating cell or tissue-specific conditional Cre-mediated Dicer KO or by knocking out specific miRNAs. These studies revealed that miRNAs play crucial roles in brain development (Fig. 15). Special roles during mouse brain development were attributed to miR-9, -124 and the -17-92 cluster (Petri et al., 2014).

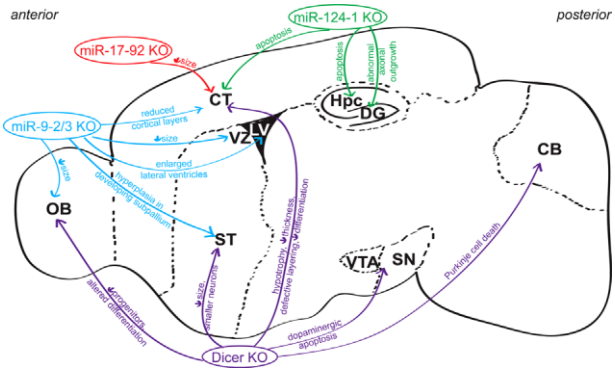


Figure 15: Dicer and miRNA knockouts, and their effect on brain development. A summary figure on the outcome of KOs for Dicer, miR-9-2/3, miR17-92 and miR-124-1. The figure gives an overview of effects on the olfactory bulb (OB), cortex (CT), striatum (ST), ventricular zones (VZ), lateral ventricles (LV), hippocampus (Hpc), the dentate gyrus (DG) of the Hpc, the cerebellum (CB) and the dopaminergic populations of the ventral tegmental area (VTA) and substantia nigra (SN) (Petri et al., 2014).

Table 8 summarizes the pathways found implicated in brain development that are described in the paragraphs above.

Table 8: Pathways contributing to brain development: lessons from neurobiology/neuropathology

Pathway	Function	Literature
BDNF-ERK-CREB	Proliferation and Survival Neurite growth, Dendritogenesis Synaptogenesis Neuronal plasticity GABA circuit maturation	Rev. in Ehrlich and Josselyn (2016)
RTK-PI3K-AKT activation	Increased proliferation Decreased apoptosis Increased cell growth	Rev. in Hevner (2015)
mTOR	Increased cell growth	Rev. in Hevner (2015)
FoxO	Apoptosis	Rev. in Hevner (2015)

GSK3B	Proliferation	Rev. in Hevner (2015)
PDGFR-PLCγ1	Neuronal migration	Rev. in Kang et al. (2016)
NCAM/FGFR- PLCγ1 with action on small GTPases, such as Rho A, Rac1, or Cdc42	Neurite outgrowth	Kiryushko et al. 2006
FGFR-mediated PLCγ1 - PKC activation with subsequent IP3 and AA	Neurite outgrowth	Rev. in Kang et al. (2016)
PDGFR-mediated activation of PLCγ1 with production of an intracellular DAG gradient	Neuronal migration	Rev. in Kang et al. (2016)
GDNF-RET-mediated activation of PLCγ1 and activation of ERK and CaMKII	Neuronal migration	Rev. in Kang et al. (2016)
BDNF/TrkB activates MAPK and PI3K pathways and PLCγ1-dependent IP3-mediated calcium release	Neuronal migration	Rev. in Kang et al. (2016)
PLCγ1-dependent calcium release with activation of PKC	Axon guidance, chemotactic migration	Rev. in Kang et al. (2016)
BDNF/TrkB - PLCγ1	Functional synaptic plasticity	Rev. in Kang et al. (2016)
Ephrin-A1/EphA4 - PLCγ1	Structural synaptic plasticity	Rev. in Kang et al. (2016)
mTOR- GSK3β	Maintenance of NPC stemness, self-renewal and proliferation	Rev. in Lee (2015)
mTORC1 hyperactivation	Migration, neuronal differentiation	Rev. in Lee (2015); Lafourcade et al. (2013)
Decreased mTORC1-STAT3 signalling	Decreased NSC proliferation and astrocyte differentiation	Rev. in Lee (2015)
Stimulated mTORC2 signalling	Increased gliogenesis	Rev. in Lee (2015)
PIP metabolism	Dendritic spine formation, synaptic plasticity, LTP,	Rev. in Dotti et al. (2015)

	LTD	
TH	Neuron and Oligodendrocyte formation and/or maturation	López-Espíndola et al. (2014), Rev. in Bernal et al. (2015)
Prostaglandins (cyclooxygenases, EP receptors)	NPC proliferation, neuronal apoptosis, differentiation, dendrite formation, synaptic plasticity	Rev. in Wong et al. (2014)
Wnt signalling	neural stem cell self-renewal, expansion, asymmetric cell division, maturation and differentiation	Rev. in Bengoa-Vergniory & Kypta (2015)
PGE_2 – wnt signalling	Neural migration, proliferation	Wong et al. (2014)
Notch	Lateral inhibition (inhibition of neurogenesis and promotion of gliogenesis in neuron neighboring NSC)	Rev. in Imayoshi et al. (2013)
miRNA-17-92 cluster	Radial glia proliferation, neuronal generation during corticogenesis	(Petri et al., 2014)
miRNA-9	NPC proliferation, differentiation	(Petri et al., 2014)
miRNA-124	Neurogenesis-gliogenesis, neuronal fate determinant	(Petri et al., 2014)

3.3 PATHWAY TO FUNCTION ANALYSES

In the next step the disturbed/necessary neurodevelopmental 'Functions' identified via assessment of DNT compounds as well as by studying the basic biology literature (displayed in tables 3 & 8) were evaluated. These analyses revealed that pathways affecting neurodevelopmental functions depicted in Fig. 16 are necessary for proper brain development. Hence, assessment of toxicity that a substance might exert on these processes will inform about a potential DNT hazard.

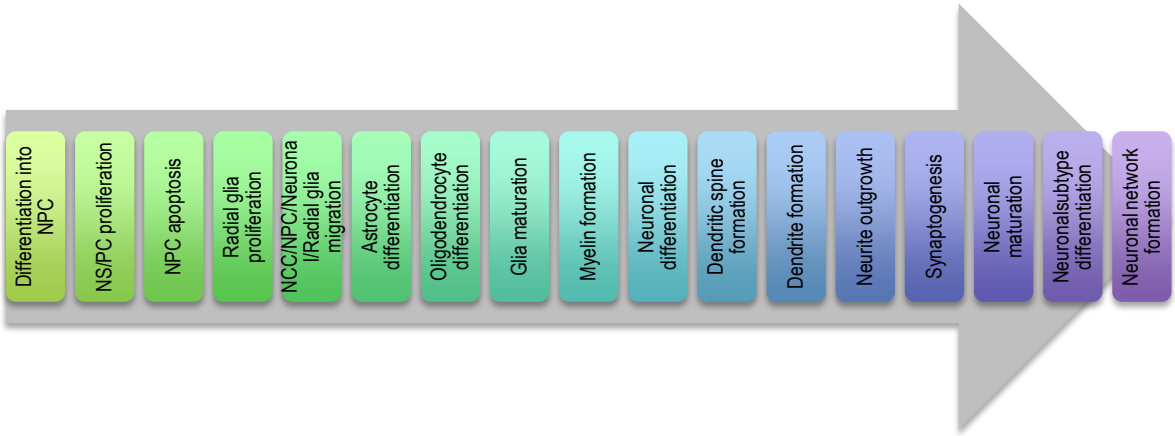


Figure 16: Processes necessary for brain development (extracted from tables 3 & 8).

The analyses of modes-of-action of DNT compounds as well as of the pathways contributing to essential neurodevelopmental processes showed that multiple pathways merge into most of such processes. These are summarized in table 9 and support the concept of neurodevelopmental process-oriented in vitro testing because these pathways were identified based on triggers for human DNT (e.g. by human DNT compounds or mutations causing neurodevelopmental syndromes). With reference to the AOP concept one can envision these processes as late cellular KE, which – most likely – in the future will be identified as 'common KE' as described in Bal-Price et al. (2015a).

Table 9: Signalling pathways involved in neurodevelopmental processes extracted and summarized from tables 3 & 8

Cellular Function	Pathway Contribution
NPC proliferation	**SH-group maintenance; Redox balance; Histone acetylation/deacetylation; Prostaglandin signalling; mTORC1-STAT3, mTOR-GSK3ß, activation RTK-PI3K-AKT signalling; PGE_2 – wnt signalling; BDNF-ERK-CREB**
NPC apoptosis	**ROS generation; FoxO activation; RTK-PI3K-AKT inhibition; BDNF-ERK-CREB;**

	RXR activation, GPCR30 inhibition
NEP differentiation	
Radial glia proliferation	**miRNA-17-92**
NPC/Neuronal/Radial glia migration	**PLCγ1, GDNF-RET, BDNF/TrkB, PDGFR, FGFR, mTORC1 signalling; MAP kinase and Reelin-Dab pathways, PGE_2 – wnt signalling; PLCγ1-dependent calcium release with activation of PKC**
NCC migration	**HDAC, microtubules, mitochondria**
Astrocyte differentiation	**mTORC1-STAT3 signalling, Notch signalling; miRNA-124; mTORC2 signalling**
Oligodendrocyte differentiation/maturation	**TH signalling**
Glia maturation	**Wnt signalling; Histone acetylation/deacetylation**
Myelin formation	**TH signalling**
Neuronal differentiation	**mTORC1, prostaglandin, Histone acetylation/deacetylation, miRNA-9, miRNA-17-92 cluster, miRNA-124, Notch signalling, wnt signalling**
Dendritic spine formation	**RYR sensitization, PIP metabolism**
Dendrite formation	**BDNF-ERK-CREB: Prostaglandins (cyclooxygenases, EP receptors); BDNF-ERK-CREB**
Axon guidance	**PLCγ1-dependent calcium release with activation of PKC**
Neurite outgrowth	**FGFR-mediated PLCγ1- PKC activation with subsequent IP3 and AA formation; NCAM/FGFR- PLCγ1 with action on small GTPases, such as Rho A, Rac1, or Cdc42; cytoskeleton maintenance; CREB signaling**
Neuronal migration	**PDGFR-PLCγ1; BDNF/TrkB activates MAPK and PI3K pathways and PLCγ1-dependent IP3-mediated calcium release; GDNF-RET-mediated activation of PLC□1 and activation of ERK and CaMKII; PDGFR-mediated activation of PLCγ1 with production of an intracellular DAG gradient**
Synaptogenesis	**NMDA-receptor activation, BDNF-Trk signalling, calcium signalling, inositol**

	metabolism, Phospholipase D activity with generation of phosphatidic acid; BDNF-ERK-CREB
Neural/neuronal apoptosis	**NMDA-receptor activation; Prostaglandins (cyclooxygenases, EP receptors)**
Neuronal maturation	**Wnt signalling**
Neuronal subtype differentiation	**Interference with calcium signalling; miRNA-124; TH signalling**
Neuronal network formation, synaptic plasticity	**Inositol metabolism; PIP metabolism; Prostaglandins (cyclooxygenases, EP receptors); TH signalling; Ephrin-A1/EphA4 - PLCγ1 (structural synaptic plasticity); BDNF/TrkB - PLCγ1 (functional synaptic plasticity); BDNF-ERK-CREB; ; BDNF-ERK-CREB (GABA circuit maturation)**

This pathway-to-function analysis is thought to be helpful for identifying predictive cell methods for DNT. For one, cell methods are needed that are able to assess functional endpoints depicted in Fig. 16 and Table 9 (Cellular Function). Secondly, high content assays might be preferred if they are predictive for DNT because the more endpoints an assay can predict the more economically feasible the testing becomes. Although not mandatory, the pathways identified to determine cellular function can help in scientifically validate in vitro methods for specific endpoints. This is especially the case as the pathways depicted in table 9 are all related to human neurodevelopmental disorders and/or DNT. Previously published examples of such pathway-based scientific validations of neurodevelopmental processes in vitro are (1) inhibition of neurite outgrowth by inhibition of PKC (Harrill et al., 2010), (2) inhibition of NPC migration by a MEK inhibitor (Moors et al., 2007) or (3) by interference with integrin-based NPC adhesion (Barenys et al., 2016). In the next chapter, individual cell methods that might be suitable for contributing to a DNT testing battery as well as already proposed DNT testing strategies from the literature are reviewed. In addition, results of a search for indicated pathways functional in identified cell methods will be displayed.

4. DNT ENDPOINTS MEASURED BY IN VITRO TESTING METHODS

A systematic literature review concerning methods to assess DNT was published in 2015 by Fritsche et co-workers. Within this publication assays were identified that had

the ability to predict adverse effects of compounds on neurodevelopmental processes correctly (a human DNT compound affecting specific neurodevelopmental processes at lower concentrations than cell viability). By evaluating methods that tested effects of human DNT compounds (Grandjean and Landrigan, 2006) on such endpoints it was found that most studies being able to identify adverse effects on the most neurodevelopmental endpoints were primary rat cells (Fig. 17). Cells with the least numbers of endpoints, mainly neuronal endpoints like neurite outgrowth were tumor cells. As tumor as well as rodent cells are not necessarily the ideal cell systems to study the majority of endpoints, e.g. for early brain cell proliferation a tumor cell line will not reflect physiology correctly, the systematic review in accordance with other literature, e.g. (Gibb, 2008) identified human stem/progenitor cells as the most promising cell type a) reflecting human physiology, b) being able to assess a variety of different neurodevelopmental endpoints and c) reflecting different developmental stages.

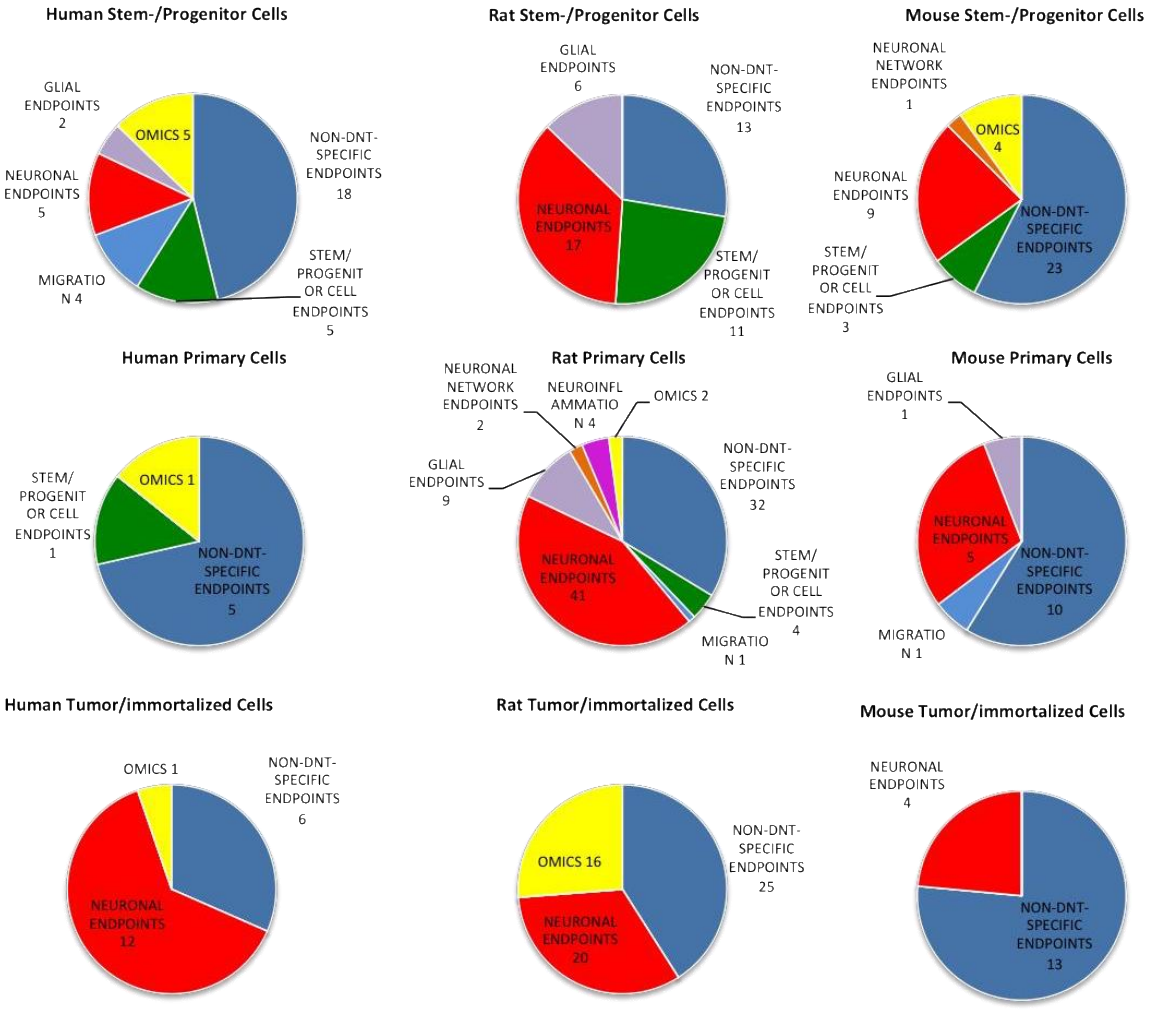


Figure 17: Numbers of studies identifying adverse effects of known DNT compounds on neurodevelopmental endpoints correctly. Comparison of stem/progenitor, primary, and tumor/immortalized cell lines from human, rat and mouse (Fritsche et al., 2015).

In addition to the systematic review, which only considered publications until March 2014, another literature search was performed for creating this document starting from April 2014 until December 2015 with the same keywords and search strategy than the systematic review performed from 1996-2014 (Fritsche et al. 2015). The procedure of the search strategy can be found in Appendix I.

Briefly, this second search (04/2014-12/2015) revealed 315 in vitro and alternative organism studies dealing with DNT after the keyword search. Of these, 77 publications were zebrafish studies and 4 studies used hiPSC. 8 papers proposed more general concepts on DNT testing using different cell methods. All studies were screened for utilizing human DNT compounds (Grandjean and Landrigan, 2006, Grandjean and Landrigan, 2014). The search revealed 1 paper on Arsenic, 2 papers on cadmium, 3 on chlorpyrifos, DDT, ethanol and lead each, 15 publications on methylmercury, 7 on organophosphorous compounds, 3 on PBDE and 1 on PCB.

A summary of human cell systems-based methods investigating DNT compounds, which will be grouped by neurodevelopmental relevant endpoints, will be discussed followed by analyses of more general studies either proposing concepts for DNT testing based on methods or studying multiple compounds. In the latter one, not only human cell-based papers, but a portfolio of available studies will be presented. An emphasis is put on newer studies as they likely reflect the current state of the art in these methodologically fast moving times. E.g. no toxicity study was identified until April 2014 employing hiPSC, while in the last two years already 4 iPSC-based studies were published. A summary of human stem/progenitor cell-based test methods with the capability to predict endpoints reflecting neurodevelopmental processes in vitro is provided in Table 10. These data is taken from Fig. 17 and Table 9, where contribution of signalling pathways affecting neurodevelopmental processes is outlined. Here, it has to be pointed out that a large variety of studies based on rodent primary cells are published for all endpoints listed in table 10. However, due to the current understanding of the benefit using human-based cell systems for in vitro testing approaches to avoid the uncertainty of species differences, the rodent literature was not reviewed here.

In addition to these papers, a recent literature review on generation of oligodendrocytes for in vitro drug screening purposes as treatments for multiple sclerosis is taken into consideration (Madill et al., 2016) as it summarizes the latest advances in stem/progenitor cell-based oligodendrocyte generation.

Table 10: Neurodevelopmentally relevant endpoints measured by human stem/progenitor cell-based methods in vitro.

Endpoint (cellular function)	Method	Citation
1. ESC differentiation to neuroepithelial precursors (NEP)/induction of neuronal rosettes	hESC -> hNEP	(Colleoni et al., 2011, Colleoni et al., 2012, Shinde et al., 2015, Stummann et al., 2009, Waldmann et al., 2014)

2. NPC proliferation	hNPC (Lonza)	(Baumann et al., 2015, Baumann, 2014, Gassmann et al., 2010, Gassmann et al., 2012, Moors et al., 2009, Schreiber et al., 2010, Tofighi et al., 2011)
	hUCBNSC,	(Buzanska et al., 2005); (Buzanska et al., 2009, Zychowicz et al., 2014)
	hESC,	(Bai et al., 2013, Nash et al., 2012, Talens-Visconti et al., 2011)
	FNC-B4 (primary human long-term olfactory bulb cells),	(Gulisano et al., 2009)
	fetal human astrocytes	(Guizzetti et al., 2003)
	ReNcell CX (Millipore)	(Breier et al., 2008, Radio et al., 2015)
3. NPC apoptosis	hESC,	(Bai et al., 2013, Nash et al., 2012, Talens-Visconti et al., 2011)
	FNC-B4	(Gulisano et al., 2009)
	hNPC, (Lonza)	(Moors et al., 2009, Fritsche et al., 2011)
	hNSC	(Hao et al., 2003)
	HUCB-NSC	(Buzanska et al., 2009)
	Primary human fetal astrocytes	(Mense et al., 2006)
	ReNcell CX (Millipore)	(Culbreth et al., 2012)
4. Radial glia		

	proliferation		
5.	**NPC/Neuronal/Radial glia migration**	Radial hNPC (Lonza) migration out of the neurosphere	(Moors et al., 2007) (Gassmann et al., 2010) (Barenys et al., 2016) (Baumann et al., 2015, Baumann, 2014)
6.	**Neural Crest Cell (NCC) migration (MINC assay)**	hESC-derived NCC	(Dreser et al., 2015, Hirsch et al., 2016, Pallocca et al., 2016, Zimmer et al., 2012, Zimmer et al., 2014)
7.	**Astrocyte differentiation**	Human neuroblast long term cell line, hUCBNSC, hESC hNPC, (Lonza)	(Gulisano et al., 2009) (Zychowicz et al., 2014) (Nash et al., 2012, Talens-Visconti et al., 2011) (Moors et al., 2012)
8.	**Oligodendrocyte differentiation/maturation**	hNPC, (Lonza) hESC hESC-derived neural cells hUCVNSC fetal neurospheres, hiPSC, hESC,	(Schreiber et al., 2010) (Kang et al., 2013, Talens-Visconti et al., 2011) (Nash et al., 2012) (Buzanska et al., 2005) rev. in (Madill et al., 2016)
9.	**Glia maturation**		
10.	**Myelin formation**	hiPSC hESC	(Wang et al., 2013) (Hu et al., 2009)
11.	**Neuronal differentiation**	hESC hNPC, (Lonza)	(Bai et al., 2013, He et al., 2012, Nash et al., 2012, Palmer et al., 2012, Stummann et al., 2009, Talens-Visconti et al., 2011) (Barenys et al., 2016,

		Baumann et al., 2015, Baumann, 2014, Moors et al., 2012, Moors et al., 2010, Schreiber et al., 2010)
	hUCBNSC,	(Buzanska et al., 2005, Buzanska et al., 2009) (Singh and Kashyap, 2015, Singh et al., 2012, Singh et al., 2013, Zychowicz et al., 2014)
	Human neuroblast long term cell line	(Gulisano et al., 2009)
	LUHMES	(Krug et al., 2013a, Scholz et al., 2011, Stiegler et al., 2011)
	Human fetal brain tissue	(Bai et al., 2013)
	hESC-derived 3-D in vitro model	(Hoelting et al., 2013, He et al., 2012);
	hESC-derived dopaminergic neurons	(Zeng et al., 2006)
	Primary fetal brain	(Rahman et al., 2011); (Pavlov et al., 1995); (Chao and Hu, 1994)
	hiPSC	(Hogberg et al., 2013)
12. Dendritic spine formation		
13. Dendrite formation		
14. Axonal growth		
15. Neurite outgrowth	hESC-derived neurons (hN2™)	(Harrill et al., 2010, Harrill et al., 2011a, He et al., 2012)
	LUHMES	(Krug et al., 2013a, Scholz et al., 2011, Stiegler et al., 2011)
	hESC-derived 3-D in vitro model	(He et al., 2012)

	hiPSC-derived dorsal root ganglia cells	
	hNPC	(Schmuck et al. in press)
16. Neuronal migration	hNPC	(Schmuck et al. in press)
17. Synaptogenesis	LUHMES	(Scholz et al., 2011)
18. Neural/neuronal apoptosis	hESC	(Lee et al., 2014a) (Talens-Visconti et al., 2011) (Bai et al., 2013)
	hNPC, (Lonza)	(Moors et al., 2009)
	hESC (H1)-derived hNSC, hNPC, primary human neuroblast long-term culture (olfactory bulb)	(Gulisano et al., 2009) (Gulisano et al., 2009)
19. Neuronal maturation		
20. Neuronal subtype differentiation	hESC	(Stummann et al., 2009) (Talens-Visconti et al., 2011)
	hiPSC, hESC (H1 cell line)-derived hNSC; hESC (H9)	(Hogberg et al., 2013)
21. Neuronal network formation, synaptic plasticity	hESC	(Kapucu et al., 2012); (Kiiski et al., 2013)
22. Peripheral neurotoxicity (PeriTox Assay)	hiPSC-derived immature dorsal root ganglia cells	(Hoelting et al., 2016)

Collectively, the data shown in Table 10 indicates that multiple human stem-/progenitor cell methods exist that have the ability to predict several neurodevelopmental endpoints. However, there is a lack of data concerning the endpoints radial glia proliferation, glia maturation, dendritic spine formation, dendrite formation, axonal growth, neuronal migration and neuronal maturation.

4.1 EVALUATION OF THE LEVEL OF READINESS OF THE IN VITRO ASSAYS FOR DNT TESTING

Assays/Test systems (Table 10) for the evaluation of DNT in vitro forming a potential battery that covers (i) timing of development and (ii) different key neurodevelopmental processes will now be evaluated for their readiness. So far 22 neurodevelopmentally-relevant endpoints that can be tested *in vitro* were identified by a total of 6 cell methods thereby not every cell method covering all endpoints. These methods summarized in Table 11 only contain human stem/progenitor cell-based methods leaving out primary human cells that are not commercially available due to practical application reasons. However, some neurodevelopmental endpoints, which are currently not covered by human cell-based assays, can be covered by rodent cells. Such include e.g. dendritic spine formation. Such non human-based methods are not covered here. These 22

endpoints can be assessed in overlapping cell systems (Table 10) meaning that multiple endpoints might measured e.g. by high content analyses to reduce the actual number of tests necessary for a DNT testing strategy. Within this report we identified the following cell systems for endpoint evaluations:

Table 11: Human stem/progenitor cell-based methods with the ability to mimic neurodevelopmental processes in vitro. Primary human cells that are not commercially available were disregarded.

Cell Method	Endpoints (EP)	EP group	Literature
I. hESC	NEP differentiation Induction of neuronal rosettes	NEP EP	(Colleoni et al., 2011, Colleoni et al., 2012, Shinde et al., 2015, Stummann et al., 2009, Waldmann et al., 2014)
	NPC proliferation	NPC EP	(Bai et al., 2013, Nash et al., 2012, Talens-Visconti et al., 2011)
	NPC Apoptosis		(Lee et al., 2014b, Talens-Visconti et al., 2011)
	NCC differentiation/migration	NCC EP	(Dreser et al., 2015, Hirsch et al., 2016, Pallocca et al., 2016, Zimmer et al., 2012, Zimmer et al., 2014)
	Astrocyte differentiation	Astroglia EP	(Talens-Visconti et al., 2011)
	Oligodendrocyte differentiation/maturation	Oligodendroglia EP	(Talens-Visconti et al., 2011, Nash et al., 2012) rev. in (Madill et al., 2016) (Hu et al., 2009)
	Neuronal differentiation	Neuronal EP	(Colleoni et al., 2011, Stummann et al., 2009, Lee et al., 2014a); (Gulisano et al., 2009, Talens-Visconti et al., 2011, Kiiski et al., 2013); (Bai et al., 2013); (Kapucu et al., 2012); (Nash et al., 2012); (Kang et al., 2013); (Palmer et al., 2012); (He et al., 2012); Schwartz et al. 2015

	Neuronal apoptosis		(Bai et al. 2013)
	Dopaminergic neuron differentiation		(Zeng et al., 2006)
.....	Neurite outgrowth		(Harrill et al., 2010, Harrill et al., 2011a, He et al., 2012)
	Neuronal apoptosis		(Lee et al., 2014a) (Talens-Visconti et al., 2011) (Bai et al., 2013)
	Neuronal subtype differentiation		(Stummann et al., 2009) (Talens-Visconti et al., 2011)
	Neuronal network formation, synaptic plasticity		(Kapucu et al., 2012, Kiiski et al., 2013)
	Peripheral neurotoxicity	Peripheral NS EP	(Hoelting et al., 2016)
II. hNPC, Neurospheres (Lonza)	NPC proliferation	NPC EP	(Baumann et al., 2015, Baumann, 2014, Fritsche et al., 2011, Gassmann et al., 2010, Gassmann et al., 2012, Moors et al., 2009, Schreiber et al., 2010, Tofighi et al., 2011)
	NPC apoptosis		(Fritsche et al., 2011, Moors et al., 2009)
	NPC/radial glia migration		(Moors et al., 2007) (Gassmann et al., 2010) (Barenys et al., 2016) (Baumann et al., 2015, Baumann, 2014)
	Astrocyte differentiation	Astroglia EP	(Baumann et al., 2015, Baumann, 2014, Moors et al., 2012, Moors et al., 2010)
	Oligodendrocyte differentiation	Oligodendroglia EP	(Baumann, 2014, Schreiber et al., 2010)
	Neuronal	Neuronal EP	(Barenys et al., 2016,

	differentiation		Baumann et al., 2015, Baumann, 2014, Moors et al., 2012, Moors et al., 2010, Schreiber et al., 2010)
	Neurite outgrowth		Schmuck et al. in revision
	Neurite outgrowth		Schmuck et al. in revision
III. human NPC line (ReNCell Cx, Millipore)	NPC proliferation	NPC EP	(Breier et al., 2008, Radio et al., 2015)
	NPC apoptosis		(Culbreth et al., 2012)
IV. hUCBNSC	NPC proliferation	NPC EP	(Buzanska et al., 2005, Buzanska et al., 2009)
	NPC apoptosis		(Buzanska et al., 2009)
	Astrocyte differentiation	Astroglia EP	(Zychowicz et al. 2014)
	Oligodendrocyte differentiation	Oligodendroglia EP	(Buzanska et al., 2005)
	Neuronal differentiation	Neuronal EP	(Buzanska et al., 2005, Buzanska et al., 2009, Singh et al., 2012, Singh et al., 2013)
V. hiPSC	Neuronal differentiation	Neuronal EP (here models are available, yet Hogberg et al. without chemical testing and Pei et al. only assessing cytotoxicity	(Hogberg et al., 2013); (Pei et al., 2015)
	Oligodendrocyte differentiation and myelin formation	Oligodendroglia EP	Rev. in Madill 2016
	Peripheral neurotoxicity	Peripheral NS EP	(Hoelting et al. 2016)
VI. LUHMES dopaminergic neuronal precursor cells	Neuronal differentiation	Neuronal EP	(Krug et al., 2013a, Scholz et al., 2011, Stiegler et al., 2011)
	Neurite outgrowth		(Krug et al., 2013a, Scholz et al., 2011,

		Stiegler et al., 2011)
Synaptogenesis (no compounds tested)		(Scholz et al., 2011)

Table 11 summarizes data presented in table 10 from the cell type/model point of view. However, primary human cells that are not commercially available were left out here as there is no regulatory application to be seen with such cells. Human cell methods that have the ability to study neurodevelopmental endpoints in vitro condense to 6 individual cell types: hESC, hNPC, ReNcell CX, hUCBNSC and LUHMES cells. Thereby, in some cases hESC are differentiated to hESC-NPC and subsequently used for further DNT studies; or differentiation of hESC cells into a certain neural cell type, like NCC, was the targeted neurodevelopmental process. This is also true for some of the other cell methods listed in table 11. Due to this fact and because of the issue that many different stem cell lines, differentiation protocols, cultivation methods and experimental set-ups were used across the many different studies, it is very difficult to evaluate the readiness of the methods according to the methods applied in the OECD scoping document ENV/JM/MOMO(2014)23. The only exceptions are the hNPC (Lonza), the hUCBNSC and the LUHMES cells as these publications are generated from one laboratory each. Thus, protocols are generally very similar across the different publications. This only bears the lack in lab-to-lab reproducibility (for hNPC and hUCBNSC one publication from a different lab each). However, an attempt will be made to address assay readiness for endpoint groups that were already defined earlier (Fritsche et al. 2015, Table 11) according to Table 12 taken from ENV/JM/MOMO(2014)23.

Table 12: Ranking Parameters for Evaluation of the Readiness of Assays for Inclusion in the TG Work plan (Table 1.1 in ENV/JM/MOMO(2014)23).

CATEGORY 1 **Initial High Priority Considerations**	**CATEGORY 2** **Assay Performance Considerations**
1. Biological Plausibility 2. Extrapolation to humans, or broadly applicable across vertebrates/phyla 3. Availability of Resources 4. Reference Chemicals	5. Within-laboratory reproducibility 6. Between-laboratory reproducibility 7. Assay Variability 8. Accuracy 9. Assay Specificity 10. Assay sensitivity
CATEGORY 3 **Technical Capabilities**	**CATEGORY 4** **Other Practical Considerations**
11. Dynamic Range 12. Concentration test range 13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias 14. Response Characterization:	15. Technological Transferability/Proprietary elements 16. Transparency of the method 17. Documentation of development and utility of the method.

According to the OECD document ENV/JM/MOMO(2014)23, the individual parameters of these categories should be weighted as follows:

Category 1: Initial High Priority Considerations

The parameters in this category are considered of highest priority. In addition, each parameter within this category is considered to have equal weight and all are essential

for an acceptable assay, i.e. a poor rating on any one is considered too severely impair the validation or regulatory acceptance of the assay.

Category 2: High Priority Assay Performance Considerations
These parameters relate to the reliability and efficacy of the assay itself. Generally, these parameters would have high priority in considerations of the potential for development of a protocol for a candidate assay into an OECD Test Guideline. All six parameters within this category are considered to have equal weight.

Category 3: Technical Capabilities
The parameters in this category also relate to assay performance so the same limitations described for Category 2 parameters apply. However the particular performance issues considered under this category of parameters were identified to be of lesser significance compared to the Category 2 performance issues.

Category 4: Other Practical Considerations
This category lists parameters which may present some challenges to validation or broad acceptance of the protocol as an OECD Test Guideline but are not insurmountable. Consequently, these were identified as being of lowest priority. All of the parameters in this list are of equal importance.

4.1.1 NEP ENDPOINT

In this assay (UNK1), hESC differentiate to neuroepithelial precursor cells (NEP) within 6 days, and they were exposed to VPA during the entire period of differentiation (Fig. 18). Readout: cell viability (Shinde et al., 2015, Waldmann et al., 2014)
and global gene expression changes as changes in GO terms with development of a transcriptomics-based teratogenicity index (Waldmann et al., 2014). This index helps distinguishing between DNT-relevant and cytotoxic concentrations and promotes performing transcriptome-based DNT studies at non-cytotoxic concentration. This protocol seems to be a further developed protocol from the same lab where NEP were generated during 12 days and effects of MeHgCl were tested (Stummann et al., 2009).

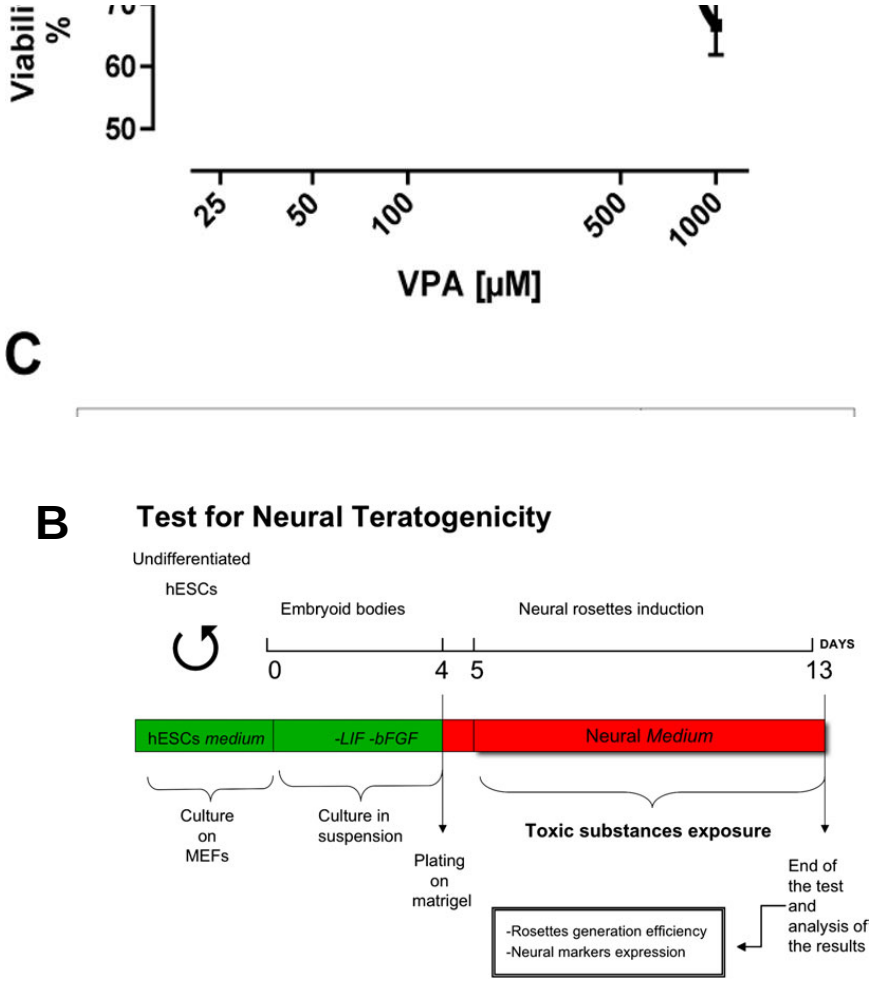


Figure 18: Schematic representation of A the NEP-Assay (Waldmann et al., 2014) and B the Neural Teratogenicity Assay (Colleoni et al., 2011).

Alternatively, the Neural Teratogenicity assay was established using hESC (Colleoni et al., 2011, Colleoni et al., 2012). Retinoic acid (RA) as well as VPA disturb neural rosette formation in a concentration-dependent manner. This was accompanied by changes in gene expression necessary for normal neural tube patterning. Readouts: Cell viability (Alamar blue), morphology of neural rosette formation, gene expression.

These assays are now evaluated due to the ranking parameters indicated above and in Table 12:

1. Biological Plausibility	The NEP assay investigates effects on biological process necessary for nervous system development. NEP are the cells forming the neural tube, which later give rise to the brain and the spinal cord. Neural tube defects are one of the most common congenital birth defects with a prevalence of 1 in 1000 births. Compounds producing neural tube defects	Category 1

	include the antiepileptic drug Valproic Acid and Retinoic acid.	
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assays use human cells.	
3. Availability of Resources	Different hESC lines are available, however, one needs special permission to work with them.	
4. Reference Chemicals	Valproic Acid (Colleoni et al. 2012; Waldmann et al. 2014) MeHgCl (Stummann et al. 2009; Shinde et al. 2015) Retinoic acid (Colloni et al. 2012)	
5. Within-laboratory reproducibility	yes	Category 2
6. Between-laboratory reproducibility	possibly, as Colloni et al. 2011,2012 report on specific genes known to be involved in neural tube formation within their RA- or VPA-induced transcriptome changes, while Waldmann et al. 2014 report on GO_Terms in their VPA-induced transcriptome changes. Stummann et al. 2009 study MeHgCl effects on hESC to NEP differentiation. Although different compounds were used, e.g. HOX genes were identified as targets for different compounds across labs.	
7. Assay Variability	n.d.	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Cytotoxicity from 0 – 100%	Category 3
12. Concentration test range	Valproic Acid: 25-1000 µM (Waldmann et al. 2014) VPA: 3 – 300 µM (Colleoni et al. 2012) VPA TSA SAHA Belinostat Entinostat Panobinostat MeHgCl Thimerosal $HgCl_2$ $HgBr_2$ PCMB PMA (concentrations 0,01 – 10 µm each; Shinde et al. 2015) MeHgCl: 10 nM – 200 nM (Stummann et al. 2009) RA: 2 nM – 2 µM (Colleoni et al. 2011)	
13. Detection/Adjustment of confounding factor and/or	Viability measures in the same cultures	

incorrect/ inconclusive measurements and/or other bias		
14. Response Characterization	Cytotoxicity: - Transcriptome: The category was considered only if it was annotated with more than 100 genes, and the minimal enrichment p-value across all concentrations was below 0.001 (Waldmann et al. 2014)	
15. Technological Transferability/ Proprietary elements	Allowance to work with hESC needed	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

n.d. = not determined

The NEP differentiation assay fulfils Category 1 criteria, which are essential, with the limitation of the need of allowance of working with hESC (3.). Moreover, the number of reference compounds is very limited (4.). Category 2 criteria, which deal with the reliability and efficacy of the assay itself, cannot be addressed sufficiently at this point due to lack of data and needs more experimental work. However, the method seems to be transferable from lab to lab. Category 3, technical assay criteria, are partially addressed by the current available data and also need more experimental work. Especially the usage of changes in gene expression data for regulatory purposes has to receive special attention. Also category 4 needs more information.

4.1.2. NPC ENDPOINTS

NPC endpoints divide into (2.1) NPC proliferation, (2.2) NPC apoptosis and (2.3) NPC/radial glia migration. Several cell methods are available for studying these endpoints and such will be evaluated separately.

4.1.2.1 NPC PROLIFERATION

- hESC-NPC proliferation assay

As already mentioned above, there are a variety of different protocols available from different groups for studying neurodevelopmental endpoints by using hESC. The first one grew H9-hESCs on a feeder layer and were then transferred to low-attachment plates to obtain embryonic bodies (EBs). EBs were plated onto Matrigel- coated plates, allowing differentiation into ‘‘rosettes.’’ Cells from the rosettes were plated onto poly-ornithine/laminin-coated plates and grown in neural proliferating medium [NPM, containing basic fibroblast growth factor (bFGF)] (Fig. 19, Talens-Visconti et al. 2011). For the cell proliferation assay, BrdU incorporation was assessed using a commercial kit (Roche). Quantification of the number of BrdU-incorporated cells was carried out by flow cytometry after 14, 21, 28 and 35 days. Reference chemical: ethanol.

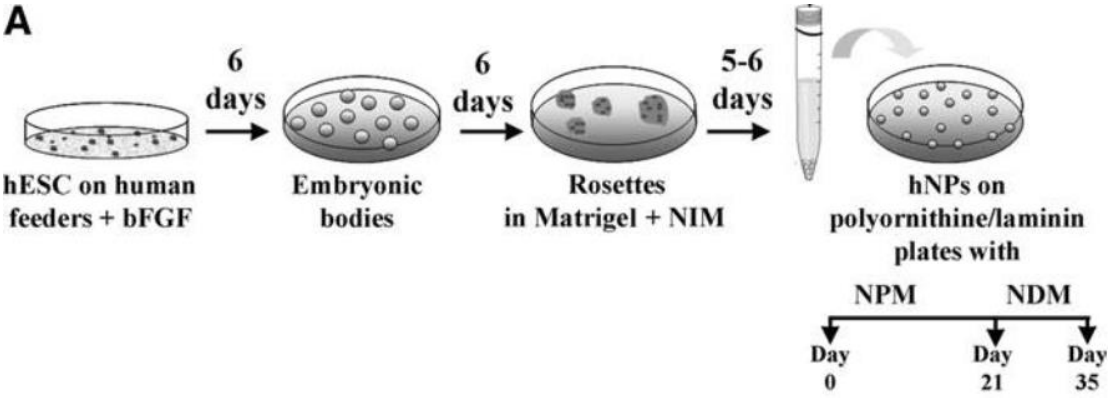


Figure 19: Schematic representation of hESC differentiation into neural cells(Talens-Visconti et al., 2011).

In a different protocol, H1 (male) or H9 (female) human embryonic stem cell-derived neural progenitor cells were purchased from Aruna Biomedical, Athens GA and grown in Neurobasal Medium (Gibco, Invitrogen) supplemented with L-glutamine, B27, and leukemia inhibitory factor (LIF), on laminin-coated plates (10 µg/ml, Sigma) in 3% O2. These neural stem cells do not express Oct-4; they uniformly express nestin and Sox-2. Cells grown as monolayers were incubated with BrdU for 4 h, then the cells were fixed in PFA and Immunohistochemical stainings with an anti-BrdU antibody was performed (Nash et al., 2012). In this work, the effects of treatment were not quantified but evaluated just by eye evaluation. Reference chemical: ethanol.

The third protocol grew hESCs (H1 cell line, WiCell Research Institute Inc.) on mitotically inactivated mouse embryonic fibroblasts (MEFs) in 0.1% gelatin-coated plates. hESC culture medium consisted of DMEM/F12 supplemented with 20% KnockoutTM serum replacement (Gibco), 1% non-essential amino acids, 1% penicillin-

streptomycin, 1 mM L-glutamine (Chemicon), 0.1 mM ß-mercaptoethanol (Sigma), and 4 ng/mL human recombinant basic fibroblast growth factor (bFGF; Invitrogen). hESCs underwent a three-step progression that includes embryonic body (EB) culture, rosette cell formation and NSC expansion. (1) EB culture. hESCs in the culture were digested using dispase (1.5 unit/mL) (Invitrogen) for 30 min. Digested hESCs were then transferred to 60 mm ultra- low-attachment dishes (Corning) and cultured in hESC medium without bFGF under normoxic conditions. The medium was changed every day. EBs were visible one day after culturing. Four days later, EBs were switched to neural induction medium consisting of DMEM/F12 supplemented with 1% N2 (Invitrogen), 1% non-essential amino acids, 5 ng/ mL bFGF, and 1mg/mL heparin (Sigma) for 4 days. (2) Rosette formation. EBs were transferred to matrigel-coated 60 mm culture dishes and cultured with neural induction medium at day 8. The medium was changed every other day. EBs attached on the dishes and formed neural tube-like rosettes with radial arrangements of columnar cells within 5 days. (3) NSC expansion. Two days after rosette formation, rosette cells were gently blown off with a 5 ml serological and then transferred to other dishes containing DMEM/F12 supplemented with 1% N2, 2% B27 (Invitrogen), 1% non-essential amino acids, 20 ng/mL bFGF, and 1mg/mL heparin. One day later, rosette cells rolled up to form round spheres called NSCs. Half of the medium was changed every other day. NSCs were passaged every 5–6 days by digestion with accutase (Innovative Cell Technology). NSC proliferation was analyzed by two methods: Ki67 staining and bromodeoxyurindine (BrdU) assay. (1) NSCs (4×10^4 per 12 mm coverslip) were cultured in NSC medium with or without 100 μM reference compound for 3 and 6 h. Ki67 expression in NSCs was visualized using immunofluorescence staining. Ki67-positive stem cells were counted from 6 random fields per coverslip in each of three independently differentiated NSC samples. (2) BrdU incorporation as an indicator of cell proliferation was studied using a colorimetric BrdU kit (Roche Diagnostics) according to the manufacturer's instructions. The incorporation of BrdU into cellular DNA is then detected using anti-BrdU antibody, allowing assessment of cell proliferation rate by using a microplate reader. Reference compound: Ketamine (Bai et al., 2013).

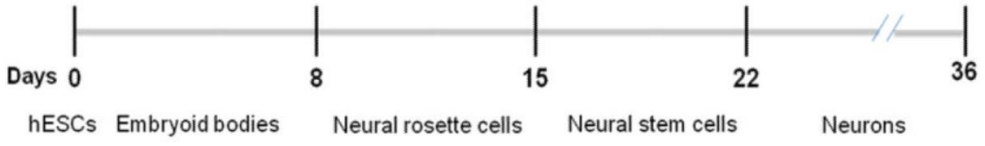


Figure 20: Schematic representation of hESC differentiation into neural cells (Bai et al., 2013).

These protocols for hESC-derived NPC proliferation are examples that we retrieved out of our literature search that was described above and are not be completely

comprehensive. Because there is no study evaluating different compounds with the same protocol, the assay in general is assessed with the ranking parameters indicated above and in Table 12 although different protocols are used for NPC generation:

1. Biological Plausibility	The hESC-NPC proliferation assay is based on NPC that were differentiated from hESC. Biological relevance of NPC proliferation is well established. However, how similar hESC-NPC are to 'real' NPC is not clear and such a comparison has so far not been done. However, some NPC markers like nestin and SOX-2 are studied in hESC-NPC and this in general is considered as a cell characterization.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human cells.	
3. Availability of Resources	Different hESC lines are available, however, one needs special permission to work with them.	
4. Reference Chemicals	Ethanol (Talens-Visconti et al. 2011; (Nash et al., 2012) Ketamine (Bai et al., 2013)	
5. Within-laboratory reproducibility	-	Category 2
6. Between-laboratory reproducibility	Ethanol (20 mM, 7 days) increased proliferation, no: however without quantification, just by eyesight (Nash et al. 2012), while 25 & 50 mM ethanol decreased proliferation after 28 & 35 days in culture (Talens-Visconti et al. 2011).	
7. Assay Variability	Contradicting results after ethanol treatment between Nash et al. (2012) and Talens-Visconti et al. (2011).	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	30-40% ± 10% BrdU+ cells. Increase to 80% (Bai et al. 2013), decrease to 10% (Talens-Visconti et al. 2011)	Category 3
12. Concentration test range	Ethanol: 25 & 50 mM for 14, 21, 28, 35 days (Talens-Visconti et al. 2011) Ethanol: 20 mM, 7 days Ketamine: 100 µM, 3 & 6 hrs (Bai et al. 2013)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Talens-Visconti et al. (2011): cell death determination by annexinV (apoptosis) and 7-amino-actinomycin D (necrosis) detection by FACS analyses Nash et al. (2012): no viability measures Bai et al. (2013): viability measures via mitochondrial membrane potential assessment	
14. Response Characterization	Statistical significance	
15. Technological	Allowance to work with hESC needed	

Transferability/ Proprietary elements		Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

- Primary hNPC proliferation assay

Primary hNPC (Lonza) grown as neurospheres in 3D were a) monitored in size over 14 days (Baumann et al., 2015, Baumann, 2014, Gassmann et al., 2010, Gassmann et al., 2012, Moors et al., 2009, Schreiber et al., 2010, Tofighi et al., 2011) or b) underwent BrdU incorporation after 3 days (Baumann et al., 2015, Baumann, 2014) as part of the 'Neurosphere Assay'. Evaluation of proliferation was performed by a) phase-contrast image analysis or b) a luminescence-based BrdU Assay (Roche) with a luminometer (Fig. 21).

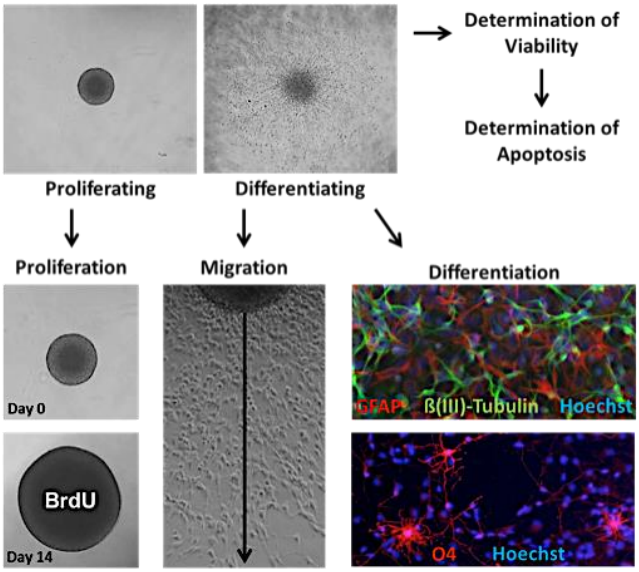


Figure 21: Schematic set-up of the 'Neurosphere Assay' including NPC proliferation by size determination or BrdU incorporation (modified from: Breier et al., 2009)

1. Biological Plausibility	The hNPC proliferation assay is based on primary NPC. Biological relevance of NPC proliferation is well established. Cell's growth in 3D is advantageous.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses primary human cells.	
3. Availability of Resources	hNPC are commercially available from Lonza. The group has been publishing with these cells since 2005.	
4. Reference Chemicals	positive substances:	

	methylmercurychloride (MeHgCl) sodium (meta)arsenite (NaAsO2) methylazoxy methanol acetate (MAM) valproic acid sodium salt (NaVPA) chlorpyrifos ethyl (CPF) parathion ethyl (parathion) negative substances: L(+)-Monosodium glutamate monohydrate (glutamate) 4-Acetamidophenol (paracetamol) Penicillin G sodium salt (PenG) (Baumann et al. 2016) 3-methylcholanthrene (3-MC) 3-methoxy-4-nitroflavone (MNF) 2,3,7,8-tetrachlorodibenzodioxine (TCDD) (Gassmann et al. 2010) brominated diphenylether (BDE)-47 brominated diphenylether (BDE)-99 (Schreiber et al. 2010)	
5. Within-laboratory reproducibility	yes	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Approx. ±10%	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Endpoint-specific control is deficiency of growth factors reducing proliferation to 10-20% of control BrdU+ cells after 3 days (Baumann et al. 2015,2016)/ 0-10% of sphere diameter after 14 days (Moors et al. 2009, Schreiber et al. 2010)	Category 3
12. Concentration test range	MeHgCl 0.004-3 µM, methylazoxymethanol 2.058 -1500 µM, valproic acid 15.432-11250 µM (Baumann et al. 2015)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Viability is assessed from the identical cultures by the AlamarBlue Assay.	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	transferable, protocols described in Baumann et al. 2014	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

- hUCB-NSC proliferation assay

The HUCB-NSC cell line was routinely cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 2% fetal bovine serum, 1% insulin-transferrin-selenium, and 1% antibiotic-antimycotic suspension. The mixed population of adherent progenitors and free-floating undifferentiated cells present in this culture was seeded into 96- well plates at a density of $2*10^4$ cells/cm2. For assessing proliferation, cells were incubated with test compounds for 48 hours and were then stained with an antibody against Ki67 (Buzanska et al., 2009, Zychowicz et al., 2014).

1. Biological Plausibility	The hUCB-NSC proliferation assay is based on a nestin-positive NSC line (Buzanska et al., 2005) that was differentiated from human umbilical cord blood stem cells. Biological relevance of NPC proliferation is well established. However, how similar hUCB-NSC are to 'real' NPC is not clear and such a comparison has so far not been done. This group has only one publication on this proliferation assay.	
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based cells.	
3. Availability of Resources	The line is not commercially or broadly available.	
4. Reference Chemicals	MeHgCl 0.01-0.5 µM (Buzanska et al. 2009) MeHgCl 0.065-1 µM (Zychowicz et al. 2014)	
5. Within-laboratory reproducibility	-	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Approx. ±10%	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	MeHgCl reduces 50% Ki67+ cells to 20% Ki67+ cells	Category 3
12. Concentration test range	MeHgCl 0.01-0.5 µM	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Viability is assessed, method not clear (Buzanska et al. 2009). Alamar blue assay (Zychowicz et al. 2014), effects on proliferation only at concentrations where viability was disturbed	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

- ReNcell CX-based proliferation assay

ReNcell CX cells were obtained commercially from Millipore (Temecula, CA). This cell line was derived from a 14-week gestation human fetal cortex obtained from Advanced Bioscience Resources. For all experiments, cells frozen at passage 3 were thawed and expanded on laminin-coated T75 cm^2 tissue culture flasks in ReNcell NSC Maintenance Medium (Millipore) supplemented with epidermal growth factor (EGF) (20 ng/ml) and basic fibroblast growth factor (FGF-2) (20 ng/ml). Three to four days after plating (e.g., prior to reaching 80% confluency), cells were passaged by detaching with accutase, centrifuging at 300 x g for 5 min and resuspending the cell pellet in fresh maintenance media containing EGF and FGF-2. For all experiments, cells were replated in laminin-coated costar 96-well plates. ReNcell CX cell proliferation was determined by quantifying DNA replication in ReNcell CX cells using the Cellomics BrdU Cell Proliferation Kit for high-content screening (Thermo- Fisher Scientific, Pittsburgh, PA) by using the Cellomics ArrayScan. Proliferation was assessed after 4, 24, and 48 hours of compound treatment in a high content format (Breier et al., 2008, Radio et al., 2015).

1. Biological Plausibility	The ReNcell CX-based proliferation assay is based on a nestin-/SOX2 positive NSC line (Breier et al. 2008) that is commercially available from Millipore. These cells were generated from human fetal brains by myc-immortalization and grow in monolayers in 2D. Biological relevance of NPC proliferation is well established. However, the myc gene is a cell cycle regulator and thus the usage of ReNcells for studying effects of compounds on proliferation is questionable.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based cells, yet mcy-immortalized.	
3. Availability of Resources	The line is commercially available from Millipore.	
4. Reference Chemicals	Aphidicolin Hydroxyurea Cytosine Arabinoside 5-Fluorouracil Ochratoxin A D-Amphetamine Cadmium chloride hydrate Dexamethasone 5,5-Diphenylhydantoin Lead acetate Methyl mercury chloride trans-Retinoic acid Valproic acid Acetaminophen Amoxicillin Dimethyl phthalate Diphenhydramine hydrochloride Glyphosate	

	Omeprazole Saccharin sodium salt hydrate D-Sorbitol (Breier et al. 2008) ToxCast I Library with 309 compounds (Radio et al. 2015)	
5. Within-laboratory reproducibility	yes	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Approx. 9% (Radio et al. 2015)	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	BrdU incorporation can be reduced from 100 to 0%	Category 3
12. Concentration test range	10 nM – 1 mM for the non-ToxCast compounds (Breier et al. 2008) For the ToxCast compounds, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 40 µM in a single well per concentration (n = 1; Radio et al. 2015)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	HCA reduces personal bias, viability is assessed at the same time	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	possible as cells are commercially available from Millipore	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

Collectively, NPC proliferation can be measured with hESC-generated (2.1.1), primary hNPC (2.1.2), hUCB-NSC line (2.1.3) or ReNcell CX (2.1.4)-based systems. Category 1 criteria, which are essential, are met differently across the systems. hESC-generated NPC bear the problem that there are many different protocols for generating such cells. A protocol harmonization is urgently needed. Moreover, one needs a special permission to work with such cells and the number of reference chemicals is fairly low (2). More compounds need to be tested. Primary hNPC meet Catergory 1, a total of 14 compounds was tested. ReNcell CX over all fulfil Category 1 criteria as many compounds were studied and the cells are commercially available. However, the mcy-immortalization critically has to be considered. Most lack in Category 1 criteria displays the hUCB-NSC line. Over all, there are few publications with this line, it is restricted to one group, not readily available and only one compound was tested for NSC proliferation. During the last 7 years, no new data was generated with this line. Category 2 criteria, which deal with the reliability and efficacy of the assay itself, is difficult for the hESC-NPC assay as depending on the protocol ethanol exhibits opposite effects on proliferation. No intra-

lab reproducibility is shown. The primary hNPC proliferation assay shows inter-lab reproducibility and uses an endpoint-specific control. Intra-lab reproducibility is missing. The ReNcell CX-based NPC proliferation assay is intra-lab reproducible, inter-lab reproducibility is not shown. Moreover, assay performance with regards to sensitivity and specificity would be needed, however, this information would require knowledge on the MoA of compounds, i.e. through which process they interfere with normal brain development. As for the most compounds this information is not known, it is not possible to determine such information. For the hUCB-NSC proliferation assay, Category 2 criteria are not met. Category 3 criteria, technical assay criteria, are generally met by the hESC-, primary hNPC- and ReNcell CX- based proliferation assay. For Category 4 criteria, detailed protocols are published for the hNPC- and ReNcell CX - based proliferation assay (Baumann et al., 2015, Breier et al., 2008), respectively.

4.1.2.2 NPC APOPTOSIS ASSAY

Apoptosis is a crucial event during brain development underlying regulatory processes. As too much apoptosis can detriment the NPC pool in the developing brain, a reduction in apoptosis can lead to morphological defects like hyperplastic brains (Takahashi et al., 2001). Apoptosis can be measured by different methods in vitro ranging from early events like mitochondria calcium or cytochrome c release, intermediate processes like caspase activation or late apoptotic activities like nuclear condensation, micronucleus formation or chromatin disintegration.

- hESC-NPC apoptosis assay

hESC-NPC apoptosis was measured by counting apoptotic nuclear morphology (Lee et al. 2014), annexin V+ cells via FACS analyses (Talens-Visconti et al., 2011), cytochrome c release into the cytoplasm by Western blot (Lee et al., 2014b), by studying caspase-3 with a substrate-based colorimetric assay with a plate reader (Talens-Visconti et al., 2011), caspase-3 and -9 cleavage by Western blot (Lee et al., 2014b) and by quantifying caspase-3 mRNA expression by RT-PRC analyses (Talens-Visconti et al. 2011). Cells were generated as described under 2.1.1.

1. Biological Plausibility	That the right balance of NPC apoptosis is crucial for brain development is well established from the biological side. E.g. mice deficient in caspase function exert brain hyperplasia during development, which is lethal to the animals.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based cells, yet the apoptosis machinery of hESC-derived NPC has not been compared to primary hNPC.	
3. Availability of Resources	Different hESC lines are available, however, one needs special permission to work with them.	
4. Reference Chemicals	Ethanol (Talens-Visconti et al. 2011) Chlorpyrifos (Lee et al. 2014)	
5. Within-laboratory reproducibility	-	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Approx. 5-10% depending on the assay	
8. Accuracy	n.d.	

9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Caspase activity (the only platereader assay) can be induced from 0 to 100%	Category 3
12. Concentration test range	Ethanol (25 – 50 mM, Talens-Visconti et al. 2011) Chlorpyrifos (25 – 100 µM; Lee et al. 2014)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Platereader Assay for Caspase activity, can be multiplexed with viability assay. Western blot not suited for screening purposes. Nuclear morphology possibly a good readout for HCA (not shown here).	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	The lab needs the allowance to work with hESC.	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

- Primary hNPC apoptosis assay

Primary hNPC (Lonza) grown as neurospheres in 3D were plated for differentiation (see Fig. 21). Cell death (LDH release), caspase-3/-7 activity (platereader) and TUNEL assay (fluorescence microscopy) were performed after treatment with staurosporin or hydrogen peroxide (Moors et al., 2009, Fritsche et al., 2011).
Primary fetal hNSC were isolated from 11-21 week foetuses. Monolayer cells were treated and a TUNEL assay was performed with the monolayer cells and TUNEL+ labelled cells counted. In parallel cell death (LDH assay) and cell viability (MTT assay) was assessed (Hao et al., 2003).

1. Biological Plausibility	That the right balance of NPC apoptosis is crucial for brain development is well established from the biological side. E.g. mice deficient in caspase function exert brain hyperplasia during development, which is lethal to the animals.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses primary human cells.	
3. Availability of Resources	hNPC are commercially available from Lonza. The group has been publishing with these cells since 2005.	
4. Reference Chemicals	Staurosporin H_2O_2 (Moors et al. 2009) Ethanol (Hao et al. 2003)	
5. Within-laboratory reproducibility	-	
6. Between-laboratory reproducibility	-	

7. Assay Variability	Approx. ±20% for caspase-3/-7 activity ±10% for TUNEL assay	Category 2
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Caspase activity (the only platereader assay) can be induced from 0 to 100%	Category 3
12. Concentration test range	Staurospporin 1 µM, H_2O_2 1 mM (Moors et al. 2009) Ethanol 1-10 mM (Hao et al. 2003)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Cell death is assessed from the identical cultures by quantifying LDH release (Moors et al. 2009; Hao et al. 2003) and viability via the MTT assay (Hao et al. 2003).	
14. Response Characterization	-	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

- ReNcell CX-based neural apoptosis assay

ReNcell CX culture is described under 2.1.4. For assessment of apoptosis, cells were stained with an antibody against cleaved caspase 3 after 24 h treatment. HCA was used for assessment of data (Culbreth et al., 2012).

1. Biological Plausibility	The ReNcell CX-based apoptosis assay is based on a nestin-/SOX2 positive NSC line (Breier et al. 2008) that is commercially available from Millipore. These cells were generated from human fetal brains by myc-immortalization and grow in monolayers in 2D. Biological relevance of NPC proliferation is well established.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based mcy-immortalized cells.	
3. Availability of Resources	The line is commercially available from Millipore.	
4. Reference Chemicals	Aphidicolin Hydroxyurea Cytosine Arabinoside 5-Fluorouracil Ochratoxin A Actinomycin D Camptothecin Paclitaxel	

	Staurospoine D-Amphetamine Cadmium chloride hydrate Dexamethasone 5,5-Diphenylhydantoin Lead acetate Methyl mercury chloride trans-Retinoic acid Valproic acid Acetaminophen Amoxicillin Dimethyl phthalate Diphenhydramine hydrochloride Glyphosate Omeprazole Saccharin sodium salt hydrate D-Sorbitol (Culbreth et al. 2012)	
5. Within-laboratory reproducibility	yes	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Approx. 9% (Radio et al. 2015)	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Caspase3 can be activated from 0% to 100% (positive control, e.g. staurosporine)	Category 3
12. Concentration test range	pM - µM	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	HCA reduces personal bias, viability is assessed at the same time. Assay can be multiplexed with BrdU assay	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	possible as cells are commercially available from Millipore	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

- hUCBNSC-based apoptosis assay

Cell culture is described under 2.1.3 (Buzanska et al. 2009). For measurement of apoptosis, cells were stained with DAPI after 48 hrs treatment and chromatin condensation and cell nuclei fragmentation were counted manually.

1. Biological Plausibility	The hUCB-NSC apoptosis assay is based on a nestin-positive NSC line (Buzanska et al. 2002) that was differentiated from human umbilical cord blood stem cells. Biological relevance of NPC apoptosis is well established. However, how similar hUCB-NSC are to 'real' NPC is not clear and such a comparison has so far not been done. This group has only one publication on this apoptosis assay.	
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based cells.	
3. Availability of Resources	The line is not commercially or broadly available.	
4. Reference Chemicals	MeHgCl 0.01-0.5 µM (Buzanska et al. 2009)	
5. Within-laboratory reproducibility	-	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Up to 50 %	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	MeHgCl induces apoptotic cells from 0.5 to 6% of all cells. No positive control used.	Category 3
12. Concentration test range	MeHgCl 0.01-0.5 µM	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Viability is assessed, method not clear.	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

Chemical-induced NPC apoptosis can be measured with hESC-generated (2.2.1), primary hNPC (2.2.2), ReNcell CX (2.2.3) or hUCB-NSC line (2.2.4) -based systems. Category 1 criteria, which are essential, are met differently across the systems. Besides the ReNcell CX cells no method has a sufficient number of reference chemicals tested. ReNcell CX over all fulfil Category 1 criteria as many compounds were studied and the cells are commercially available. However, the myc-immortalization critically has to be

considered. Most lack in Category 1 criteria displays the hUCB-NSC line especially because there is no positive control compound used. Category 2 criteria, which deal with the reliability and efficacy of the assay itself, is also best for the ReNcell CX cell as the apoptosis assay seems to be reproducible in the lab. hESC-NPC and hNPC have no intra-lab reproducibility, but assay variation is comparable to the ReNcell CX cells. Assay variation is very high in the hUCB-NSC line and no intra-lab reproducibility is shown leading to this line having the worst Category 2 criteria. Category 3 criteria, technical assay criteria, are met best again by ReNcell CX-based NPC apoptosis assay due to the high number of compounds tested and the number of positive controls amongst the comounds. They are also generally met by the hESC- and primary hNPC- based apoptosis assays, yet the dynamic range of hUCB-NSC assay seems rather small and a positive control is missing. For Category 4 criteria, detailed protocols are published for the ReNcell CX -based apoptosis assay (Culbreth et al. 2012). Work with hESC needs special permission.

4.1.3 RADIAL GLIA ENPOINT

4.1.3.1 NPC/RADIAL GLIA MIGRATION

Radial glial cells play key roles during cerebral cortex development, as primary stem and progenitor cells that give rise to neurons and glia, but also acting as scaffold for the cerebral cortex architecture and migrating neurons. Also, migration of radial glia is crucial for formation of cortical layers (Borrell and Gotz, 2014). Radial glia cells belong to the NPC population with specific functions in humans compared to rodent species (Borrell and Gotz, 2014, Fish et al., 2008). E.g. these cells are responsible for folding of the human cerebral cortex.

- NPC/radial glia migration assay

So far, the human 'Neurosphere Assay' based on primary NPC is the only assay allowing studying self-organized radial migration of radial glia cells *in vitro*. These nestin/GFAP double positive cells show a typical radial glia-like morphology when stained by immunocytochemistry. Moreover, their migration is dependent on laminin-integrin interaction (Barenys et al., 2016) that is also known to be crucial for radial migration *in vivo* (Belvindrah et al., 2007). In addition, several signalling pathways known to contribute to cellular migration processes are involved in hNPC migration (Moors et al., 2007). One of these pathway inhibitors, PP2, a src kinase inhibitor, is thus used as a negative endpoint-specific control for this assay while epidermal growth factor (EGF) increases migration and thus serves as a positive endpoint-specific control (Baumann et al., 2015, Baumann, 2014). For migration analyses, Neurospheres with a diameter of 300µm are plated in presence or absence of compounds. After 24, 48 or 72 hours migration is measured manually from the sphere core to the furthest migrated cell (Fig. 22) or after staining the nuclei with DAPI by HCA with a Cellomics Array Scan or a similar device. The self-written algorithm Omnisphero (www.omnisphero.com) then automatically analyses migration distance (Schmuck et al. in press).

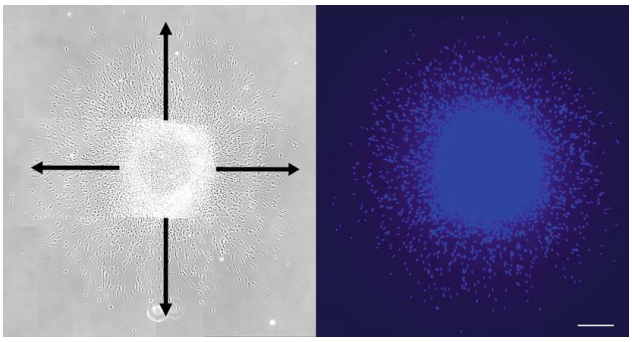


Figure 22: Measuring migration within the "Neurosphere Assay." (Left) Phase-contrast image of the whole migration area of a human neurosphere after 24 h of culture on a PDL/laminin coated surface of a well filled with differentiation medium. Arrows exemplify the assessment of the migration distance by measuring four perpendicular radii from the neurosphere edge to the end of the migration area. (Right) Fluorescent picture of the whole migration area of the same human neurosphere, where migrated nuclei stained with Hoechst can be counted automatically, scale bar 1/4 100 µm. From: Baumann et al. 2015

1. Biological Plausibility	That NPC, i.e. radial glia, migration is crucial for cortical development is well established. Recently, also species differences in radial glia cell-based cortical layer formation e.g. by radial glia cell types and functions have been elucidated (Borrell and Götz 2014) stressing the importance for using human cells for evaluating this endpoint in vitro.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses primary human cells.	
3. Availability of Resources	hNPC are commercially available from Lonza. The group has been publishing with these cells since 2005.	
4. Reference Chemicals	MeHgCl Phorbolester PMA Ethanol cAMP Phosphokinase C inhibitor Bisindolylmaleimide I Erk1/2 inhibitor PD98059 Epidermal Growth Factor Receptor (EGFR) inhibitor AG1478 Src Kinase inhibitors SU6656 and PP2 (Moors et al., 2007) *positive substances:* methylmercurychloride (MeHgCl) sodium (meta)arsenite (NaAsO2) methylazoxy methanol acetate (MAM) valproic acid sodium salt (NaVPA) chlorpyrifos ethyl (CPF) parathion ethyl (parathion) *negative substances:* L(+)-Monosodium glutamate monohydrate (glutamate) 4-Acetamidophenol (paracetamol) Penicillin G sodium salt (PenG)	

	(Baumann et al., 2015) 3-methylcholanthrene (3-MC) 3-methoxy-4-nitroflavone (MNF) 2,3,7,8-tetrachlorodibenzodioxine (TCDD) (Gassmann et al., 2010) β1-integrin functional inhibiting antibody β4-integrin functional inhibiting antibody Epigallocatechin gallate (Barenys et al., 2016) Epidermal growth factor Acrylamide (Schmuck et al. in press)	
5. Within-laboratory reproducibility	yes	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Migration distance varies ±5-10%	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Migration can be decreased to 0% of control and increased by using EGF.	Category 3
12. Concentration test range	nM-mM range depending on the compound	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Cell viability is assessed from the identical cultures by the Alamar Blue Assay (Baumann et al. 2015; and all papers mentioned above for the individual compounds). Endpoint can be detected with HCA and the Omnisphero program (Schmuck et al. in press)	
14. Response Characterization	Statistical evaluation	
15. Technological Transferability/ Proprietary elements	Detailed protocols published (Baumann et al. 2014, Fritsche et al. 2011)	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

Category 1-4 criteria are met, yet more chemicals with known effects on migration need to be tested.

4.1.4 NEURAL CREST CELL ENDPOINT

4.1.4.1. NEURAL CREST CELL MIGRATION

Neural crest cells (NCC) are essential for normal human development. During development, NCC differentiate into cell types of various tissues (e.g. bone, cartilage, neurons, melanocytes, etc.). To fulfil their specific roles in the appropriate tissues, they have to migrate correctly to different parts of the fetus (Dupin and Sommer, 2012).

- Neural crest cell migration assay (MINC assay)

The MINC assay is based on hESC. After differentiation of cells into NCC, cells are frozen, thawed and the migration assay is performed by the scratch assay (Dreser et al., 2015, Pallocca et al., 2016, Zimmer et al., 2012, Zimmer et al., 2014) or the stamp assay (Hirsch et al., 2016). For the scratch assay, NCC were thawed and 50,000 cells/cm^2 were seeded in 48 well plates and were grown to a monolayer. A confluent layer of cells is typically reached 2 days after plating. Once the cells have reached 100% confluence a cell free gap (scratch) was created using a 20 ml pipette tip. After scratching, the medium was changed to fresh media containing the test compounds for 48 h. Immediately after scratching the width of the cell free area was determined in a control plate. After 48 h cytotoxicity was analyzed using the resazurin reduction assay. In order to determine migrated cells, the nuclei were stained with the DNA dye H-33342 and unbiased images (3–4) along the scratch were taken at 4x magnification. The number of cells in the region of interest was counted automatically. The assay's timeline is depicted in Fig. 23
In Pallocca et al. (2016) microarray data is added to the migration data from Zimmer et al. (2014).

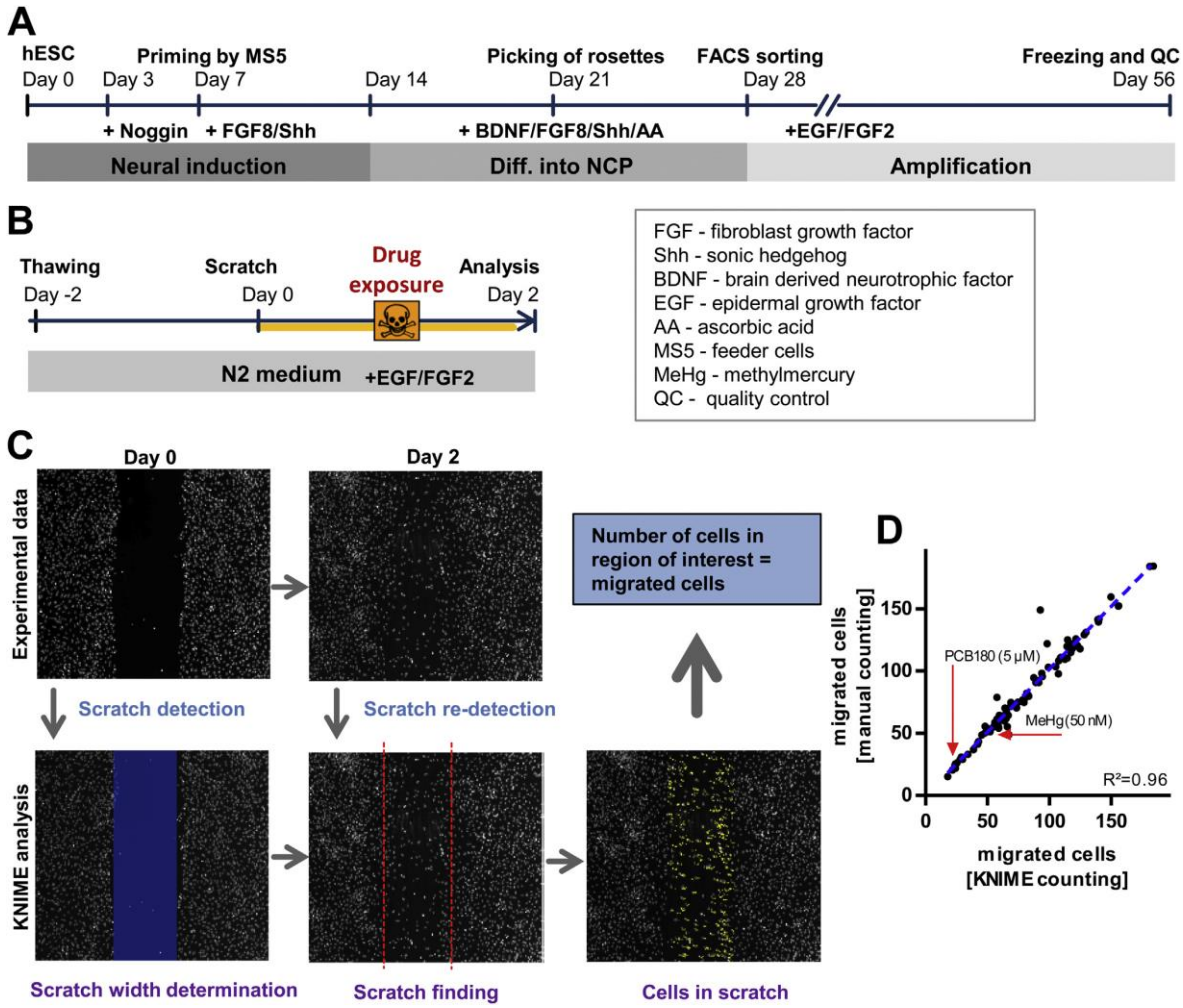


Figure 23: 'Migration inhibition of neural crest cell" (MINC) assay combined with automatic analysis. (A) Schematic illustration of the differentiation protocol of human embryonic stem cells (hESC) to neural crest cell (NCC). (B) Treatment protocol of the MINC assay. (C) Image analysis by the KNIME workflow. On day 0 the scratch was detected and the average width of the scratch was determined from three test wells. After a 2 day incubation period unbiased pictures were taken. The software automatically redetected the scratch in the pictures by using the information from day 0 and determined the position of the scratch and thereby the region of interest (ROI). The cells in the ROI were detected and counted automatically by the software. Finally, the number of cells migrated into the ROI was defined as the total number of migrated cells. (D) Validation of the KNIME image analysis vs. manual counting: the MINC assay was performed in the absence and presence of eight compounds at different concentrations. The number of cells migrating into the scratch was analyzed either manually (as described by Zimmer et al. (2014)) or by using the KNIME analysis tool, based on the same recorded images (n = 85) (Dreser et al., 2015).

1. Biological Plausibility	The NCC migration assay (MINC) studies the migration of NCC. This endpoint is very well established as essential for embryonic development.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human hESC-derived cells.	
3. Availability of Resources	Different hESC lines are available, however, one needs special permission to work with them. However, when cells are already differentiated to NCC they can be frozen down and one could	

	work with them without special permission.	
4. Reference Chemicals	MeHgCl $HgCl_2$ Thimerosal Triadimefon Triadimenol PbAc VPA (Zimmer et al. 2012) Teriflunomide Nintedanib/BIBF 1120 Telaprevir Sitagliptin Abiraterone Roflumilast Exenatide Gefitinib/Iressa Rivaroxaben Aliskiren Galnon Neuregulin Erythropoietin Geldanacycin G-CSF IFNb Sildenafil Amiodarone Chlorpromazine Methoxyacetic acid Cyproconazole PCR-153 Arsenic Trioxide PBDE-99 Triadimefon TSA VPA (Zimmer et al., 2014) MeHgCl Thimerosal PbAc Cadmium chloride Arsenic Trioxide PCB 153 PCB 170 PCB 180 Triadimefon Triadimenol Cyproconazole Rotenone VPA	

	TSA SAHA Tubacin (Dreser et al., 2015)	
5. Within-laboratory reproducibility	Well proven	Category 2
6. Between-laboratory reproducibility	This is the only group publishing this assay. However, the assay is very well published.	
7. Assay Variability	Migration distance has up to approx. 12% variability	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Migration distance from 0 – 100%	Category 3
12. Concentration test range	See individual papers	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Cytotoxicity is measured within the identical cultures.	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	Allowance to work with hESC needed unless NCC are used directly.	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

The MINC assay is very well established. Catergory 1 Criteria met, especially when one does not need a permission to work with hESC, but starts directly from NCC. A lot of test compounds have been tested. For category 2 criteria, between-laboratory reproducibility has to be shown, otherwise criteria met. Sensitivity/specificity analyses should be done with the sum of the compounds. Category 3 and 4 criteria are also met, over all it seems to be a reliable method.

4.1.5 ASTROGLIA ENDPOINT

4.1.5.1 ASTROCYTE DIFFERENTIATION

Astroglia differentiation is a crucial event during brain development as the functions of astroglial cells are many; astrocytes create the brain environment, build up the micro-architecture of the brain parenchyma, maintain brain homeostasis, store and distribute energy substrates, control the development of neural cells, synaptogenesis and synaptic maintenance and provide for brain defence. An archetypal morphological feature of astrocytes is their expression of intermediate filaments, which form the cytoskeleton.

The main types of astroglial intermediate filament proteins are glial fibrillary acidic protein (GFAP) and vimentin; expression of GFAP is commonly used as a specific marker for the identification of astrocytes. It works well in cultured astrocytes, but in situ the levels of GFAP expression vary quite considerably: for example, GFAP is expressed by virtually every Bergmann glial cell in the cerebellum whereas only about 15–20 % of astrocytes in the cortex of mature animals express GFAP. There is a large variety of different astrocytes present in brains. Protoplasmic astrocytes are present in gray matter, while fibrous astrocytes are present in white matter. The second big group of astroglial cells are the radial glia, which are bipolar cells each with an ovoid cell body and elongated processes. Radial glia usually have two main processes, one of them forming endfeet at the ventricular wall and the other at the pial surface. Radial glia are a common feature of the developing brain, as they are the first cells to develop from neural progenitors; from very early embryonic stages radial glia also form a scaffold, which assist in neuronal migration. After maturation, radial glia disappear from many brain regions and transform into stellate astrocytes (Adapted from: (Kettenmann and Verkhratsky, 2011).
According to this background information, astrocyte differentiation can be measured in developing mixed cell cultures by counting the percentage of e.g. GFAP+ cells from the total number of differentiated cells (number of nuclei). This was done by Talens-Visconti et al. (2011) with differentiated hESC treated for 14 days (day 21 – 35 of plating during differentiation) and by Moors et al. (2010; 2012) with primary hNPC grown as neurospheres differentiated for 4 days.

- hESC-NPC astrocyte differentiation assay

hESC-NPC were generated as described under 2.1.1.. On day 0 -21 cells were cultured in proliferation medium changing to differentiation medium for 14 days from day 21-35. Cells differentiated into 20% ±12% GFAP+ astrocytes. For assessment of compound effects on astrocyte differentiation, cells were treated during the 14 days of differentiation with the test compound (Talens-Visconti et al., 2011).

1. Biological Plausibility	That astrocytes are crucial for a functioning brain due to their many functions is well established.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based cells, hESC-derived NPC.	
3. Availability of Resources	Different hESC lines are available, however, one needs special permission to work with them.	
4. Reference Chemicals	Ethanol (Talens-Visconti et al. 2011)	
5. Within-laboratory reproducibility	-	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Control cultures develop 20% ±12% GFAP+ astrocytes.	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	

11. Dynamic Range	Treatment with EtOH reduced development of GFAP+ astrocytes to 5%, yet no variance was given for the ethanol treated cells and cytotoxicity was observed leaving it open if GFAP+ cell died or did not develop to begin with.	Category 3
12. Concentration test range	Ethanol (25 – 50 mM, Talens-Visconti et al. 2011)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Manual counting of cells with a high deviation of controls.	
14. Response Characterization	No statistics	
15. Technological Transferability/ Proprietary elements	The lab needs the allowance to work with hESC.	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

- Primary hNPC astrocyte differentiation assay

Primary hNPC (Lonza) grown as neurospheres in 3D were plated for differentiation (see Fig. 21). After 3 or 4 days, cells were stained for GFAP and the number of GFAP+ cells were visualized/counted (Baumann et al., 2015, Baumann, 2014, Moors et al., 2012, Moors et al., 2010). Immunocytochemichal stainings indicate the presence of GFAP+ cells with radial glia-like morphology (close to the sphere core) and with more stellate appearance in the periphery of the neurosphere migration area (Baumann, 2014). Compound treatment during differentiation resulted in accelerated morphological maturation (BMP7, (Baumann, 2014) or increased number of GFAP+ cells (Dexamethasone, (Moors et al., 2012); IL-7 (Moors et al., 2010).

1. Biological Plausibility	That astrocytes are crucial for a functioning brain due to their many functions is well established.	
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses primary human cells.	
3. Availability of Resources	hNPC are commercially available from Lonza. The group has been publishing with these cells since 2005.	
4. Reference Chemicals	IL-7 (Moors et al. 2010) Dexamethasone (Moors et al. 2012) BMP-7 (Baumann et al. 2015)	
5. Within-laboratory reproducibility	+	Category 2
6. Between-laboratory reproducibility	+	

7. Assay Variability	Approx. ±6% number of GFAP+ cells (Moors et al. 2012)	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Dexamethasone and IL-7 induced number of GFAP+ cells by 100% and 10%, respectively	Category 3
12. Concentration test range	Dexamethasone (1 μM) IL-7 (1.25 pg/μg)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	GFAP+ cells were counted manually.	
14. Response Characterization	Statistically	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

For astrocyte differentiation, hESC-NPC or primary hNPC are differentiated into neurons, astrocytes and oligodendrocytes. The % of GFAP+ cells was determined. For hESC there was just one publication found studying the effects of noxae on this endpoint, while for hNPC three publications are available. Catergory 1 criteria are better met by the hNPC assay than by the hESC-NPC assay as there is no special permit necessary for working with hNPC and three publications covered this endpoint. Moreover, Category 2 criteria are also covered better by primary hNPC than by hESC-NPC as the hNPC-astrocyte differentiation method was transferred to a different lab. For category 3, the hESC-NPC-based assay no dynamic range could be defined as the deviation of the treatment group was not given. In contrast, hNPC data revealed deviation amongst treatment (Dexamethasone ±5%, IL-7 ±15%).

- hUCB-NSC astrocyte differentiation

hUCB-NSC were differentiated as described above.
When grown on fibronectin-coated surfaces, HUCB-NSC differentiate into GFAP+, β-III-tubulin+ cells, cells negative and cells double positive for these molecular markers (35%, 25%, 25% and 10%, respectively).

1. Biological Plausibility	The hUCB-NSC assay is based on a nestin-positive NSC line (Buzanska et al. 2002) that was differentiated from human umbilical cord blood stem cells. Biological relevance of NPC differentiation into astrocytes is well established. However, how similar hUCB-NSC are to 'real', primary NPC is not clear and such a comparison has so far not been done.	

2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based cells.	
3. Availability of Resources	The line is not commercially or broadly available.	
4. Reference Chemicals	MeHgCl 0.0625 - 1 µM (Zychowicz et al. 2014)	
5. Within-laboratory reproducibility	-	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Up to 10 %	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	MeHgCl (0.0125) reduces the 35% GFAP+ cells by 40%, while viability of the cells was reduced by 20% at the same concentration after 48 hrs.	Category 3
12. Concentration test range	MeHgCl 0.01-0.5 µM	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Viability is assessed by the Alamar Blue Assay	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

Catergory 1 criteria is limited due to non-availability of the cells and restriction of test compounds (just MeHgCl). Category 2 criteria is limited by the missing inter- and intra-lab reproducibilities. For category 3, it is not clear if reduction in GFAP+ cells is due to cytotoxicity or if it is a specific effect on glia differentiation. Category 4 is not met due to non-availability of cells.

4.1.6 OLIGODENDROGLIA ENDPOINTS

Oligodendrocyte formation is crucial for brain functioning as they myelinate axons (Fig. 24), a necessity for proper nerve cell function. Multiple processes are involved in generation of sufficient numbers of oligodendrocytes: oligodendrocyte formation from oligodendrocyte progenitor cells, oligodendrocyte maturation, generation of myelin and finally proper wrapping around axons. Some human diseases based on myelination dysfunction due to multiple reasons are called leukodystrophia and can be accompanied by problems with movement, vision, hearing, balance, ability to eat, memory, behavior, and thought thus strengthening the role of oligodendrocytes for proper brain function.

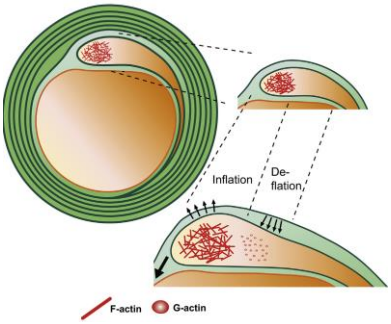


Figure 24: During CNS development, oligodendrocytes wrap their plasma membrane around axons to generate multilamellar myelin sheaths. To drive growth at the leading edge of myelin at the interface with the axon, mechanical forces are necessary that are thought to be based on F-actin(Nawaz et al., 2015).

4.1.6.1 OLIGODENDROCYTE FORMATION AND MATURATION

Different cell types describe the differentiation of NPC and stem cell-generated NPC into cells that express different oligodendrocyte markers like O4 or GalC. This is in general an oligodendrocyte formation assay because maturation over time is not evaluated. Oligodendrocytes are in these publications formed as a fraction of all differentiated cells.

- Oligodendrocyte assay based on hNPC

hNPC grown as neurospheres are cultured and plated as described above. After 5 days of differentiation they are stained with an antibody against O4 and nuclei are counterstained with DAPI. The number of oligodendrocytes is assessed by manual counting and related to the total number of nuclei in the migration area (Schreiber et al. 2010, Baumann et al. 2014). In addition, this cell system can also be used to study oligodendrocyte maturation as a function of myelin basic protein (MBP) gene expression over time. Thyroid hormone accelerates MBP expression in hNPC after 5 days of differentiation and serves as an endpoint-specific control for oligodendrocyte maturation (Baumann et al. 2014).
Neurospheres generated from embryonic/fetal cortices (GW 10-13) also contain oligodendrocyte precursor cells (OPCs; (Lu et al., 2015). supporting the methodology of primary human neurospheres for oligodendrocyte formation.

1. Biological Plausibility	That oligodendrocytes are crucial for brain function is well established. Without myelin sheets neuronal electrical conduction is not functioning properly.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses primary human cells.	
3. Availability of Resources	hNPC are commercially available from Lonza.	

	The group has been publishing with these cells since 2005.	
4. Reference Chemicals	Polybrominated diphenyl ether (PBDE)-47 and -99 (Schreiber et al., 2010) PBDE-99 Ascorbic Acid (Dach et al. in revision) Bone morphogenic protein (BMP)-7 as endpoint-specific control (Baumann, 2014)	
5. Within-laboratory reproducibility	yes	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Number of O4+ oligodendrocytes ±6-8%	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Oligodendrocytes can be decreased to 0% of control (BMP-7; Baumann et al. 2015) or increased to 200% (Ascorbic Acid, Dach et al. in revision).	Category 3
12. Concentration test range	nM-µM range	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Cell viability is assessed from the identical cultures by the Alamar Blue Assay (Schreiber et al. 2010; Baumann et al. 2014; Dach et al. in revision).	
14. Response Characterization	Statistical evaluation	
15. Technological Transferability/ Proprietary elements	Detailed protocols published (Baumann et al. 2014, Fritsche et al. 2011)	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

Category one is met, endpoint-specific controls are established. However, the number of reference compounds is not high enough. More substances need testing in the assay. For category 2, mainly the inter-lab variability is missing and the assay's accuracy assessment. Therefore, more reference compounds need to be tested. Category 3 criteria are met. Category 4 cannot be sufficiently evaluated. Over all, the assay is ready for compound testing.

- Oligodendrocyte formation/maturation assay with hESC

Different groups have differentiated hESC into oligodendrocytes, yet there is very sparse data on chemicals' effects on hESC-derived oligodendrocytes. Talens-Visconti et al. (2011) differentiated oligodendrocytes during 14 – 28 days in culture. Ethanol inhibited number of Olig1- and GalC+ cells. However, cells were not quantified in this study, therefore, assay parameters cannot be evaluated. Moreover, Nash et al. 2012 studied hESC-based oligodendrocyte formation after 20 mM ehanol exposure for one week on

the basis of Western blot analyses using the NG2 antibody, that recognizes an integral membrane chondroitin sulfate proteoglycan present specifically in oligodendrocyte progenitors. However, also the Western blots were not quantified.
In addition, data from the review article on disease models for multiple sclerosis therapy will be displayed here (Madill et al. 2016). Briefly, neural induction was induced through embryoid bodies or in monolayer by treatment with LDN193189/SB431542. Shh and RA patterned differentiating cells to pMN regional identity expressing OLIG2/NKX2.2. OPC- promoting factors (PDGF, NT-3, T3, IGF-1, and HGF) then used to differentiate neural stem cells to OPCs. Withdrawal of growth factors results in terminal differentiation/maturation of OPCs to MBP+ OLs (Douvaras and Fossati, 2015, Stacpoole et al., 2013). In addition, hiPSC were differentiated to OPCs. The most rapid protocol published to date yields 30% O4+ OPCs in 55 days differentiation from iPSCs (rev. in (Madill et al., 2016)).
Because in these models stem cells are differentiated to OPC these assays might be used for OPC differentiation. One drawback of the method is the long time cells need for differentiation just into OPCs. Afterwards, OPCs also mature in culture possibly allowing to study OPC maturation with such systems (Hu et al., 2009).

- Oligodendrocyte formation assay with hUCB-NSC

After differentiation of hUCB-NSC cells seem to express the oligodendrocyte marker GalC (Buzanska et al. 2005). Yet, the number of oligodendrocytes was not quantified. None of the compounds tested, MeHgCl, L- and D-glutamate, acetaminophen, theophylline altered number of GalC+ cells in the cultures (Buzanska et al. 2009). Others studied oligodendrocyte formation in culture without using test compounds. However, such protocols might be useful in the future (rev. in Madill et al., 2016, Chen et al., 2013, Leite et al., 2014).
Due to the lack of compound testing data with hUCB-NSC the method cannot be evaluated as a toxicity test.

1. Biological Plausibility	That oligodendrocytes are crucial for brain function is well established. Without myelin sheets neuronal electrical conduction is not functioning properly.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based cells.	
3. Availability of Resources	The line is not commercially or broadly available.	
4. Reference Chemicals	No compound used altered number of GalC+ oligodendrocyte in culture, all were negative for this endpoint: MeHgCl, L- and D-glutamate, acetaminophen, theophylline (Buzanska et al., 2009)	
5. Within-laboratory reproducibility	Yes, but no quantification of cells	
6. Between-laboratory	-	

reproducibility		Category 2
7. Assay Variability	Up to 100 % (Buzanska et al., 2009)	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Not enough concentrations of compounds tested	Category 3
12. Concentration test range	MeHgCl	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Viability is assessed, method not clear.	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

4.1.7 NEURONAL ENDPOINTS

Neurogenesis is another crucial endpoint for proper brain function. Neurogenesis actually starts from generation of neuroblasts, however, in this context the endpoints covered here represent neuronal features from NPC differentiation into neurons, neurite formation up to cell-cell connections by forming (functioning) synapses.

4.1.7.1 NEURONAL DIFFERENTIATION

Neurons are necessary for brain function as they carry information via transport of electrical activity. Different neuronal subtypes confer to this function forming inhibitory and excitatory synapses by extending axons and dendrites. A vast amount of work on neuron development has been done using primary rodent neurons (Fritsche et al., 2015). As this paper solely reports on human-based methods, these will not be reviewed and evaluated here.

- Neuronal differentiation using hESC

Compound effects on neuronal differentiation is the most studied endpoint in hESC (Bai et al., 2013, He et al., 2012, Nash et al., 2012, Palmer et al., 2012, Stummann et al., 2009, Talens-Visconti et al., 2011), some of them also measuring neuronal apoptosis. Above (under 2.1.1) we gave three detailed examples of different methods for NPC generation from hESC (Talens-Visconti et al., 2012; Nash et al., 2012; Bai et al., 2013). Although within the assays reviewed for neuronal differentiation also each lab used its specifically adapted protocols, these will not be specifically listed here in detail as it goes beyond the scope of this report. However, again, there is an urgent need for harmonization of protocols to get comparable results of DNT testing across different labs. To briefly summarize studies investigating compounds' effects on neurogenesis:

Mercury reduced mRNA expression of MAP2 in hESC-derived neurons that were generated through the NPC pathway, and treated between day 21 and 33, only at

cytotoxic concentrations. Other cells in cultures were not specified. Cultures were not stained at day 33 when RNA samples were taken, but at day 42. Thus, it does not seem to be a differentiation, but rather a cytotoxic effect on differentiating neurons (Stummann et al., 2009).

He et al. (2012) differentiated hESC-NPC for 24 days with 10 – 1000 nM of MeHgCl. >100 nM MeHgCl decreased viability (MTT assay) after 13 days of exposure, while concentrations ≥1 nM reduced number of Map-2 positive neurons and neurite length significantly as determined by HCA. mRNA expression for NANOG and HOXB4 was increased with concentrations ≥10 nM and for PAX6 and EMX2 decreased for concentrations ≤10 and 1 nM, respectively. These data show differentiation effects of MeHgCl on neuronal differentiation.

Directly from hESC differentiated neurons (92% pure cultures) became apoptotic upon treatment with ketamine (24 hrs, 100 μM) (Bai et al., 2013).

Ethanol treatment (25 & 50 mM) was performed for 14 days between day 21 and 35 during neuronal differentiation from hESC-based NPC. Ethanol treated cells showed less differentiated neurons (Tuji), less Map-2 staining and GABA-A- and Glutamate-positive neurons. However, not a single immunofluorescent marker was quantified. This group also measured neuronal apoptosis (Talens-Visconti et al., 2011).

Ethanol exposure for 7 days (20 mM) did not alter neuronal differentiation of hESC-neuroblasts. However, immunostaining was not quantified. Apoptosis was not measured in differentiated neurons, just in undifferentiated hESC (Nash et al., 2012).

Four day treatment with 0,1 and 0,3% ethanol did not alter number of β-III-tubulin+, tyrosine hydroxylase+ or SV2A+ (synaptic vesicle protein) differentiated cells as determined by immunofluorescence. Yet, some images are not shown. Metabolome changes were observed in alcohol treated neurons and disturbed tryptophan metabolism by ethanol speculated as a mechanism of ethanol developmental neurotoxicity (Palmer et al., 2012).

1. Biological Plausibility	That neuronal differentiation is essential for brain development is well established.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human cells.	
3. Availability of Resources	Different hESC lines are available, however, one needs special permission to work with them.	
4. Reference Chemicals	MeHgCl (Stumman et al. 2009; He et al. 2012) Ethanol (Talens-Visconti et al. 2011; Nash et al., 2012) Ketamine (Bai et al., 2013)	
5. Within-laboratory reproducibility	-	Category 2
6. Between-laboratory reproducibility	Neuronal differentiation protocols different, exposure times different, no quantification of	

	data, etc. makes evaluation very difficult, yet, every lab differentiated hESC into β-III-tubulin+ cells. Ratio of neurons and glia not always clear, strong differences between protocols	
7. Assay Variability	Cannot be determined without quantification of results	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Cannot be determined without quantification of results	Category 3
12. Concentration test range	Methylmercury: 3 – 300 nM for 12 days (Stummann et al. 2009) Methylmercury: 10-1000 nM for 24 days (He et al. 2012) Ethanol: 25 & 50 mM for 14 days (Talens-Visconti et al. 2011) Ethanol: 20 mM, 7 days (Nash et al. 2012) Ethanol: 0,1 and 0,3% for 4 days (Palmer et al. 2012) Ketamine: 100 µM, 24 hrs (Bai et al. 2013)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Stummann et al. (2009) measured viability via the alamar blue assay He et al. (2012) measured viability by MTT and neuroal endpoints by HCA Talens-Visconti et al. (2011): cell death determination by annexinV (apoptosis) and 7-amino-actinomycin D (necrosis) detection by FACS analyses Nash et al. (2012): no viability measures Palmer et al. (2012): no viability measures Bai et al. (2013): viability measures via mitochondrial membrane potential assessment, also measures of apoptosis by cleaved caspase-3	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	Allowance to work with hESC needed	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

The neuronal differentiation assay based on hESC is very heterogeneous across research groups and has mainly been used for mechanistic or hazard assessment studies. For Category 1 criteria, more reference compounds are needed and a harmonization protocol is obligatory. Category 2 is not fulfilled as in most studies there was no quantification performed. Moreover, the reproducibility is difficult with the different protocols. For Category 3 criteria are also not met because concentration-response curves are needed that were only provided by Stummann et al. (2009). Viability assays must be mandatory to differentiate effects of compounds on neuronal cell death from

interference with neuronal differentiation. Category 4 criteria also need clear harmonization of procedures with detailed documentation.

- Neuronal differentiation using hNPC

One more endpoint that the 'Neurosphere Assay' can detect is differentiation from NPC to β-III Tubulin+ neurons (Barenys et al., 2016, Baumann et al., 2015, Baumann, 2014, Moors et al., 2012, Moors et al., 2010, Schreiber et al., 2010). Neurospheres of a defined size are plated onto poly-D-lysin/laminin coated matrices. Neuronal differentiation is assessed after 3 or more days by counting number of β-III Tubulin+ neurons in the migration area. Number of neurons is divided by the number of total migrated nuclei resulting in the percentage of cells. Neurons in the neurosphere migration area can also be assessed by HCA using a self-developed algorithm (Schmuck et al., in press). This assay is not suited for studying neuronal maturation with the current protocol, just neuron formation, because the cells stay at a relatively immature, bipolar state that they obtain in vivo during migration and cortical formation (Borell and Gotz 2014).

1. Biological Plausibility	That neuronal differentiation is essential for brain development is well established.	
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses primary human cells.	
3. Availability of Resources	hNPC are commercially available from Lonza. The group has been publishing with these cells since 2005.	
4. Reference Chemicals	PBDE-47 PBDE-99 (Schreiber et al. 2010) Epigallocatechin gallate (Barenys et al. 2016) *positive substances:* methylmercurychloride (MeHgCl) sodium (meta)arsenite ($NaAsO_2$) methylazoxy methanol acetate (MAM) valproic acid sodium salt (NaVPA) chlorpyrifos ethyl (CPF) parathion ethyl (parathion) *negative substances:* L(+)-Monosodium glutamate monohydrate (glutamate) 4-Acetamidophenol (paracetamol) Penicillin G sodium salt (PenG) (Baumann et al. 2015) Epidermal growth factor (EGF) (Baumann et al. 2014) Dexamethasone (Moors et al. 2012)	
5. Within-laboratory reproducibility	+	
6. Between-laboratory reproducibility	-	

7. Assay Variability	Approx. ±15-20% variation in number of β-III-Tubulin+ cells	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Dexamethasone and EGF reduced number of β-III-Tubulin+ cells by 90 %.	Category 3
12. Concentration test range	nM-mM range depending on the compound Dexamethasone (1 µM) IL-7 (1.25 pg/µg)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	β-III-Tubulin+ cells were counted manually. Automated counting meanwhile established (Schmuck et al. in press) Cell viability is assessed from the identical cultures by the Alamar Blue Assay (Baumann et al. 2014; and all papers mentioned above for the individual compounds).	
14. Response Characterization	Statistically	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

Category 1 criteria are met, yet more chemicals with known effects on neuronal differentiation need to be tested. Category 2 criteria lack the lab-to-lab transferability. Category 3 criteria are met, Category 4 criteria needs more work. Methods are published in detail in Fritsche et al. 2011, Baumann et al. 2014.

- Neuronal differentiation using hUCB-NSC

hUCB-NSC were differentiated as described above.
When grown on fibronectin-coated surfaces, HUCB-NSC differentiate into GFAP+, β-III-tubulin+ cells, cells negative and cells double positive for these molecular markers (35%, 25%, 25% and 10%, respectively).

1. Biological Plausibility	The hUCB-NSC assay is based on a nestin-positive NSC line (Buzanska et al. 2002) that was differentiated from human umbilical cord blood stem cells. Biological relevance of NPC differentiation into astrocytes is well established. However, how similar hUCB-NSC are to 'real', primary NPC is not clear and such a comparison has so far not been done.	
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human-based cells.	

3. Availability of Resources	The line is not commercially or broadly available.	
4. Reference Chemicals	MeHgCl 0.0625 - 1 µM (Zychowicz et al. 2014)	
5. Within-laboratory reproducibility	-	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	Up to 10 %	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	MeHgCl (0.0125) reduces the 25% β-III-tubulin+ cells by 30%, while viability of the cells was reduced by 20% at the same concentration after 48 hrs.	Category 3
12. Concentration test range	MeHgCl 0.01-0.5 µM	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Viability is assessed by the Alamar Blue Assay	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

Catergory 1 criteria is limited due to non-availability of the cells and restriction of test compounds (just MeHgCl). Category 2 criteria is limited by the missing inter- and intra-lab reproducibilities. For category 3, it is not clear if reduction in β-III-tubulin+ cells is due to cytotoxicity or if it is a specific effect on glia differentiation. Category 4 is not met due to non-availability of cells.

4.1.7.2 NEURONAL SUBTYPE DIFFERENTIATION

Two models were published for neuronal subtype (dopaminergic) differentiation: hESC and LUHMES cells.
For dopaminergic differentiation hESCs were seeded at a density of approximately 1000 clumps/3cm dish on a confluent layer of the mouse stromal cell line PA6 feeder cells in Glasgow Minimum Essential Media supplemented with 10% KSR, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM b-mercaptoethanol. hESCs were allowed to differentiate into dopaminergic neurons for 3 weeks on PA6 cells before drug treatment. A colony was counted as a TH+ colony if at least 25 individual cells in the colony were stained for tyrosine hydroxylase (TH). Model compounds: MPP+ (0,5 – 5 mM), GDNF as neuroprotectant; Readouts: number of TH+ colonies, LDH, ROS (Zeng et al., 2006).

Generation of purely dopaminergic LUHMES cells from progenitor cells has been published (Scholz et al., 2011). LUHMES cells (available through ATCC) were derived from female human fetal (8 weeks) brain by clonal selection of conditionally immortalized (tetracycline-controlled v-myc) ventral mesencephalic cells. These cells could be used to study also dopaminergic differentiation.
There are not enough publications for this endpoint available to analyse assay readiness.

4.1.7.3 NEURITE OUTGROWTH

Neurite outgrowth is a critical process of nervous system development in which neurons extend a compliment of specialized processes for the purpose of establishing contacts sites (i.e. synapses) and facilitating the directional flow of information throughout a neural network. Abnormalities in neurite morphology have been observed following in vivo exposure of developing laboratory rodents to developmental neurotoxicants (Alfano and Petit, 1982). Neurite outgrowth can be studied *in vitro* using a variety of neural culture models.

- Neurite outgrowth assay using hESC/hNPC-based methods

Neurite outgrowth was measured with hESC-based methods and HCA (Neural Profiling Bioapplication). This possibility of data evaluation makes this endpoint fairly reliable and suitable for higher throughput applications (Harrill et al., 2010, Harrill et al., 2011a, He et al., 2012). Harrill et al. (2011a) uses hESC-derived neurons (hN2™), which are single-cell type cultures. In contrast, He et al. (2012) used 3D aggregated hESC-derived neural cells, which differentiate in mixed culture. Still, neurite outgrowth can be measured.
With the neurosphere assay and a self-written algorithm (www.omnisphero.com) in comparison to the Neural Profiling Bioapplication neurite parameters can also be measured in a sphere set-up (Schmuck et al. in press).

1. Biological Plausibility	That neurite outgrowth is essential for brain development as it determines cell-cell interaction and synaptogenesis is well established.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human cells.	
3. Availability of Resources	Different hESC lines are available, however, one needs special permission to work with them. hNPC are commercially available from Lonza.	
4. Reference Chemicals	Bis1 U0126 LiCl Na3VO4 Bref A (Harrill et al. 2010) *Positives* Methylmercury chloride t-retinoic acid Bis-indolylmaleimide 1 (Bis-1) Lead acetate	

	U0126 Dexamethasone *Negatives* Saccharin sodium salt Acetaminophen Glyphosate Dimethyl Phthalate Amoxicillin D-Sorbitol (Harrill et al. 2011a) MeHgCl (He et al. 2012) MeHgCl Acrylamide EGF (Schmuck et al. in press)	
5. Within-laboratory reproducibility	+	Category 2
6. Between-laboratory reproducibility	Many labs have established hESC-based neuronal differentiation. Thus, one can assume good transferability in case there is a common, stringent and well-documented protocol (SOP). The 3D protocol has not been transferred. The neurosphere assay needs transfer in a different lab.	
7. Assay Variability	± 10-20% depending on the method	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Up to 100% reduction in neurite length	Category 3
12. Concentration test range	Methylmercury: 1 – 100 nM for 23 days (He et al. 2012) 0.003–10 µM for Bis1, 0.01–30 µM for all other compounds (Harrill et al. 2011a)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Harrill et al. (2011a) measured cell count as a measure for viability. He et al. (2012) performed MTT assay. Schmuck et al. performed Alamar blue assay in the same cultures.	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	Allowance to work with hESC needed None for hNPC.	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

- LUHMES-based Neurite outgrowth assay

As the LUHMES culture and differentiation (Scholz et al., 2011) is special and differs from other stem/progenitor cell methods, it will be described here in detail. In the proliferation state, LUHMES cells expressed v-myc and had a doubling time of 24 h. Differentiation followed a 3-step procedure: For preparation of the differentiation, 8 million cells of passage 5–15 were seeded in a Nunclon T175 flask and were grown for 24 h in proliferation medium. In a second step, the differentiation process was initiated by changing the medium to differentiation medium I consisting of advanced DMEM/F12 supplemented with 2mM L-glutamine, 1 3 N2, 2.25µM tetracycline, 1mM dibutyryl-cAMP, and 2 ng/ml recombinant human GDNF. Under these conditions, v-myc was switched off rapidly and cells became postmitotic. In the third step, LUHMES cells predifferentiated for 2 days were trypsinized and seeded on dishes precoated with 50 µg/ml poly-L-ornithine (PLO) and 1 µg/ml fibronectin under the continued presence of tetracycline but without cAMP and GDNF (differentiation medium II 1/4 DM II). Cells were seeded at a density of 30,000 cells per well in 50 µl DM II on PLO/fibronectin coated 96-well dishes. Compounds were serially diluted in DM µI, and 50 µl were added to the cells 1 h after seeding. Cells were stained with calcein-AM and nuclei with Hoechst. Neurites were quantified with HCA (Cellomics ArrayScan).

1. Biological Plausibility	That neurite outgrowth is essential for brain development as it determines cell-cell interaction and synaptogenesis is well established.	Category 1
2. Extrapolation to humans, or broadly applicable across vertebrates/phyla	The assay uses human cells, that are c-myc immortalized and consist of dopaminergic neural progenitors.	
3. Availability of Resources	LUHMES cells are available through ACTT	
4. Reference Chemicals	U0126 BisI MeHg Brefeldin A Cycloheximide Flavopiridol Na3VO4 Y-27623 (Stiegler et al., 2011) Acrylamide, antimycin A, acetylsalicylic acid, blebbistatin, brefeldin A, buthionine sulfoximine (BSO), calcein-AM, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), chlorpyrifos, cisplatin, colchicine, cycloheximide, cytoch- alasin B, dibutyryl-cAMP (cAMP), 2,4-dinitrophenol, diquat dibromide, etoposide, bronectin, pronil, a-vopiridol, hoechst bisbenzimide H-33342, honokiol, IPA3, potassium chromate (K_2CrO_4), mannitol, menadione, methylmercury (II) chloride (MeHg), mevastatin, narciclasine, nocodazole, oligomycin, paraquat dichloride, puromycin, resazurin sodium salt, rotenone,	

	saponin, sodium orthovanadate (Na_3VO_4), SP600125, tert-butyl hydroperoxide (tBuOOH), tetracycline, vincristine, FGF-2, GDNF ,Bisindolylmaleimide I (Bis1), dimethyl sulfoxide (DMSO), 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), oka- daic acid potassium salt, PTP inhibitor IV, H1152, simvastatin, U0126, Y-27632, tween-20, sodium dodecyl sulfate (SDS), HA-1077, thiazovivin, chlorpyrifos oxon, piericidin, methamphetamine (Krug et al., 2013a)	
5. Within-laboratory reproducibility	+	Category 2
6. Between-laboratory reproducibility	-	
7. Assay Variability	± 10-15%	
8. Accuracy	n.d.	
9. Assay specificity	n.d.	
10. Assay sensitivity	n.d.	
11. Dynamic Range	Up to 100% reduction in neurite length	Category 3
12. Concentration test range	Depending on the compound (Krug et al., 2013a)	
13. Detection/Adjustment of confounding factor and/or incorrect/ inconclusive measurements and/or other bias	Resazurin reduction was measured as aviability assay in the same cultures.	
14. Response Characterization	Statistical significance	
15. Technological Transferability/ Proprietary elements	-	Category 4
16. Transparency of the method	n.d.	
17. Documentation	n.d.	

Category 1 criteria are fulfilled, just caution has to be taken as these cells differentiate into pure dopaminergic neurons and thus do not resemble a broader spectrum.
Category 2 the between lab reproducibility has to be shown, other than that criteria are met also for categories 3 and 4. Sensitivity and specificity needs evaluation, which should be possible with such a large compound test set.

4.1.7.4 SYNAPTOGENESIS/NEURONAL NETWORK FORMATION

Synaptogenesis is crucial for brain formation as well as learning and memory. Therefore, this is an important endpoint to study. So far there are not many studies available measuring this endpoint. There is more literature to find on such methods for basic research than for chemical testing. One existing testing assay uses primary rat cells but shows that in principle a 96-well plate assay is suitable to study synaptogenesis (Harrill et al., 2011b) in developing brain cells. A second study investigates synaptogenesis in LUHMES cells.
In addition to measuring synaptogenesis, one can grow neurons on multielectrode arrays (MEAs) to study functionality of synapses. This has also recently been done for chemical testing purposes using rat cells (Robinette et al., 2011) and is currently up-graded into a multi-well format. Moreover, this system has undergone a ring trial for acute neurotoxicity lately using rat cells (Vassallo et al., 2016) and been used in a testing strategy for NT/NDT (Behl et al., 2015).

With human cells, labs that intend chemical testing and have therefore started to establish human-based cell systems on MEAs or study synaptogenesis are the following:
Susanna Narkilahti established hESC on MEAs and is currently also working on hiPSC (Kapucu et al., 2012, Kiiski et al., 2013).
Marcel Leist established the LUHMES cell system in a way that electrical activity can be measured by patch-clamp (Scholz et al., 2011).
Ellen Fritsche has established hiPSC-derived neurospheres to exert electrical activities on MEAs (Hofrichter et al., submitted).
To the best of my knowledge, also Tim Shafer from the US-EPA and Anna Bal-Price from the JRC (Hogberg et al., 2011) are working on hiPSC-based MEA recordings for DNT testing.
Also Helena Hogberg and David Pamies from CAAT are working on hiPSC-derived mini-brains. They detected the synaptic protein synapsin in the 3D cultures yet no quantification or reproducibility was shown.
Over all, the endpoint synaptogenesis/network activity is the DNT endpoint that needs the most establishment and chemical testing before human-based methods can be used. However, with rat cultures this is already possible.

4.1.8 PERIPHERAL NERVOUS SYSTEM ENDPOINTS

With an elaborate protocol hESC can be differentiated into immature dorsal root ganglia cells (Hoelting et al., 2016). These can be used as a test system to screen for compounds affecting peripheral nervous system development. Although this system was primarily established for testing for acute, peripheral neurotoxicity, it might also find its place in a DNT testing strategy.

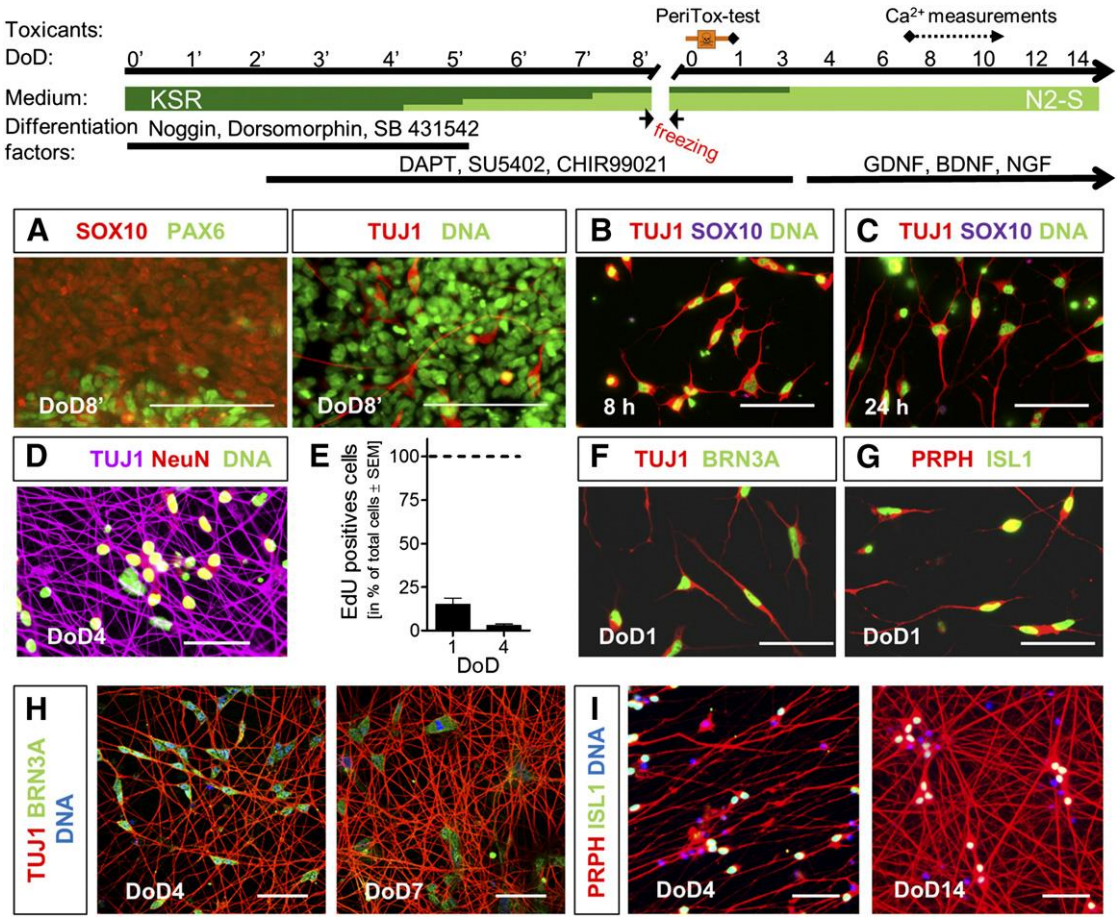


Figure 25: Generation of human immature dorsal root ganglia neuron (iDRG) cells for toxicity testing. Pluripotent stem cells were differentiated in a two-step procedure, as indicated. (A–D): Immunocytochemical characterization of hESC-derived cells. Labels are color keyed to images. (E): Proliferating cells (EdU+) were quantified (mean 6 SEM; n = 3). (F–I): Immunocytochemical characterization of iDRG cells after thawing. Labels are color keyed to images. Scale bars = 100 mm (A) and 50 mm (B–D, F–I). Abbreviations: BDNF, brain-derived neurotrophic factor; DoD, day of differentiation; GDNF, glia-derived neurotrophic factor; hESC, human embryonic stem cell; NGF, nerve growth factor (Hoelting et al., 2016).

Next, the more general, conceptual studies identified are discussed. A summary of each will be given, endpoints and number of compounds described and strength and weaknesses of the studies will be addressed.

4.2 STUDIES PRESENTING CONCEPTS FOR DNT TESTING

4.2.1

Dreser et al. (2015) suggested the MINC (migration inhibition of neural crest cells) assay in combination with gene expression data as a DNT assay with a functional readout relevant for human development and ideas for the mode-of-action of compounds. This approach should facilitate read-across approaches by generation of molecular data within a functional assay (Fig. 26). It is suggested that this approach can be an immediate step to support hazard assessment by read-across and for early grouping of

potential toxicants, long before the technology and resources are ready to determine full AOPs/toxicity pathways.

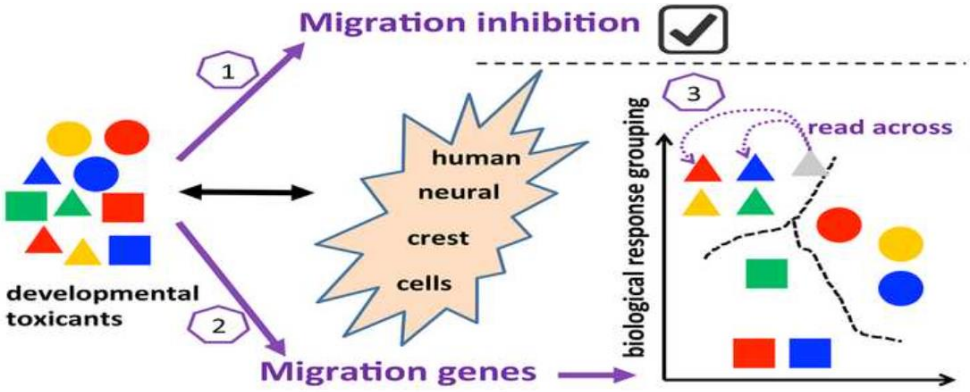


Figure 26: Concept of the MINC assay in combination with gene expression readouts to facilitate grouping and read-across of compounds (Dreser et al., 2015).

Readouts:

- Neural crest cell (NCC) migration in the MINC assay by using hESC-derived NCC
- Gene expression analyses

Number of compounds tested:

- 16 DNT positive compounds including their structural analogues, no negative or non-DNT compounds

Advantages:

- Medium throughput
- Combination of functional and molecular information
- Primary stem cell-based assay
- Mechanism-based approach by focusing on HDAC inhibition

Disadvantages

- hESC-based (possibly ethical issue for testing purposes)
- Scratch assay not specific for migration during development

Study conclusion:
The MINC assay might be part of a larger DNT testing strategy covering migration during early development relevant for neural tube formation as well as generation of the peripheral nervous system and migration of other cell types like melanocytes. Later cell stages are also needed as well as other endpoints. This publication is supported by other studies of the authors (Zimmer et al., 2012, Zimmer et al., 2014, Pallocca et al., 2016).

4.2.2

Baumann et al. (2015) employed hNPC in a multiplexed neurosphere assay to study NPC proliferation, migration, neuronal differentiation and viability. Results from hNPC were compared to time-matched rat NPC (Fig. 28). These assays were performed using SOPs. Results of assays (IC_{50} values) were related to published internal concentrations of respective compounds causing adverse neurodevelopmental outcomes in children and rats. This approach helped defining the application domain of the neurosphere assay and placed the assay in a larger framework of DNT testing mainly addressing timing and processes (Fig. 27). The assay can be used for medium throughput when spheres are plated automatically with a Copas Biosorter (Gassmann et al., 2012) and HCA can be applied (Schmuck et al. in press).

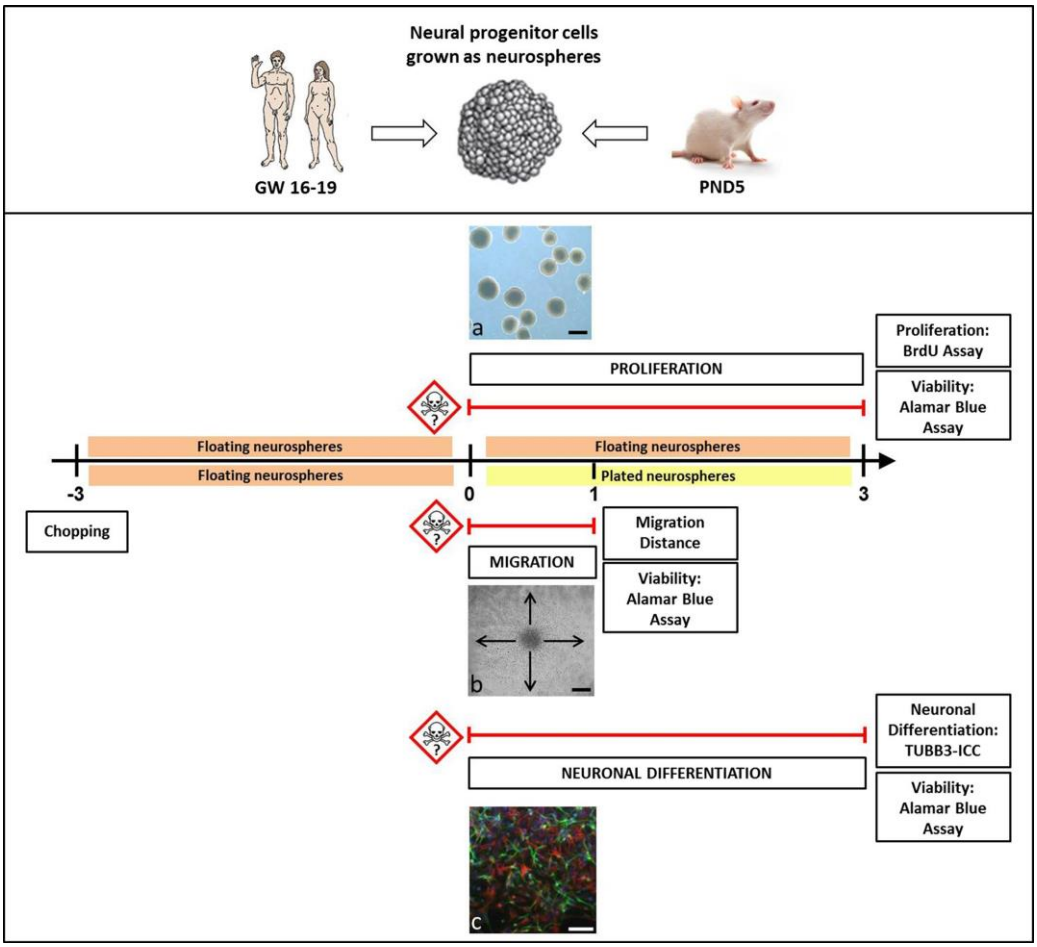


Figure 27: Schematic overview of the experimental setup and chemical treatment periods of human and rat neurospheres. Human and rat neurospheres are exposed to test compounds (indicated in *red*) as oating neurospheres for assessing proliferation (days 0–3) or as plated neurospheres to assess either migration (days 0–1) or neuronal differentiation (days 0–3). For all endpoints, viability is investigated in parallel. Timeline is in days. *Scale bars* a and b 300 µm, c 100 µm. c *Red* GFAP-positive cells, *green* βIII-tubulin-positive cells, *blue* cell nuclei (Baumann et al., 2015).

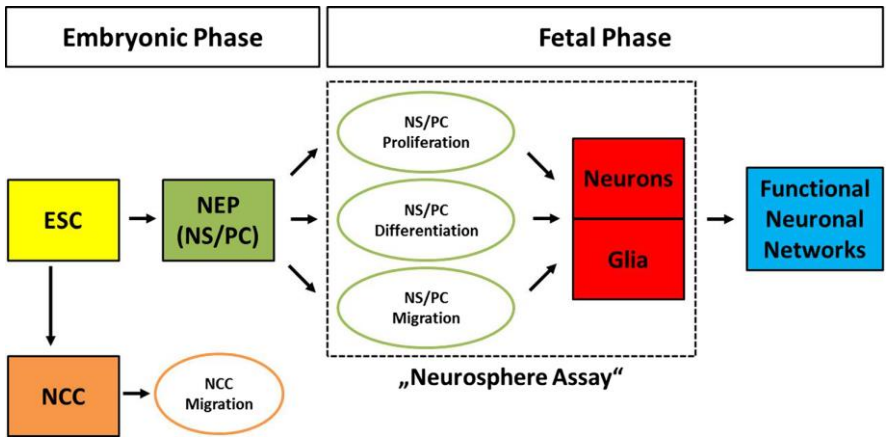


Figure 28: Testing strategy for in vitro DNT testing. The assessment of different early and late neurodevelopmental key events provides a comprehensive approach for developmental neurotoxicity testing. Thereby, the endpoints evaluated within the neurosphere assay integrate into early fetal development. *ESC* embryonic stem cell, *NCC* neural crest cell, *NEP* neuroepthelial precursor cell, *NS/PC* neural stem/progenitor cell (Baumann et al., 2015).

Readouts:

- Proliferation: BrdU incorporation
- Migration: measurements of distance NPC travel out of the sphere
- Neuronal differentiation: immunocytochemical staining for ß(III)tubulin & nuclei (Hoechst) in mixed neuronal-glia cultures
- Multiple publications by the group on these endpoints studying different pathways

Number of compounds tested:

- 9 (6 DNT compounds (Methylmercury, Arsenic, Valproic Acid, Methylazoxymethanol (MAM), Chlorpyrifos, Parathion; 3 negative compounds)

Advantages:

- Medium throughput
- Multiple endpoint measures in an SOP-based testing set-up
- Species comparison for parallelogram approach possible: human NPC, rat NPC
- Defined media without FCS
- Human cells commercially available (Lonza)
- Endpoint-specific controls included

Disadvantages

- Primary cells with donor variability

Study conclusion:
The comparative neurosphere assay might be part of a larger DNT testing strategy because not all modes-of-action are assessed in this cellular stage (early fetal). Embryonic-based cells as well as later endpoints like synaptogenesis and neuronal

network formation are needed. Also oligodendrocytes need assessment, which the cell system is also capable of as shown by earlier publications of the group (Schreiber et al. 2010, Dach et al. in Revision).

4.2.3

In the paper from Behl et al. (2015), they showed the results of testing flame retardants in a DNT/DT testing strategy. The DNT assays were: hNPC proliferation, rat primary and human stem cell-based neurite outgrowth assays. For the proliferation assay, hESC-derived NPC (hNP1TM; ArunA Biomedical, Athens, GA) were cultured in 96-well culture plates pre-coated with poly-L-ornithine and laminin at a density of 10,000 cells/well. Two hours after plating, hNP1 neuroprogenitor cells were exposed to chemicals at a concentration range of 0.003 μM to 30 μM. Twenty-four hours later BrdU was added and analysed via HCA. Cell count was taken as a measure for cytotoxicity. For measuring neurite outgrowth primary mixed cortical neural cultures and hESC-derived neurons (hN2™) obtained from ArunA Biomedical were plated at 10,000 cells/well and exposed to each chemical at concentrations of 0.003 μM to 30 μM 2 h post-plating. After 48 h, the cells were fixed and stained for β-III tubulin, and neurite outgrowth was determined using HCI on a Cellomics Arrayscan VTI (Software: NeuronalProliferation Bioapplication, V4). Total cell number was counted and a decrease used as an indication of cytotoxicity.

Readouts:

- DNT: Proliferation: BrdU incorporation via HCA
- DNT: Neurite outgrowth: measurements of b-III-Tubulin neuritis via HCA
- Acute Neurotoxicity: MEA recordings of rat neuronal networks in a multi-well format
- Developmental tox (mEST, C. elegans, Zebrafish)

Number of compounds tested:

11 (2,2'-4,4'-Tetrabromodiphenyl ether, 3,3',5,5'-Tetrabromobisphenol A, Triphenyl phosphate, Phenol, isopropylated, phosphate (3:1), 2-Ethylhexyl diphenyl phosphate, Tricresyl phosphate, Isodecyl diphenyl phosphate tert-Butylphenyl diphenyl phosphate, Tri-O-cresyl phosphate, Tris(1,3-dichloro-2-propyl)phosphate, Tris(2-chloroethyl) phosphate)

Advantages:

- Large battery for DNT, NT, DT with different assays
- Based on HCA, higher throughput
- Species comparison for parallelogram approach
- Defined media without FCS
- Human cells commercially available (Aruna)
- Endpoint-specific controls included

Disadvantages

- Species comparison for parallelogram approach is good, yet rat cells are mixed cortical cultures and human cells are hESC-based pure neurons and thus not comparable due to presence/lack of glia
- DNT not covered comprehensively
- No negative compounds were tested
- Unknown compounds were tested in a not yet 'validated' testing battery, known positive and negative substances are missing

Study conclusion:
With this battery, toxicity of flame retardants is trying to be assessed. Therefore, the developmental and neurotoxicity of TBBPA and phased out BDE-47 are compared and contrasted to replacement organophosphate-based flame retardants using a rapid screening paradigm. The results indicate that some of the replacement OPFRs shows activity comparable to BDE-47 and TBBPA in these assays, thereby warranting further hazard characterization of these emerging FRs.
However, battery needs validation by known positive and negative compounds.

4.2.4
Druwe et al. (2015) published a multiplex assay using the ApoLive-GloTM Assay kit (Promega, Madison, WI) in 96-well plates for identification of apoptosis measuring caspase-3/-7 activity and viability based on protease activity. They compared 4 different cell types with this assay.

Cells	mCNS	ReNcell CX	hNP1	iCells
Characteristics	Mouse derived neuroprogenitor cells isolated from cortices of embryonic day 15–18 C57/BL6 mice	Human cortical neural stem cells derived from a 14-week sample of human cortex and immortalized with a c-myc oncogene	Human embryonic neural stem cells derived from a neuroepithelial cell lineage of WA09 hESC	Differentiated human neurons derived from human induced pluripotent stem cells
Provider	Millipore	Millipore	ArunA Biomedical	Cellular Dynamics International

Readouts:
- ApoLive-GloTM Assay kit (Promega, Madison, WI) in 96-well plates. Multiplex assay assessing for apoptosis measuring caspase-3/-7 activity and viability based on protease activity

Number of compounds tested:

- 12 (5 DNT compounds (methylmercury, Arsenic, Manganese, Chlorpyrifos-oxon, Ketamine), 3 negative compounds amongst them)

Advantages:

- High Throughput
- Easy, multiplexed readout (luciferase)
- Cell type comparison: mouse NPC, human NPC myc-immortalized, human NPC from ESC, human neurons
- Defined media without FCS
- Cells commercially available

Disadvantages

- mCNS: Mouse cells
- ReNcell CX: myc-immortalized
- iCell: pure neurons, no astroglia

Study conclusion:
Cell types responded differently towards apoptosis-inducing agents. Therefore, non-transformed, human model system may be the most appropriate for screening chemical effects on apoptosis in neuroprogenitor cells. Further research comparing the robustness and sensitivity of human neuroprogenitor cells derived from different sources is warranted.

4.2.5

Kashyap et al. 2015 proposes a DNT testing strategy based on human umbilical cord blood stem cells (hUCBSC). The scheme of the testing strategy is shown in Fig. 29 and Fig. 30.

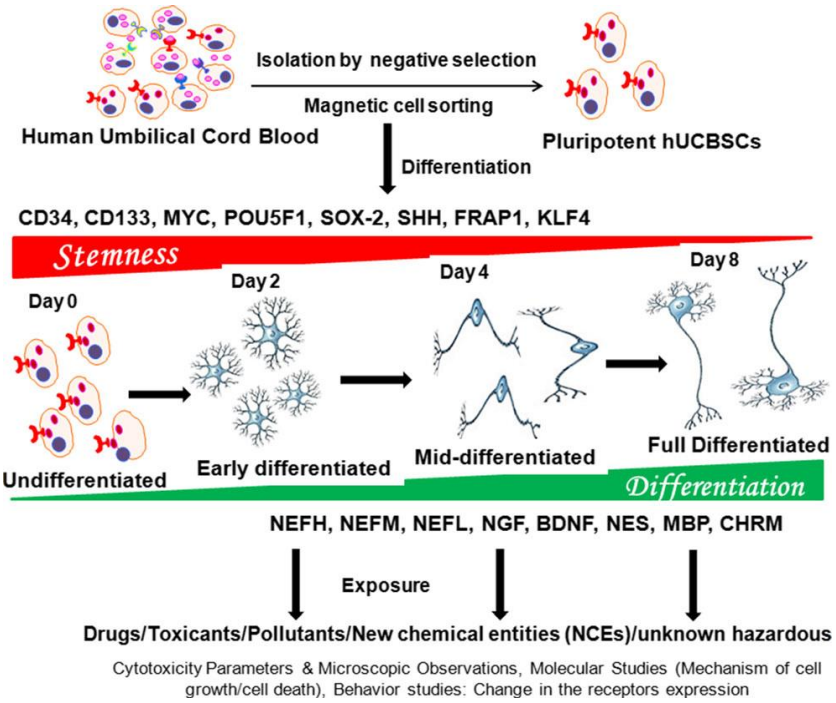


Figure 29: A general approach to show the use of human umbilical cord blood stem cell (hUCBSC)-derived neuronal cell-based in vitro model to study developmental neurotoxicity. The applicability of hUCBSC- derived differentiating neuronal cell-based in vitro model to assess the developmental neurotoxicity of chemicals/drugs/xenobiotics/NCEs is un- paralleled. Umbilical cord blood could be used as an enriched source for the isolation of pluripotent haematopoietic stem cells. During their neu- ronal differentiating, these cells may be exposed to unknown chemicals/ drugs/xenobiotics/NCEs, and the effects of these compounds can be assessed by studying different markers involved in cell proliferation, neu- ronal differentiation, neuronal injuries and receptors at various stages of neuronal maturity such as days 2, 4 and 8. These neuronal cells derived from human umbilical cord stem cells can be used as a powerful tool to assess the developmental neurotoxicity in human beings (Singh & Kashyap, 2015).

hUCBSCs of 95 donors were isolated, characterized, and cultured for the bulk production and differentiated into the neuronal cells as described earlier by the same group:

Kashyap MP, Singh AK, Kumar V, Yadav DK, Khan F, Jahan S, Khanna VK, Yadav S, Pant AB (2013) Pkb/Akt1 mediates Wnt/ GSK3beta/beta-catenin signalling-induced apoptosis in human cord blood stem cells exposed to organophosphate pesticide monocrotophos. Stem Cells Dev 22(2):224–238

Singh AK, Kashyap MP, Jahan S, Kumar V, Tripathi VK, Siddiqui MA, Yadav S, Khanna VK, Das V, Jain SK, Pant AB (2012) Expression and inducibility of cytochrome P450s (CYP1A1, 2B6, 2E1, 3A4) in human cord blood CD34+ stem cell-derived differentiating neuronal cells. Toxicol Sci 129:392–410

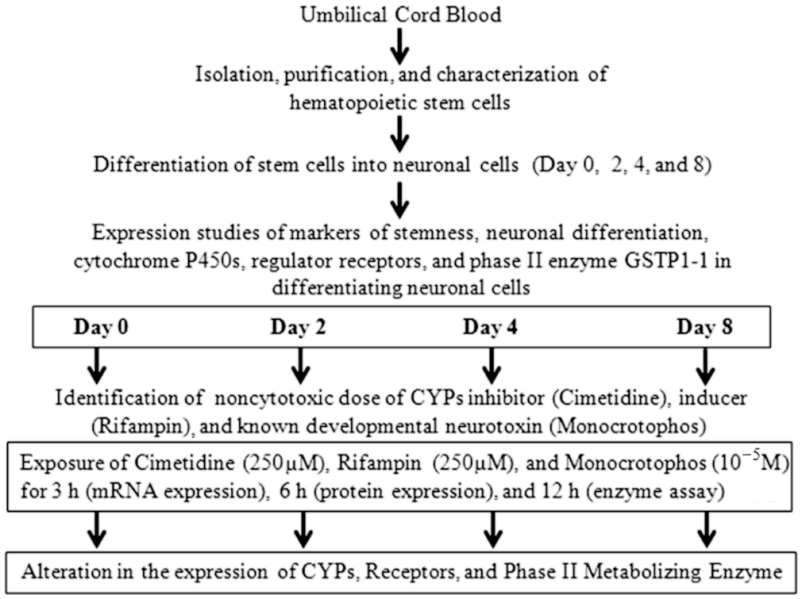


Figure 30: Exposure scheme of hUCBSC for DNT assessment.

Readouts:

- Gene expression (stemness, neuronal, oxidative stress)
- Protein expression (stemness, neuronal, oxidative stress)

- Apoptosis (TUNEL assay detected by flow cytometry)
- Acetylcholinesterase activity
- Acetylcholine
- Neuronal receptors (cholinergic and dopaminergic)
- Choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH) via immunocytochemistry

Number of compounds tested:
In Kashyap et al. (2015) one compound, monocrotophos was tested. No quantified results for protein expression data was presented.

Advantages:
- Human stem cell-based material assessable
- No ethical concerns

Disadvantages
- No quantification of protein readouts
- interpretation of PCR data for single transcripts difficult for DNT assessment
- No assay for proliferation, neuronal differentiation
- No endpoint-specific controls
- Study not scientifically reliable

Issues: how reliable is characterization? Here 'Fig' refers to figures in Kashyap et al. (2015) paper:
Fig. 3 Western blots are not quantified and it seems that housekeeping proteins also change over time.
In Fig. 4 all cells are stained positive for different markers.
Fig. 9 immunofluorescence of choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH) is quantified without normalization e.g. to cell number.

4.2.6
A fairly sophisticated in vitro method for DNT assessment based on genome changes measured by RNA-Sequencing (RNA-Seq) was published by Schwartz et al. (2015) in the Proc. Nat. Acad. Sci. U.S.A.. This paper contains a thorough characterization of H1-ESC-based 3D organoids growing in hydrogels. RNA-Seq data was produced for 60 DNT positive and negative compounds after a 7 day in vitro exposure to a single concentration. Machine learning was used from global gene expression data from these treatments. Algorithms were then utilized to assess a test set of 10 positive and negative compounds. This paper summarized a humongous data set.

Readouts:
- The models containing NPC, microglia and vascular cells were characterized extensively by immunocytochemistry using 18 antibodies for relevant epidtopes and RNA-seq data.

- Global gene expression (RNA-seq) was the readout for chemical exposure

Number of compounds tested:
70 (39 positive, 31 negative)

Advantages:
- Human stem cell-based material which can be expanded and used for generation of medium-throughput highly sophisticated in vitro methods
- Models very well characterized
- Standardized production of organoids containing neural as well as microglia and vasculature cells
- Large amount of RNA-seq data presented

Disadvantages
- ESC-based methods bear ethical concern, a hiPSC-approach would be preferred
- Machine learning algorithm is based on compounds that are not entirely human DNT compounds, e.g. dioxin (2,3,7,8-TCDD does not cause DNT in humans)
- No cytotoxicity assay was performed
- Two of the best studied human DNT compounds, methylmercury and valproic acid, were not assessed in this paper
- A human DNT compound, cadmium was not correctly identified
- It is unexplored to what content the organoids reflect human brain development
- Gene expression was not related to functional/morphological endpoints
- No endpoint-specific controls

The model seems very promising, but purely relying on gene expression data with a machine learning algorithm as a prediction model using compounds that might be misclassified seems difficult.

4.2.7
Robinson et al. (2016) published a highly sophisticate approach for using genomics as readouts for DNT (Fig. 31). Briefly, they compared in a hESC-neural differentiation model transcripts that change during neurodevelopment in vitro with transcripts that change during human embryogenesis in vivo via a meta analysis. They identified 304 enriched transcripts that are regulated in vivo and in vitro during neural development. Changes of these transcripts during 7 days exposure of the hESC-neuronal differentiation model revealed 13 genes that were regulated amongst the 304 transcripts enriched in vivo and in vitro. These 13 genes are thought to be able to serve as DNT-biomarkers.

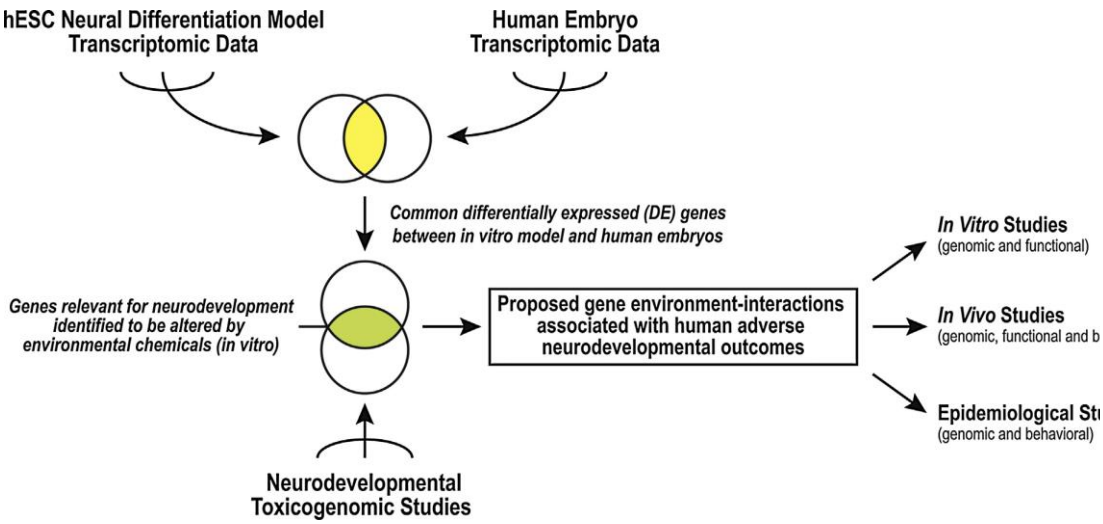


Figure 31: An analysis framework for integrating human transcriptomic datasets to identify biomarkers of neurodevelopmental toxicity.
Meta-analyses of transcriptomic data generated in human embryonic stem cell (hESC) neural differentiation models revealed signatures at the various stages, which were compared to datasets that interrogated gene expression at specific days of human embryonic development, including neurogenesis. Differentially expressed (DE) genes that were shared between the two models were chosen as candidate biomarkers of neural development. They were applied to toxicogenomic studies that employed hESCs to identify potential gene-environment interactions associated with adverse neurodevelopmental outcomes. These results could inform the design of future studies that use in vitro and in vivo models or investigate effects at a population level. (Robinson et al., 2016).

Readouts:

- Gene expression of 13 human in vivo – in vitro validated biomarkers (see explanation of procedure above) with 80-90% concordance across species (rat and zebrafish)

Number of compounds tested:
3 DNT positive

Advantages:

- Human stem cell-based material which can be expanded and used for medium-throughput
- Models well characterized by genomics
- Comparison to human, rat and zebrafish in vivo mRNA arrays was performed for gene expression changes during differentiation

Disadvantages

- ESC-based methods bear ethical concern, a hiPSC-approach would be preferred

- Only 3 compounds with individual modes-of-actions tested, different compounds acting on different targets would likely cause different changes in gene expression patterns
- Changes in gene expression were not related to changes in neurodevelopmental processes, thus, their functional relevance need to be assessed.

The model seems promising, however, sensitivity and specificity of more compounds for indicated biomarkers have to be assessed.

4.2.8

Colaianna et al. (2016) published a luciferase-based neuronal differentiation assay, CGR8 2-Luc differentiation Assay. The assay assesses compound's effects on early neuronal differentiation by studying neuronal versus general promoter activities via luciferase readouts. The schematic layout of the DNT assay is depicted in Fig. 32. The characteristics as well as strength and limitations are listed below.

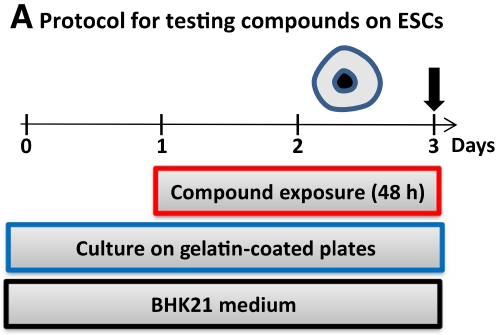


Figure 32: Scheme for DNT-CGR8 2-Luc Assay (Colaianna et al., 2016).

Readouts:

- Dual Luciferase Assay (T□1- & EF1□-Luc steered promoters for neuronal and general transcription, respectively)
- PI Assay for cell number as a measure for cytotoxicity

Number of compounds tested:

- 37 (5 DNT compounds (methylmercury, VPA, PBDE-99, PCB-153, Arsenic), 7 negative compounds amongst them)

Advantages:

- High Throughput
- Easy readout (luciferase)

Disadvantages

- Mouse cells
- Pure neurons, no astroglia

- Cell availability restricted as not commercially available
- Medium contains FCS
- Testing results of 5 human DNT compounds identifies 1/5 as active below concentrations causing cytotoxicity (Table 10)
- Methylmercury not identified as a positive

Table 10: ESC-Luc LOAEL (µM) taken from Colaianna et al. (2015):

	ESC- T□1	ESC- EF1□□	ESC-PI	ESC- T□1/PI
Methylmercury	0.5	0.5	0.5	1
Valproic Acid	5000	5000	5000	1
PBDE-99	50	>50	>50	<1
PCB-153	>50	>50	>50	1
Arsenic	0.5	0.5	0.5	1

Questions: basal reporter activity over time? How far developed are the neurons after 3 days of differentiation?

4.2.9

Hirsch et al. 2016 published a multiparameter toxicity assessment of novel DOPO-derived organophosphorus flame retardants (Hirsch et al., 2016). This study assessed three novel bis-DOPO-derived flame retardants in direct comparison with their parental compound DOPO and polybrominated diphenyl ether PBDE-99 with respect to their acute cytotoxic potential, their interaction with epidermal barriers, and their influence on neuronal systems.

Readouts:

- Acute toxicity lung and immune system (A549 cells and macrophages)
- Epidermal uptake of compounds with a 3D human epidermal in vitro model
- Irritative potential of the compounds on the cellular level was analyzed in a keratinocyte assay
- Nervous system effects were studied with in vitro models of central (LUHMES) and peripheral (hESC-based) neurons (acute or developmental exposure)
- Manipulation of early-stage developmental processes was tested in the human NCC migration assay (MINC)

Number of compounds tested:

- 4 (BDE-99, 9,10-dihydro-9-oxa-10-phospha- phenanthrene-10-oxide (DOPO), 6-(2-((6-oxido-6H- dibenzo[c,e][1,2]oxaphosphinin-6-yl)amino)ethoxy)-6H-dibenzo[c,e][1,2]oxaphosphinine 6-oxide (ETA-DOPO), 6,6'-(ethane-1,2-diylbis(oxy))bis(6H-dibenzo[c,e] [1,2]oxaphosphinine-6-oxide) (EG-DOPO), 6,6′ -(ethane-1,2- diylbis(azanediyl))bis(6H-dibenzo[c,e][1,2]oxaphosphin- ine-6-oxide) (EDA-DOPO))

Advantages:

- broad variety of test systems
- for central (LUHMES neurite outgrowth) and peripheral (PeriTox) as well as NCC (MINC) assays data-rich assays were used that are well described via transcriptome data and many compounds have been tested before (see sections above)
- HCA assays for neurotox-dependent endpoints

Disadvantages

- LUHMES cells are pure, dopaminergic neurons
- No astro- or oligodendroglia in the assays
- unknown compounds tested in a battery with so far not specified application domain
- Positive control BDE-99 effects at fairly high concentrations (in comparison to calculated internal exposure)

The assays used here seem to be promising as part of a larger DNT testing strategy.

4.3 'OMICS' AS READOUTS FOR DNT TESTING

In addition to the cell biological endpoints, there has been a great effort to study changes in transcriptomes in different cell systems mainly from the group of Marcel Leist for LUHMES cells, hESC-derived peripheral neurons and NCC (Scholz et al., 2011, Zimmer et al., 2011a, Zimmer et al., 2011b, Balmer et al., 2012, Krug et al., 2013b, Waldmann et al., 2014, Zimmer et al., 2014, Dreser et al., 2015, Shinde et al., 2015, Hoelting et al., 2016, Pallocca et al., 2016) as well as (Schulpen et al., 2015). These studies linked modes-of-action of compounds to transcriptome changes in the different systems. From the current perspective, making use of transcriptome studies for regulatory purposes is difficult. However, as additional information on the assays, such data can be very useful, e.g. for verification on the molecular level that cell biological endpoints are reflected by certain pathways in the in vitro systems. Therefore, not only compound effects on developing in vitro systems are of interest, but especially molecular changes within the developing in vitro systems over time are of interest as well (Hoelting et al., 2016). Such information is a prerequisite to understand the biological application domains of the assays, which in the end, when the whole testing battery is assembled, should sum up to all possible pathways relevant for brain development at a function of neurodevelopmental processes over time. Such developmental profiles in gene expression can also be compared with existing expression profiles of human fetal brains that were published earlier. Hoelting et al. (2016) did compare different in vitro systems with human brain samples from a data base (http://cellnet.hms. harvard.edu/), however, these were not developing brains and thus makes comparison difficult as timing is crucial in neurodevelopment (Fig. 33). Also Robinson et al. (2016) had a similar approach comparing in vitro to in vivo transcriptomes. This study identified neurodevelopmental biomarkers, however, these have to be validated in corresponding functional assays. Such data is necessary for creating confidence in the novel methods that are promising for creating a DNT testing strategy. As in general strategies for using omics technologies in risk assessment processes evolve, these will also reach out to the field of DNT.

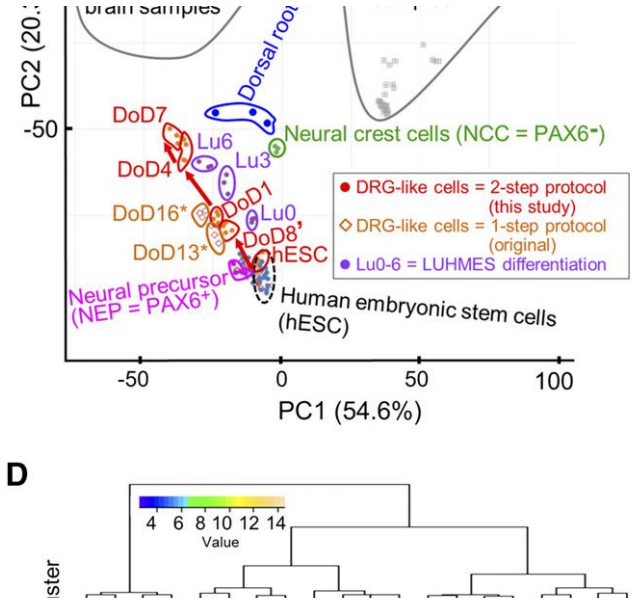


Figure 33: Differentiation tracking by transcriptome analysis of iDRG cells. Samples were obtained at different developmental stages for whole transcriptome analysis; data are displayed as a principal component analysis (PCA) map, together with legacy data from cell cultures or from human dorsal root ganglion, brain, and liver tissue. The red arrow indicates the cell differentiation track of the two-step iDRG cell differ- entiation protocol. Note: DoD7 of the two-step protocol is 16 days older than hESCs (i.e., roughly corresponding to DoD16* of the one-step protocol in differentiation time) (Hoelting et al., 2016).

5. A PROPOSAL FOR ALTERNATIVE DNT TESTING IN VITRO

Based on the recommendation published within the EFSA opinion earlier (Fritsche et al., 2015) and depicted in Fig. 34, a more detailed evaluation of human cell-based methods filling this proposed grid is proposed. According to Tables 10 and 11, and as already proposed in (Fritsche et al., 2015) certain neurodevelopmental endpoint groups are now filled with more specific endpoints and methods and the evaluations of their readiness levels (see Tables within chapter 4). Methods with the highest readiness levels are chosen for the proposed testing strategy. In case multiple assays with similar readiness levels are available, these are indicated. For some endpoints there is no data from human-based models available. However, if e.g. this endpoint belongs to a neuronal endpoint group, like dendrite formation, and there are models, which produce neurons from hES/PC methods, then it is assumed that also dendrite formation could potentially be measured. Over all, there is a high degree of readiness for methods measuring neurodevelopmental endpoints in vitro. Besides some endpoints, which are fairly data-rich (like NCC migration), there is a need for compound testing, especially over a whole proposed testing strategy. As disturbance of any endpoint could cause an adverse outcome, it is necessary to test any compound across all endpoints **(Tier 1)**. This will reveal the 'most sensitive endpoint' (MSE) across all assays as was already proposed

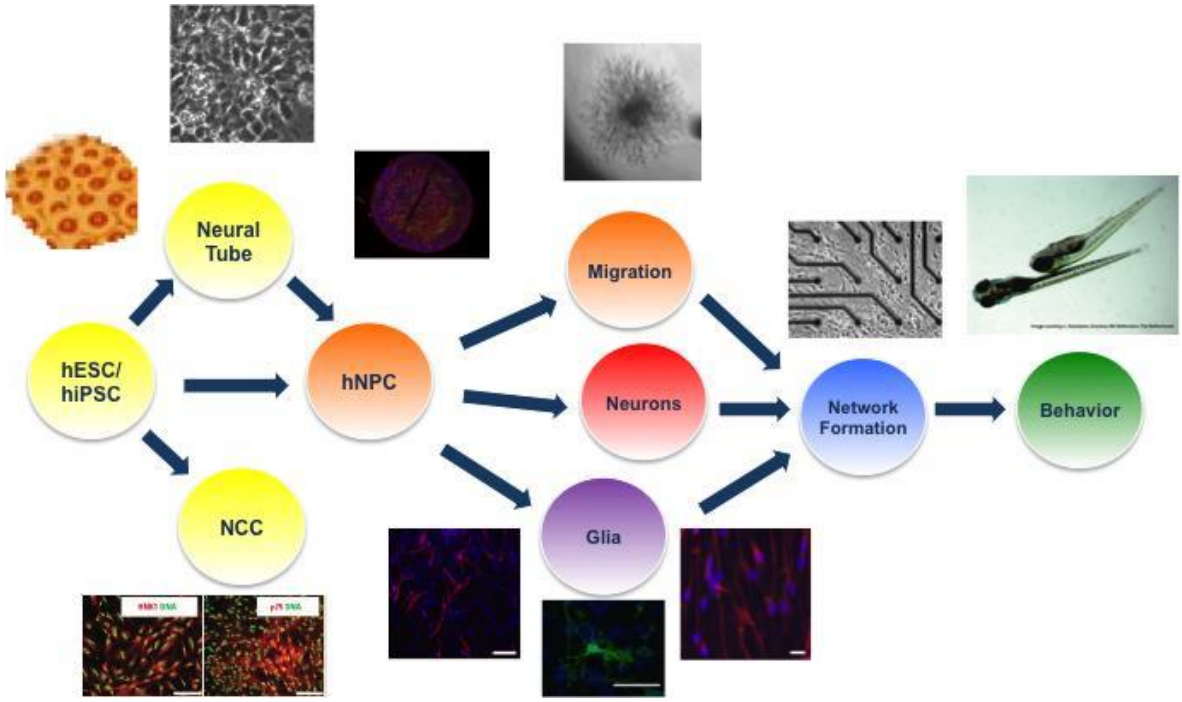


Figure 34: Rough outline of a proposed in vitro testing strategy for DNT in vitro testing (Fritsche et al., 2015).

earlier and performed for a smaller set of endpoints (Baumann et al., 2015). This MSE will point towards the most probable mode-of-action for neurodevelopmental disturbance. E.g. if a compound affects oligodendrocyte formation/maturation with the lowest observed adverse effect concentration across all endpoints, further testing can be streamlined towards these effects e.g. in the zebrafish **(Tier 2)** or even if necessary in a targeted approach in the rat in vivo. Before going towards the rat in vivo however, it is recommended to study in a translational approach if the effect is even likely to be seen in rats in vivo by using complementary rat in vitro methods **(Tier 3)**. Such species-specific compound effects in complementary in vitro methods were described earlier (Baumann et al., 2015, Gassmann et al., 2010, Harrill et al., 2011a). In case the effects are human-specific and thus cannot be detected in the rodent, there is no need for a detailed rodent in vivo study. If, even at different potencies, effects are seen in rat cultures, then an in vivo rat study might be necessary to confirm whole organism relevance of in vitro results **(Tier 4)**. This should only be done for the time being when confidence in in vitro testing is limited. Gaining more information of such in vitro-in vivo approaches with the parallelogram approach will strengthen confidence in the in vitro testing strategy and might possibly pave the road for risk assessment based solely on human in vitro data. For any risk assessment approaches, exposure data is crucial. As the risk assessment process in the EU is hazard-based, one would like to minimize over-sensitivity of in vitro assays. Therefore, as a **Tier 0**, it would be advised to receive kinetic data on compounds e.g. through pharmacokinetic modelling. These data can be used i) to determine test concentration ranges and ii) to receive information on possible metabolites that might be tested instead of the parent compound. Concerning the test concentration ranges, one possibility would be to start testing at 2 orders of magnitude higher than the expected in

vivo concentration (safety margin in vivo). This approach would help minimizing false-positive data due to overloading the in vitro systems with far exceeding realistic exposure scenarios. Information on the metabolites will help eliminating false-negatives due to lack of maternal, placental and fetal metabolism in the in vitro systems. This approach is summarized in Fig. 35.

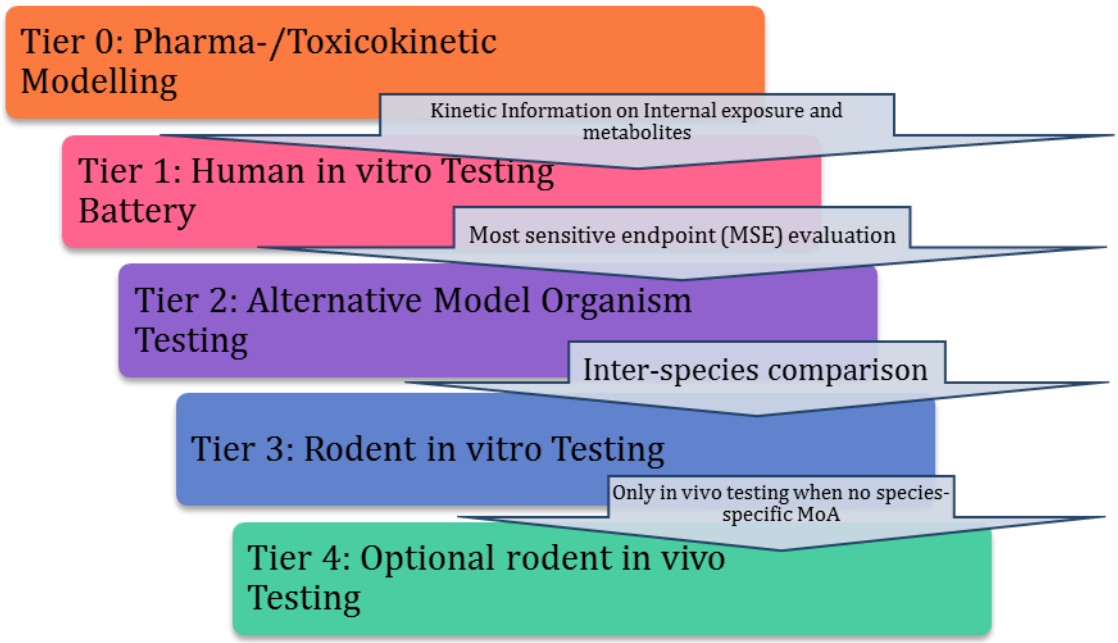


Figure 35: Proposed tiered testing scheme being the basis for an IATA for DNT evaluation. For detailed information on the strategy see text.

5.1 A PROPOSED IN VITRO TESTING STRATEGY BASED ON ASSAY READINESS

For Tier 1 of the DNT tiered testing scheme a variety of tests is necessary to cover most basic neurodevelopmental endpoints. Table 13 gives the ground for discussion of such tests:

Table 13: A selection of Assays covering neurodevelopmental endpoints as a ground for discussion to assemble an in vitro DNT testing strategy. Assay readiness was one selection criteria, which based e.g. on cell availability and confidence in the method evaluated under chapter 4.

Test Name	Test System	Endpoint measured	Develop-mental stage	Multiple end-points	Literature
NEP differentiation	hESC differentiation to NEP-containing neural rosettes	NEP differentiation resembling neural tube formation	Embryonic	possible	(Colleoni et al., 2011, Colleoni et al., 2012, Shinde et al., 2015, Stummann et al., 2009,

					Waldmann et al., 2014)
NPC proliferation (Neurosphere Assay)	hNPC	NPC proliferation necessary for brain growth	Fetal	yes	(Baumann et al., 2015, Baumann, 2014, Fritsche et al., 2011, Gassmann et al., 2010, Gassmann et al., 2012, Moors et al., 2009, Schreiber et al., 2010, Tofighi et al., 2011)
NPC proliferation	ReNcell CX	NPC proliferation necessary for brain growth	Fetal (myc immortalized)	yes	(Breier et al., 2008, Radio et al., 2015)
NPC proliferation	(hESC)	NPC proliferation necessary for brain growth	embryonic	yes	(Bai et al., 2013, Nash et al., 2012, Talens-Visconti et al., 2011)
NPC apoptosis (Neurosphere Assay)	hNPC	NPC Apoptosis	Fetal	yes	(Fritsche et al., 2011, Moors et al., 2009)
NPC apoptosis	ReNcell CX	NPC Apoptosis	Fetal (myc immortalized)	yes	(Culbreth et al., 2012)
NPC/Radial glia migration (Neurosphere Assay)	hNPC	Radial Migration	Fetal	yes	(Moors et al., 2007) (Gassmann et al., 2010) (Barenys et al., 2016) (Baumann et al., 2015, Baumann, 2014)
NCC migration (MINC Assay)	hESC-derived NCC	NCC migration	Embryonal	Yes	(Dreser et al., 2015, Hirsch et al., 2016,

					Pallocca et al., 2016, Zimmer et al., 2012, Zimmer et al., 2014)
Astrocyte differentiation	hESC-NPC astrocyte differentiation	Astrocyte differentiation	Embryonal	Yes	(Talens-Visconti et al., 2011)
Astrocyte Differentiation (Neurosphere Assay)	hNPC-based astrocyte differentiation	Astrocyte differentiation	Fetal	Yes	(Baumann et al., 2015, Baumann, 2014, Moors et al., 2012, Moors et al., 2010)
Oligodendrocyte differentiation (Neurosphere Assay)	hNPC-based oligodendrocyte differentiation	Oligodendrocyte differentiation	Fetal	Yes	(Baumann, 2014, Schreiber et al., 2010) Dach et al. in Revision
Oligodendrocyte differentiation	hESC-based methods without compound testing	Oligodendrocyte differentiation	Embryonal		Rev. in (Madill et al., 2016)
Neuronal differentiation	hESC-based neuronal differentiation	Neuron differentiation	Embryonal	Yes	(Bai et al., 2013, He et al., 2012, Nash et al., 2012, Palmer et al., 2012, Stummann et al., 2009, Talens-Visconti et al., 2011)
Neuronal differentiation (Neuroshpere Assay)	hNPC-based neuronal differentiation	Young, β-III-Tubulin+ neuron differentiation		Yes	(Barenys et al., 2016, Baumann et al., 2015, Baumann, 2014, Moors et al., 2012, Moors et al., 2010, Schreiber et al., 2010)

Neuronal differentiation (LUHMES Assay)	LUHMES cell-based neuronal differentiation	Dopaminergic Neuron differentiation	Fetal	Yes	(Krug et al., 2013a, Scholz et al., 2011, Stiegler et al., 2011)
Neurite outgrowth	hESC-based neurite assay	Neurite outgrowth	Embryonal	Yes	(Harrill et al., 2010, Harrill et al., 2011a, He et al., 2012)
Neuronal differentiation (LUHMES Assay)	LUHMES cell-based neuronal differentiation	Dopaminergic Neuron differentiation	Fetal	Yes	(Krug et al., 2013a, Scholz et al., 2011, Stiegler et al., 2011)
Neurite outgrowth (Neurosphere Assay)	hNPC-based neurite assay	Neurite outgrowth	Fetal	Yes	Schmuck et al. in press
Neuronal Network formation	hESC-based neuronal networks	Electrical Activity	Embryonal	Yes	(Kapucu et al., 2012, Kiiski et al., 2013)
Peripheral neurotoxicity	hiPSC-derived peripheral neurons	Neurogenesis	Embryonal	Yes	(Hoelting et al., 2016)

While some endpoints, like NCC migration are only covered by one assay, other endpoints, like NPC proliferation, have been covered by multiple assays. Some assays are heavily published, while others have less citations. Thus, the basis for testing is different amongst the different assays. However, the table makes obvious that for every endpoint category concerning the different endpoints across the different cell types of the developing brain – neurons and glia cells – there are assays ready to go for chemical testing. Many assays already use HCA for higher throughput testing. Endpoints where the assays are not set up yet, but in principle possible, like e.g. dendritic spine formation, rodent cells could help out until human assays are ready. These are summarized in Fritsche et al. (2015). However, human-based systems are preferred.

Now in a next step, it needs to be decided on the assays most useful and applicable for regulatory purposes and this battery needs to be challenged with a large set of test compounds across all assays. Only by actual compound testing the predictivity of such a method can be assessed. Implementation of such a test battery into an IATA based on proposed Tier 0 – Tier 4 testing will help improving the current DNT testing paradigm (OECD426).

6. REFERENCES:

ADIGUN, A. A., SEIDLER, F. J. & SLOTKIN, T. A. 2010. Disparate developmental neurotoxicants converge on the cyclic AMP signaling cascade, revealed by transcriptional profiles in vitro and in vivo. *Brain Res,* 1316**,** 1-16.

ALMEIDA, L. E., ROBY, C. D. & KRUEGER, B. K. 2014. Increased BDNF expression in fetal brain in the valproic acid model of autism. *Mol Cell Neurosci,* 59**,** 57-62.

BAI, X., YAN, Y., CANFIELD, S., MURAVYEVA, M. Y., KIKUCHI, C., ZAJA, I., CORBETT, J. A. & BOSNJAK, Z. J. 2013. Ketamine enhances human neural stem cell proliferation and induces neuronal apoptosis via reactive oxygen species-mediated mitochondrial pathway. *Anesth Analg,* 116**,** 869-80.

BAL-PRICE, A., CROFTON, K. M., LEIST, M., ALLEN, S., ARAND, M., BUETLER, T., DELRUE, N., FITZGERALD, R. E., HARTUNG, T., HEINONEN, T., HOGBERG, H., BENNEKOU, S. H., LICHTENSTEIGER, W., OGGIER, D., PAPARELLA, M., AXELSTAD, M., PIERSMA, A., RACHED, E., SCHILTER, B., SCHMUCK, G., STOPPINI, L., TONGIORGI, E., TIRAMANI, M., MONNET-TSCHUDI, F., WILKS, M. F., YLIKOMI, T. & FRITSCHE, E. 2015a. International STakeholder NETwork (ISTNET): creating a developmental neurotoxicity (DNT) testing road map for regulatory purposes. *Arch Toxicol,* 89**,** 269-87.

BAL-PRICE, A., CROFTON, K. M., SACHANA, M., SHAFER, T. J., BEHL, M., FORSBY, A., HARGREAVES, A., LANDESMANN, B., LEIN, P. J., LOUISSE, J., MONNET-TSCHUDI, F., PAINI, A., ROLAKI, A., SCHRATTENHOLZ, A., SUNOL, C., VAN THRIEL, C., WHELAN, M. & FRITSCHE, E. 2015b. Putative adverse outcome pathways relevant to neurotoxicity. *Crit Rev Toxicol,* 45**,** 83-91.

BAL-PRICE, A., LEIN, P. J., KEIL, K. P., SETHI, S., SHAFER, T., BARENYS, M., FRITSCHE, E., SACHANA, M. & MEEK, M. E. 2016. Developing and applying the adverse outcome pathway concept for understanding and predicting neurotoxicity. *Neurotoxicology*.

BALMER, N. V., WENG, M. K., ZIMMER, B., IVANOVA, V. N., CHAMBERS, S. M., NIKOLAEVA, E., JAGTAP, S., SACHINIDIS, A., HESCHELER, J., WALDMANN, T. & LEIST, M. 2012. Epigenetic changes and disturbed neural development in a human embryonic stem cell-based model relating to the fetal valproate syndrome. *Hum Mol Genet,* 21**,** 4104-14.

BARENYS, M., GASSMANN, K., BAKSMEIER, C., HEINZ, S., REVERTE, I., SCHMUCK, M., TEMME, T., BENDT, F., ZSCHAUER, T. C., ROCKEL, T. D., UNFRIED, K., WATJEN, W., SUNDARAM, S. M., HEUER, H., COLOMINA, M. T. & FRITSCHE, E. 2016. Epigallocatechin gallate (EGCG) inhibits adhesion and migration of neural progenitor cells in vitro. *Arch Toxicol*.

BARTEL, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell,* 116**,** 281-97.

BAUMANN, J., GASSMANN, K., MASJOSTHUSMANN, S., DEBOER, D., BENDT, F., GIERSIEFER, S. & FRITSCHE, E. 2015. Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events. *Arch Toxicol*.

BAUMANN, J. B., M.; GASSMANN, K.; FRITSCHE, E. 2014. Comparative Human and Rat "Neurosphere Assay" for Developmental Neurotoxicity Testing. *In:* REED, L. G. C. J. C. D. D. A. L. D. J. (ed.) *Current Protocols in Toxicology.* John Wiley & Sons.

BEHL, M., HSIEH, J. H., SHAFER, T. J., MUNDY, W. R., RICE, J. R., BOYD, W. A., FREEDMAN, J. H., HUNTER, E. S., 3RD, JAREMA, K. A., PADILLA, S. & TICE, R. R. 2015. Use of

alternative assays to identify and prioritize organophosphorus flame retardants for potential developmental and neurotoxicity. *Neurotoxicol Teratol,* 52**,** 181-93.
BELVINDRAH, R., GRAUS-PORTA, D., GOEBBELS, S., NAVE, K. A. & MULLER, U. 2007. Beta1 integrins in radial glia but not in migrating neurons are essential for the formation of cell layers in the cerebral cortex. *J Neurosci,* 27**,** 13854-65.
BENGOA-VERGNIORY, N. & KYPTA, R. M. 2015. Canonical and noncanonical Wnt signaling in neural stem/progenitor cells. *Cell Mol Life Sci,* 72**,** 4157-72.
BERNAL, J., GUADANO-FERRAZ, A. & MORTE, B. 2015. Thyroid hormone transporters-functions and clinical implications. *Nat Rev Endocrinol,* 11**,** 690.
BORRELL, V. & GOTZ, M. 2014. Role of radial glial cells in cerebral cortex folding. *Curr Opin Neurobiol,* 27**,** 39-46.
BREIER, J. M., RADIO, N. M., MUNDY, W. R. & SHAFER, T. J. 2008. Development of a high-throughput screening assay for chemical effects on proliferation and viability of immortalized human neural progenitor cells. *Toxicol Sci,* 105**,** 119-33.
BUZANSKA, L., HABICH, A., JURGA, M., SYPECKA, J. & DOMANSKA-JANIK, K. 2005. Human cord blood-derived neural stem cell line--possible implementation in studying neurotoxicity. *Toxicol In Vitro,* 19**,** 991-9.
BUZANSKA, L., SYPECKA, J., NERINI-MOLTENI, S., COMPAGNONI, A., HOGBERG, H. T., DEL TORCHIO, R., DOMANSKA-JANIK, K., ZIMMER, J. & COECKE, S. 2009. A human stem cell-based model for identifying adverse effects of organic and inorganic chemicals on the developing nervous system. *Stem Cells,* 27**,** 2591-601.
CHAO, C. C. & HU, S. X. 1994. TUMOR-NECROSIS-FACTOR-ALPHA POTENTIATES GLUTAMATE NEUROTOXICITY IN HUMAN FETAL BRAIN-CELL CULTURES. *Developmental Neuroscience,* 16**,** 172-179.
CHATTOPADHYAY, S., BHAUMIK, S., PURKAYASTHA, M., BASU, S., NAG CHAUDHURI, A. & DAS GUPTA, S. 2002. Apoptosis and necrosis in developing brain cells due to arsenic toxicity and protection with antioxidants. *Toxicol Lett,* 136**,** 65-76.
CHEN, H., ZHANG, Y., YANG, Z. & ZHANG, H. 2013. Human umbilical cord Wharton's jelly-derived oligodendrocyte precursor-like cells for axon and myelin sheath regeneration. *Neural Regen Res,* 8**,** 890-9.
CHEN, P., CULBRETH, M. & ASCHNER, M. 2016. Exposure, epidemiology, and mechanism of the environmental toxicant manganese. *Environ Sci Pollut Res Int,* 23**,** 13802-10.
CHOI, B. H. 1986. Methylmercury poisoning of the developing nervous system: I. Pattern of neuronal migration in the cerebral cortex. *Neurotoxicology,* 7**,** 591-600.
CHOI, B. H., LAPHAM, L. W., AMIN-ZAKI, L. & SALEEM, T. 1978. Abnormal neuronal migration, deranged cerebral cortical organization, and diffuse white matter astrocytosis of human fetal brain: a major effect of methylmercury poisoning in utero. *J Neuropathol Exp Neurol,* 37**,** 719-33.
COLAIANNA, M., ILMJARV, S., PETERSON, H., KERN, I., JULIEN, S., BAQUIE, M., PALLOCCA, G., BOSGRA, S., SACHINIDIS, A., HENGSTLER, J. G., LEIST, M. & KRAUSE, K. H. 2016. Fingerprinting of neurotoxic compounds using a mouse embryonic stem cell dual luminescence reporter assay. *Arch Toxicol*.
COLLEONI, S., GALLI, C., GASPAR, J. A., MEGANATHAN, K., JAGTAP, S., HESCHELER, J., SACHINIDIS, A. & LAZZARI, G. 2011. Development of a neural teratogenicity test based on human embryonic stem cells: response to retinoic acid exposure. *Toxicol Sci,* 124**,** 370-7.
COLLEONI, S., GALLI, C., GASPAR, J. A., MEGANATHAN, K., JAGTAP, S., HESCHELER, J., SACHINIDIS, A. & LAZZARI, G. 2012. Characterisation of a neural teratogenicity

assay based on human ESCs differentiation following exposure to valproic acid. *Curr Med Chem,* 19**,** 6065-71.
COSTA, L. G. 1994. Signal transduction mechanisms in developmental neurotoxicity: the phosphoinositide pathway. *Neurotoxicology,* 15**,** 19-27.
CRANEFIELD, P. F. 1962. The discovery of cretinism. *Bull Hist Med,* 36**,** 489-511.
CROFTON, K. M. 2004. Developmental disruption of thyroid hormone: correlations with hearing dysfunction in rats. *Risk Anal,* 24**,** 1665-71.
CROFTON, K. M., KODAVANTI, P. R., DERR-YELLIN, E. C., CASEY, A. C. & KEHN, L. S. 2000. PCBs, thyroid hormones, and ototoxicity in rats: cross-fostering experiments demonstrate the impact of postnatal lactation exposure. *Toxicol Sci,* 57**,** 131-40.
CROFTON, K. M. & RICE, D. C. 1999. Low-frequency hearing loss following perinatal exposure to 3,3',4,4',5-pentachlorobiphenyl (PCB 126) in rats. *Neurotoxicol Teratol,* 21**,** 299-301.
CROFTON, K. M. & ZOELLER, R. T. 2005. Mode of action: neurotoxicity induced by thyroid hormone disruption during development--hearing loss resulting from exposure to PHAHs. *Crit Rev Toxicol,* 35**,** 757-69.
CRONICAN, A. A., FITZ, N. F., CARTER, A., SALEEM, M., SHIVA, S., BARCHOWSKY, A., KOLDAMOVA, R., SCHUG, J. & LEFTEROV, I. 2013. Genome-wide alteration of histone H3K9 acetylation pattern in mouse offspring prenatally exposed to arsenic. *PLoS One,* 8**,** e53478.
CULBRETH, M. E., HARRILL, J. A., FREUDENRICH, T. M., MUNDY, W. R. & SHAFER, T. J. 2012. Comparison of chemical-induced changes in proliferation and apoptosis in human and mouse neuroprogenitor cells. *Neurotoxicology,* 33**,** 1499-510.
DACH, K., BENDT, F., HUEBENTHAL, U., GIERSIEFER, S., HEUER, H., FRITSCHE, E. BDE-99 impairs human and mouse oligodendrogenesis by species-specific modes of action. under Revision
DAS, K. P. & BARONE, S. 1999. Neuronal differentiation in PC12 cells is inhibited by chlorpyrifos and its metabolites: Is acetylcholinesterase inhibition the site of action? *Toxicology and Applied Pharmacology,* 160**,** 217-230.
DE ESCOBAR, G. M., OBREGON, M. J. & DEL REY, F. E. 2004. Maternal thyroid hormones early in pregnancy and fetal brain development. *Best Pract Res Clin Endocrinol Metab,* 18**,** 225-48.
DOTTI, C. G., ESTEBAN, J. A. & LEDESMA, M. D. 2014. Lipid dynamics at dendritic spines. *Front Neuroanat,* 8**,** 76.
DOUVARAS, P. & FOSSATI, V. 2015. Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells. *Nat Protoc,* 10**,** 1143-54.
DRESER, N., ZIMMER, B., DIETZ, C., SUGIS, E., PALLOCCA, G., NYFFELER, J., MEISIG, J., BLUTHGEN, N., BERTHOLD, M. R., WALDMANN, T. & LEIST, M. 2015. Grouping of histone deacetylase inhibitors and other toxicants disturbing neural crest migration by transcriptional profiling. *Neurotoxicology,* 50**,** 56-70.
DRUWE, I., FREUDENRICH, T. M., WALLACE, K., SHAFER, T. J. & MUNDY, W. R. 2015. Sensitivity of neuroprogenitor cells to chemical-induced apoptosis using a multiplexed assay suitable for high-throughput screening. *Toxicology,* 333**,** 14-24.
DUKHANDE, V. V., MALTHANKAR-PHATAK, G. H., HUGUS, J. J., DANIELS, C. K. & LAI, J. C. 2006. Manganese-induced neurotoxicity is differentially enhanced by glutathione depletion in astrocytoma and neuroblastoma cells. *Neurochem Res,* 31**,** 1349-57.
DUPIN, E. & SOMMER, L. 2012. Neural crest progenitors and stem cells: from early development to adulthood. *Dev Biol,* 366**,** 83-95.
EHRLICH, D. E. & JOSSELYN, S. A. 2016. Plasticity-related genes in brain development and amygdala-dependent learning. *Genes Brain Behav,* 15**,** 125-43.

ESTEVAN, C., FUSTER, E., DEL RIO, E., PAMIES, D., VILANOVA, E. & SOGORB, M. A. 2014. Organophosphorus pesticide chlorpyrifos and its metabolites alter the expression of biomarker genes of differentiation in D3 mouse embryonic stem cells in a comparable way to other model neurodevelopmental toxicants. *Chem Res Toxicol,* 27**,** 1487-95.

FERNANDES, E. C., HENDRIKS, H. S., VAN KLEEF, R. G., RENIERS, A., ANDERSSON, P. L., VAN DEN BERG, M. & WESTERINK, R. H. 2010a. Activation and potentiation of human GABAA receptors by non-dioxin-like PCBs depends on chlorination pattern. *Toxicol Sci,* 118**,** 183-90.

FERNANDES, E. C., HENDRIKS, H. S., VAN KLEEF, R. G., VAN DEN BERG, M. & WESTERINK, R. H. 2010b. Potentiation of the human GABA(A) receptor as a novel mode of action of lower-chlorinated non-dioxin-like PCBs. *Environ Sci Technol,* 44**,** 2864-9.

FISH, J. L., DEHAY, C., KENNEDY, H. & HUTTNER, W. B. 2008. Making bigger brains-the evolution of neural-progenitor-cell division. *J Cell Sci,* 121**,** 2783-93.

FLASKOS, J. 2012. The developmental neurotoxicity of organophosphorus insecticides: a direct role for the oxon metabolites. *Toxicol Lett,* 209**,** 86-93.

FLORA, S. J., BHATT, K. & MEHTA, A. 2009. Arsenic moiety in gallium arsenide is responsible for neuronal apoptosis and behavioral alterations in rats. *Toxicol Appl Pharmacol,* 240**,** 236-44.

FOTI, S. B., CHOU, A., MOLL, A. D. & ROSKAMS, A. J. 2013. HDAC inhibitors dysregulate neural stem cell activity in the postnatal mouse brain. *Int J Dev Neurosci,* 31**,** 434-47.

FRITSCHE, E., ALM, H., BAUMANN, J., GEERTS, L., HÅKANSSON, H., MASJOSTHUSMANN, S., WITTERS, H. 2015. Literature review on in vitro and alternative Developmental Neurotoxicity (DNT) testing methods. *EFSA supporting publication,* 2015:EN- 778**,** 186 pp.

FRITSCHE, E., GASSMANN, K. & SCHREIBER, T. 2011. Neurospheres as a model for developmental neurotoxicity testing. *Methods Mol Biol,* 758**,** 99-114.

FUKUCHI, M., NII, T., ISHIMARU, N., MINAMINO, A., HARA, D., TAKASAKI, I., TABUCHI, A. & TSUDA, M. 2009. Valproic acid induces up- or down-regulation of gene expression responsible for the neuronal excitation and inhibition in rat cortical neurons through its epigenetic actions. *Neurosci Res,* 65**,** 35-43.

GASSMANN, K., ABEL, J., BOTHE, H., HAARMANN-STEMMANN, T., MERK, H. F., QUASTHOFF, K. N., ROCKEL, T. D., SCHREIBER, T. & FRITSCHE, E. 2010. Species-specific differential AhR expression protects human neural progenitor cells against developmental neurotoxicity of PAHs. *Environ Health Perspect,* 118**,** 1571-7.

GASSMANN, K., BAUMANN, J., GIERSIEFER, S., SCHUWALD, J., SCHREIBER, T., MERK, H. F. & FRITSCHE, E. 2012. Automated neurosphere sorting and plating by the COPAS large particle sorter is a suitable method for high-throughput 3D in vitro applications. *Toxicol In Vitro,* 26**,** 993-1000.

GIBB, S. 2008. Toxicity testing in the 21st century: a vision and a strategy. *Reprod Toxicol,* 25**,** 136-8.

GILBERT, M. E., SANCHEZ-HUERTA, K. & WOOD, C. 2016. Mild Thyroid Hormone Insufficiency During Development Compromises Activity-Dependent Neuroplasticity in the Hippocampus of Adult Male Rats. *Endocrinology,* 157**,** 774-87.

GILBERT, M. E., SUI, L., WALKER, M. J., ANDERSON, W., THOMAS, S., SMOLLER, S. N., SCHON, J. P., PHANI, S. & GOODMAN, J. H. 2007. Thyroid hormone insufficiency

during brain development reduces parvalbumin immunoreactivity and inhibitory function in the hippocampus. *Endocrinology,* 148**,** 92-102.
GO, H. S., KIM, K. C., CHOI, C. S., JEON, S. J., KWON, K. J., HAN, S.-H., LEE, J., CHEONG, J. H., RYU, J. H., KIM, C.-H., KO, K. H. & SHIN, C. Y. 2012. Prenatal exposure to valproic acid increases the neural progenitor cell pool and induces macrocephaly in rat brain via a mechanism involving the GSK-3 beta/beta-catenin pathway. *Neuropharmacology,* 63**,** 1028-1041.
GOLDEY, E. S. & CROFTON, K. M. 1998. Thyroxine replacement attenuates hypothyroxinemia, hearing loss, and motor deficits following developmental exposure to Aroclor 1254 in rats. *Toxicol Sci,* 45**,** 94-105.
GOLDEY, E. S., KEHN, L. S., LAU, C., REHNBERG, G. L. & CROFTON, K. M. 1995a. Developmental exposure to polychlorinated biphenyls (Aroclor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. *Toxicol Appl Pharmacol,* 135**,** 77-88.
GOLDEY, E. S., KEHN, L. S., REHNBERG, G. L. & CROFTON, K. M. 1995b. Effects of developmental hypothyroidism on auditory and motor function in the rat. *Toxicol Appl Pharmacol,* 135**,** 67-76.
GRANDJEAN, P. & LANDRIGAN, P. J. 2006. Developmental neurotoxicity of industrial chemicals. *Lancet,* 368**,** 2167-78.
GRANDJEAN, P. & LANDRIGAN, P. J. 2014. Neurobehavioural effects of developmental toxicity. *Lancet Neurol,* 13**,** 330-8.
GUIZZETTI, M., MOLLER, T. & COSTA, L. G. 2003. Ethanol inhibits muscarinic receptor-mediated DNA synthesis and signal transduction in human fetal astrocytes. *Neurosci Lett,* 344**,** 68-70.
GUIZZETTI, M., PATHAK, S., GIORDANO, G. & COSTA, L. G. 2005. Effect of organophosphorus insecticides and their metabolites on astroglial cell proliferation. *Toxicology,* 215**,** 182-90.
GULISANO, M., PACINI, S., PUNZI, T., MORUCCI, G., QUAGLIATA, S., DELFINO, G., SARCHIELLI, E., MARINI, M. & VANNELLI, G. B. 2009. Cadmium modulates proliferation and differentiation of human neuroblasts. *J Neurosci Res,* 87**,** 228-37.
GYAMFI, C., WAPNER, R. J. & D'ALTON, M. E. 2009. Thyroid dysfunction in pregnancy: the basic science and clinical evidence surrounding the controversy in management. *Obstet Gynecol,* 113**,** 702-7.
HADDOW, J. E., PALOMAKI, G. E., ALLAN, W. C., WILLIAMS, J. R., KNIGHT, G. J., GAGNON, J., O'HEIR, C. E., MITCHELL, M. L., HERMOS, R. J., WAISBREN, S. E., FAIX, J. D. & KLEIN, R. Z. 1999. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *N Engl J Med,* 341**,** 549-55.
HAO, H. N., PARKER, G. C., ZHAO, J., BARAMI, K. & LYMAN, W. D. 2003. Human neural stem cells are more sensitive than astrocytes to ethanol exposure. *Alcohol Clin Exp Res,* 27**,** 1310-7.
HARRILL, J. A., FREUDENRICH, T. M., MACHACEK, D. W., STICE, S. L. & MUNDY, W. R. 2010. Quantitative assessment of neurite outgrowth in human embryonic stem cell-derived hN2 cells using automated high-content image analysis. *Neurotoxicology,* 31**,** 277-90.
HARRILL, J. A., FREUDENRICH, T. M., ROBINETTE, B. L. & MUNDY, W. R. 2011a. Comparative sensitivity of human and rat neural cultures to chemical-induced inhibition of neurite outgrowth. *Toxicol Appl Pharmacol,* 256**,** 268-80.
HARRILL, J. A., ROBINETTE, B. L. & MUNDY, W. R. 2011b. Use of high content image analysis to detect chemical-induced changes in synaptogenesis in vitro. *Toxicol In Vitro,* 25**,** 368-87.

HE, X., IMANISHI, S., SONE, H., NAGANO, R., QIN, X. Y., YOSHINAGA, J., AKANUMA, H., YAMANE, J., FUJIBUCHI, W. & OHSAKO, S. 2012. Effects of methylmercury exposure on neuronal differentiation of mouse and human embryonic stem cells. *Toxicol Lett,* 212**,** 1-10.
HERR, D. W., FREEBORN, D. L., DEGN, L., MARTIN, S. A., ORTENZIO, J., PANTLIN, L., HAMM, C. W. & BOYES, W. K. 2016. Neurophysiological assessment of auditory, peripheral nerve, somatosensory, and visual system function after developmental exposure to gasoline, E15, and E85 vapors. *Neurotoxicol Teratol,* 54**,** 78-88.
HERR, D. W., GRAFF, J. E., DERR-YELLIN, E. C., CROFTON, K. M. & KODAVANTI, P. R. 2001. Flash-, somatosensory-, and peripheral nerve-evoked potentials in rats perinatally exposed to Aroclor 1254. *Neurotoxicol Teratol,* 23**,** 591-601.
HESTER, S. D., JOHNSTONE, A. F., BOYES, W. K., BUSHNELL, P. J. & SHAFER, T. J. 2011. Acute toluene exposure alters expression of genes in the central nervous system associated with synaptic structure and function. *Neurotoxicol Teratol,* 33**,** 521-9.
HEVNER, R. F. 2015. Brain overgrowth in disorders of RTK-PI3K-AKT signaling: a mosaic of malformations. *Semin Perinatol,* 39**,** 36-43.
HIRSCH, C., STRIEGL, B., MATHES, S., ADLHART, C., EDELMANN, M., BONO, E., GAAN, S., SALMEIA, K. A., HOELTING, L., KREBS, A., NYFFELER, J., PAPE, R., BURKLE, A., LEIST, M., WICK, P. & SCHILDKNECHT, S. 2016. Multiparameter toxicity assessment of novel DOPO-derived organophosphorus flame retardants. *Arch Toxicol*.
HOELTING, L., KLIMA, S., KARREMAN, C., GRINBERG, M., MEISIG, J., HENRY, M., ROTSHTEYN, T., RAHNENFUHRER, J., BLUTHGEN, N., SACHINIDIS, A., WALDMANN, T. & LEIST, M. 2016. Stem Cell-Derived Immature Human Dorsal Root Ganglia Neurons to Identify Peripheral Neurotoxicants. *Stem Cells Transl Med,* 5**,** 476-87.
HOELTING, L., SCHEINHARDT, B., BONDARENKO, O., SCHILDKNECHT, S., KAPITZA, M., TANAVDE, V., TAN, B., LEE, Q. Y., MECKING, S., LEIST, M. & KADEREIT, S. 2013. A 3-dimensional human embryonic stem cell (hESC)-derived model to detect developmental neurotoxicity of nanoparticles. *Arch Toxicol,* 87**,** 721-33.
HOGBERG, H. T., BRESSLER, J., CHRISTIAN, K. M., HARRIS, G., MAKRI, G., O'DRISCOLL, C., PAMIES, D., SMIRNOVA, L., WEN, Z. & HARTUNG, T. 2013. Toward a 3D model of human brain development for studying gene/environment interactions. *Stem Cell Res Ther,* 4 Suppl 1**,** S4.
HOGBERG, H. T., SOBANSKI, T., NOVELLINO, A., WHELAN, M., WEISS, D. G. & BAL-PRICE, A. K. 2011. Application of micro-electrode arrays (MEAs) as an emerging technology for developmental neurotoxicity: evaluation of domoic acid-induced effects in primary cultures of rat cortical neurons. *Neurotoxicology,* 32**,** 158-68.
HOWARD, A. S., BUCELLI, R., JETT, D. A., BRUUN, D., YANG, D. & LEIN, P. J. 2005. Chlorpyrifos exerts opposing effects on axonal and dendritic growth in primary neuronal cultures. *Toxicol Appl Pharmacol,* 207**,** 112-24.
HU, B. Y., DU, Z. W. & ZHANG, S. C. 2009. Differentiation of human oligodendrocytes from pluripotent stem cells. *Nat Protoc,* 4**,** 1614-22.
IMAYOSHI, I., SHIMOJO, H., SAKAMOTO, M., OHTSUKA, T. & KAGEYAMA, R. 2013. Genetic visualization of notch signaling in mammalian neurogenesis. *Cell Mol Life Sci,* 70**,** 2045-57.
JOHNSTON, M. V., ISHIDA, A., ISHIDA, W. N., MATSUSHITA, H. B., NISHIMURA, A. & TSUJI, M. 2009. Plasticity and injury in the developing brain. *Brain Dev,* 31**,** 1-10.
KAJTA, M., LITWA, E., RZEMIENIEC, J., WNUK, A., LASON, W., ZELEK-MOLIK, A., NALEPA, I., GRZEGORZEWSKA-HICZWA, M., TOKARSKI, K., GOLAS, A., GUZIK, E.,

GROCHOWALSKI, A., SZYCHOWSKI, K. A. & WOJTOWICZ, A. K. 2014. Isomer-nonspecific action of dichlorodiphenyltrichloroethane on aryl hydrocarbon receptor and G-protein-coupled receptor 30 intracellular signaling in apoptotic neuronal cells. *Mol Cell Endocrinol,* 392**,** 90-105.

KANG, D. S., YANG, Y. R., LEE, C., KIM, S., RYU, S. H. & SUH, P. G. 2016. Roles of phosphoinositide-specific phospholipase Cgamma1 in brain development. *Adv Biol Regul,* 60**,** 167-73.

KANG, H. S., JUNG, E. M. & JEUNG, E. B. 2013. 205 assessment of developmental neurotoxicity on neural differentiation in human embryonic stem cells. *Reproduction, fertility, and development,* 26**,** 216-7.

KAPUCU, F. E., TANSKANEN, J. M., MIKKONEN, J. E., YLA-OUTINEN, L., NARKILAHTI, S. & HYTTINEN, J. A. 2012. Burst analysis tool for developing neuronal networks exhibiting highly varying action potential dynamics. *Front Comput Neurosci,* 6**,** 38.

KASHYAP, M. P., KUMAR, V., SINGH, A. K., TRIPATHI, V. K., JAHAN, S., PANDEY, A., SRIVASTAVA, R. K., KHANNA, V. K. & PANT, A. B. 2015. Differentiating neurons derived from human umbilical cord blood stem cells work as a test system for developmental neurotoxicity. *Mol Neurobiol,* 51**,** 791-807.

KETTENMANN, H. & VERKHRATSKY, A. 2011. [Neuroglia--living nerve glue]. *Fortschr Neurol Psychiatr,* 79**,** 588-97.

KIISKI, H., AANISMAA, R., TENHUNEN, J., HAGMAN, S., YLA-OUTINEN, L., AHO, A., YLI-HANKALA, A., BENDEL, S., SKOTTMAN, H. & NARKILAHTI, S. 2013. Healthy human CSF promotes glial differentiation of hESC-derived neural cells while retaining spontaneous activity in existing neuronal networks. *Biol Open,* 2**,** 605-12.

KIRYUSHKO, D., KORSHUNOVA, I., BEREZIN, V. & BOCK, E. 2006. Neural cell adhesion molecule induces intracellular signaling via multiple mechanisms of Ca2+ homeostasis. *Mol Biol Cell,* 17**,** 2278-86.

KLEIN, J. 2005. Functions and pathophysiological roles of phospholipase D in the brain. *J Neurochem,* 94**,** 1473-87.

KOLOZSI, E., MACKENZIE, R. N., ROULLET, F. I., DECATANZARO, D. & FOSTER, J. A. 2009. Prenatal exposure to valproic acid leads to reduced expression of synaptic adhesion molecule neuroligin 3 in mice. *Neuroscience,* 163**,** 1201-10.

KRUG, A. K., BALMER, N. V., MATT, F., SCHONENBERGER, F., MERHOF, D. & LEIST, M. 2013a. Evaluation of a human neurite growth assay as specific screen for developmental neurotoxicants. *Arch Toxicol,* 87**,** 2215-31.

KRUG, A. K., KOLDE, R., GASPAR, J. A., REMPEL, E., BALMER, N. V., MEGANATHAN, K., VOJNITS, K., BAQUIE, M., WALDMANN, T., ENSENAT-WASER, R., JAGTAP, S., EVANS, R. M., JULIEN, S., PETERSON, H., ZAGOURA, D., KADEREIT, S., GERHARD, D., SOTIRIADOU, I., HEKE, M., NATARAJAN, K., HENRY, M., WINKLER, J., MARCHAN, R., STOPPINI, L., BOSGRA, S., WESTERHOUT, J., VERWEI, M., VILO, J., KORTENKAMP, A., HESCHELER, J., HOTHORN, L., BREMER, S., VAN THRIEL, C., KRAUSE, K. H., HENGSTLER, J. G., RAHNENFUHRER, J., LEIST, M. & SACHINIDIS, A. 2013b. Human embryonic stem cell-derived test systems for developmental neurotoxicity: a transcriptomics approach. *Arch Toxicol,* 87**,** 123-43.

LAFOURCADE, C. A., LIN, T. V., FELICIANO, D. M., ZHANG, L., HSIEH, L. S. & BORDEY, A. 2013. Rheb activation in subventricular zone progenitors leads to heterotopia, ectopic neuronal differentiation, and rapamycin-sensitive olfactory micronodules and dendrite hypertrophy of newborn neurons. *J Neurosci,* 33**,** 2419-31.

LAFRANCHI, S. H. & AUSTIN, J. 2007. How should we be treating children with congenital hypothyroidism? *J Pediatr Endocrinol Metab,* 20**,** 559-78.

LASKY, R. E., WIDHOLM, J. J., CROFTON, K. M. & SCHANTZ, S. L. 2002. Perinatal exposure to Aroclor 1254 impairs distortion product otoacoustic emissions (DPOAEs) in rats. *Toxicol Sci,* 68**,** 458-64.
LASSITER, T. L., MACKILLOP, E. A., RYDE, I. T., SEIDLER, F. J. & SLOTKIN, T. A. 2009. Is fipronil safer than chlorpyrifos? Comparative developmental neurotoxicity modeled in PC12 cells. *Brain Res Bull,* 78**,** 313-22.
LE DOUARIN, N. M., CALLONI, G. W. & DUPIN, E. 2008. The stem cells of the neural crest. *Cell Cycle,* 7**,** 1013-9.
LEE, D. Y. 2015. Roles of mTOR Signaling in Brain Development. *Exp Neurobiol,* 24**,** 177-85.
LEE, J. E., LIM, M. S., PARK, J. H., PARK, C. H. & KOH, H. C. 2014a. Nuclear NF-kappaB contributes to chlorpyrifos-induced apoptosis through p53 signaling in human neural precursor cells. *Neurotoxicology*.
LEE, J. E., LIM, M. S., PARK, J. H., PARK, C. H. & KOH, H. C. 2014b. Nuclear NF-kappaB contributes to chlorpyrifos-induced apoptosis through p53 signaling in human neural precursor cells. *Neurotoxicology,* 42**,** 58-70.
LEITE, C., SILVA, N. T., MENDES, S., RIBEIRO, A., DE FARIA, J. P., LOURENCO, T., DOS SANTOS, F., ANDRADE, P. Z., CARDOSO, C. M., VIEIRA, M., PAIVA, A., DA SILVA, C. L., CABRAL, J. M., RELVAS, J. B. & GRAOS, M. 2014. Differentiation of human umbilical cord matrix mesenchymal stem cells into neural-like progenitor cells and maturation into an oligodendroglial-like lineage. *PLoS One,* 9**,** e111059.
LIGHT, K. E., GE, Y. & BELCHER, S. M. 2001. Early postnatal ethanol exposure selectively decreases BDNF and truncated TrkB-T2 receptor mRNA expression in the rat cerebellum. *Brain Res Mol Brain Res,* 93**,** 46-55.
LIU, J., MORROW, A. L., DEVAUD, L. L., GRAYSON, D. R. & LAUDER, J. M. 1997. Regulation of GABA(A) receptor subunit mRNA expression by the pesticide dieldrin in embryonic brainstem cultures: a quantitative, competitive reverse transcription-polymerase chain reaction study. *J Neurosci Res,* 49**,** 645-53.
LU, Y., YANG, Y., WANG, Z., WANG, C., DU, Q., WANG, Q. & LUAN, Z. 2015. Isolation and culture of human oligodendrocyte precursor cells from neurospheres. *Brain Res Bull,* 118**,** 17-24.
MADILL, M., FITZGERALD, D., O'CONNELL, K. E., DEV, K. K., SHEN, S. & FITZGERALD, U. 2016. In vitro and ex vivo models of multiple sclerosis. *Drug Discov Today*.
MANDAL, C., PARK, J. H., CHOI, M. R., KIM, S. H., BADEJO, A. C., CHAI, J. C., LEE, Y. S., JUNG, K. H. & CHAI, Y. G. 2015. Transcriptomic study of mouse embryonic neural stem cell differentiation under ethanol treatment. *Mol Biol Rep,* 42**,** 1233-9.
MARTINEZ, L., JIMENEZ, V., GARCIA-SEPULVEDA, C., CEBALLOS, F., DELGADO, J. M., NINO-MORENO, P., DONIZ, L., SAAVEDRA-ALANIS, V., CASTILLO, C. G., SANTOYO, M. E., GONZALEZ-AMARO, R. & JIMENEZ-CAPDEVILLE, M. E. 2011. Impact of early developmental arsenic exposure on promotor CpG-island methylation of genes involved in neuronal plasticity. *Neurochem Int,* 58**,** 574-81.
MAURYA, S. K., MISHRA, J., TRIPATHI, V. K., SHARMA, R. & SIDDIQUI, M. H. 2014. Cypermethrin induces astrocyte damage: role of aberrant Ca(2+), ROS, JNK, P38, matrix metalloproteinase 2 and migration related reelin protein. *Pestic Biochem Physiol,* 111**,** 51-9.
MCCREA, H. J. & DE CAMILLI, P. 2009. Mutations in phosphoinositide metabolizing enzymes and human disease. *Physiology (Bethesda),* 24**,** 8-16.
MENSE, S. M., SENGUPTA, A., LAN, C., ZHOU, M., BENTSMAN, G., VOLSKY, D. J., WHYATT, R. M., PERERA, F. P. & ZHANG, L. 2006. The common insecticides cyfluthrin and

chlorpyrifos alter the expression of a subset of genes with diverse functions in primary human astrocytes. *Toxicol Sci,* 93**,** 125-35.
MIDDLEMORE-RISHER, M.-L., ADAM, B.-L., LAMBERT, N. A. & TERRY, A. V., JR. 2011. Effects of Chlorpyrifos and Chlorpyrifos-Oxon on the Dynamics and Movement of Mitochondria in Rat Cortical Neurons. *Journal of Pharmacology and Experimental Therapeutics,* 339**,** 341-349.
MOORE, D. B., MADORSKY, I., PAIVA, M. & BARROW HEATON, M. 2004. Ethanol exposure alters neurotrophin receptor expression in the rat central nervous system: Effects of prenatal exposure. *J Neurobiol,* 60**,** 101-13.
MOORS, M., BOSE, R., JOHANSSON-HAQUE, K., EDOFF, K., OKRET, S. & CECCATELLI, S. 2012. Dickkopf 1 mediates glucocorticoid-induced changes in human neural progenitor cell proliferation and differentiation. *Toxicol Sci,* 125**,** 488-95.
MOORS, M., CLINE, J. E., ABEL, J. & FRITSCHE, E. 2007. ERK-dependent and -independent pathways trigger human neural progenitor cell migration. *Toxicol Appl Pharmacol,* 221**,** 57-67.
MOORS, M., ROCKEL, T. D., ABEL, J., CLINE, J. E., GASSMANN, K., SCHREIBER, T., SCHUWALD, J., WEINMANN, N. & FRITSCHE, E. 2009. Human neurospheres as three-dimensional cellular systems for developmental neurotoxicity testing. *Environ Health Perspect,* 117**,** 1131-8.
MOORS, M., VUDATTU, N. K., ABEL, J., KRAMER, U., RANE, L., ULFIG, N., CECCATELLI, S., SEYFERT-MARGOLIES, V., FRITSCHE, E. & MAEURER, M. J. 2010. Interleukin-7 (IL-7) and IL-7 splice variants affect differentiation of human neural progenitor cells. *Genes Immun,* 11**,** 11-20.
NASH, R., KRISHNAMOORTHY, M., JENKINS, A. & CSETE, M. 2012. Human embryonic stem cell model of ethanol-mediated early developmental toxicity. *Exp Neurol,* 234**,** 127-35.
NAWAZ, S., SANCHEZ, P., SCHMITT, S., SNAIDERO, N., MITKOVSKI, M., VELTE, C., BRUCKNER, B. R., ALEXOPOULOS, I., CZOPKA, T., JUNG, S. Y., RHEE, J. S., JANSHOFF, A., WITKE, W., SCHAAP, I. A., LYONS, D. A. & SIMONS, M. 2015. Actin filament turnover drives leading edge growth during myelin sheath formation in the central nervous system. *Dev Cell,* 34**,** 139-51.
PALLOCCA, G., GRINBERG, M., HENRY, M., FRICKEY, T., HENGSTLER, J. G., WALDMANN, T., SACHINIDIS, A., RAHNENFUHRER, J. & LEIST, M. 2016. Identification of transcriptome signatures and biomarkers specific for potential developmental toxicants inhibiting human neural crest cell migration. *Arch Toxicol,* 90**,** 159-80.
PALMER, J. A., POENITZSCH, A. M., SMITH, S. M., CONARD, K. R., WEST, P. R. & CEZAR, G. G. 2012. Metabolic Biomarkers of Prenatal Alcohol Exposure in Human Embryonic Stem Cell-Derived Neural Lineages. *Alcoholism-Clinical and Experimental Research,* 36**,** 1314-1324.
PATEL, J., LANDERS, K., LI, H., MORTIMER, R. H. & RICHARD, K. 2011. Thyroid hormones and fetal neurological development. *J Endocrinol,* 209**,** 1-8.
PAVLOV, O. V., BOBRYSHEV, Y. V., BALABANOV, Y. V. & ASHWELL, K. 1995. AN IN-VITRO STUDY OF THE EFFECTS OF LOVASTATIN ON HUMAN FETAL BRAIN-CELLS. *Neurotoxicology and Teratology,* 17**,** 31-39.
PEI, Y., PENG, J., BEHL, M., SIPES, N. S., SHOCKLEY, K. R., RAO, M. S., TICE, R. R. & ZENG, X. 2015. Comparative neurotoxicity screening in human iPSC-derived neural stem cells, neurons and astrocytes. *Brain Res*.
PETRI, R., MALMEVIK, J., FASCHING, L., AKERBLOM, M. & JAKOBSSON, J. 2014. miRNAs in brain development. *Exp Cell Res,* 321**,** 84-9.

RADIO, N. M., BREIER, J. M., REIF, D. M., JUDSON, R. S., MARTIN, M., HOUCK, K. A., MUNDY, W. R. & SHAFER, T. 2015. Use of Neural Models of Proliferation and Neurite Outgrowth to Screen Environmental Chemicals in the ToxCast Phase I Library. *Appl in vitro Toxicol,* 1**,** 131-139.

RAHMAN, A., BREW, B. J. & GUILLEMIN, G. J. 2011. Lead Dysregulates Serine/Threonine Protein Phosphatases in Human Neurons. *Neurochemical Research,* 36**,** 195-204.

RAI, A., MAURYA, S. K., KHARE, P., SRIVASTAVA, A. & BANDYOPADHYAY, S. 2010. Characterization of developmental neurotoxicity of As, Cd, and Pb mixture: synergistic action of metal mixture in glial and neuronal functions. *Toxicol Sci,* 118**,** 586-601.

REZVANI, A. H. 2006. Involvement of the NMDA System in Learning and Memory. *In:* LEVIN, E. D. & BUCCAFUSCO, J. J. (eds.) *Animal Models of Cognitive Impairment.* Boca Raton (FL).

RIOS, R., ZARAZUA, S., SANTOYO, M. E., SEPULVEDA-SAAVEDRA, J., ROMERO-DIAZ, V., JIMENEZ, V., PEREZ-SEVERIANO, F., VIDAL-CANTU, G., DELGADO, J. M. & JIMENEZ-CAPDEVILLE, M. E. 2009. Decreased nitric oxide markers and morphological changes in the brain of arsenic-exposed rats. *Toxicology,* 261**,** 68-75.

ROBINETTE, B. L., HARRILL, J. A., MUNDY, W. R. & SHAFER, T. J. 2011. In vitro assessment of developmental neurotoxicity: use of microelectrode arrays to measure functional changes in neuronal network ontogeny. *Front Neuroeng,* 4**,** 1.

ROBINSON, J. F., GORMLEY, M. J. & FISHER, S. J. 2016. A genomics-based framework for identifying biomarkers of human neurodevelopmental toxicity. *Reprod Toxicol,* 60**,** 1-10.

SACHANA, M., SIDIROPOULOU, E., FLASKOS, J., HARRIS, W., ROBINSON, A. J., WOLDEHIWET, Z. & HARGREAVES, A. J. 2014a. Diazoxon disrupts the expression and distribution of betaIII-tubulin and MAP 1B in differentiating N2a cells. *Basic Clin Pharmacol Toxicol,* 114**,** 490-6.

SCHALOCK, R. L., BROWN, W. J. & SMITH, R. L. 1977. Neonatal hypothyroidism: behavioral, thyroid hormonal and neuroanatomical effects. *Physiol Behav,* 19**,** 489-91.

SCHMUCK, M.R., TEMME, T., DACH, K., DE BOER, D., BARENYS, M., BENDT, F., MOSIG, A., FRITSCHE, E. Omnisphero: A high content image analysis (HCA) approach for phenotypic developmental neurotoxicity (DNT) screenings of organoid neurosphere cultures *in vitro*. *Arch Toxicol*, in press.

SCHOLZ, D., POELTL, D., GENEWSKY, A., WENG, M., WALDMANN, T., SCHILDKNECHT, S. & LEIST, M. 2011. Rapid, complete and large-scale generation of post-mitotic neurons from the human LUHMES cell line. *Journal of Neurochemistry,* 119**,** 957-971.

SCHREIBER, T., GASSMANN, K., GOTZ, C., HUBENTHAL, U., MOORS, M., KRAUSE, G., MERK, H. F., NGUYEN, N. H., SCANLAN, T. S., ABEL, J., ROSE, C. R. & FRITSCHE, E. 2010. Polybrominated diphenyl ethers induce developmental neurotoxicity in a human in vitro model: evidence for endocrine disruption. *Environ Health Perspect,* 118**,** 572-8.

SCHULPEN, S. H., DE JONG, E., DE LA FONTEYNE, L. J., DE KLERK, A. & PIERSMA, A. H. 2015. Distinct gene expression responses of two anticonvulsant drugs in a novel human embryonic stem cell based neural differentiation assay protocol. *Toxicol In Vitro,* 29**,** 449-57.

SCHWARTZ, M. P., HOU, Z., PROPSON, N. E., ZHANG, J., ENGSTROM, C. J., SANTOS COSTA, V., JIANG, P., NGUYEN, B. K., BOLIN, J. M., DALY, W., WANG, Y., STEWART, R.,

PAGE, C. D., MURPHY, W. L. & THOMSON, J. A. 2015. Human pluripotent stem cell-derived neural constructs for predicting neural toxicity. *Proc Natl Acad Sci U S A,* 112**,** 12516-21.
SHINDE, V., KLIMA, S., SURESHKUMAR, P. S., MEGANATHAN, K., JAGTAP, S., REMPEL, E., RAHNENFUHRER, J., HENGSTLER, J. G., WALDMANN, T., HESCHELER, J., LEIST, M. & SACHINIDIS, A. 2015. Human Pluripotent Stem Cell Based Developmental Toxicity Assays for Chemical Safety Screening and Systems Biology Data Generation. *J Vis Exp***,** e52333.
SIDIROPOULOU, E., SACHANA, M., FLASKOS, J., HARRIS, W., HARGREAVES, A. J. & WOLDEHIWET, Z. 2009. Diazinon oxon affects the differentiation of mouse N2a neuroblastoma cells. *Archives of Toxicology,* 83**,** 373-380.
SIDIROPOULOU, E., SACHANA, M., FLASKOS, J., HARRIS, W., HARGREAVES, A. J. & WOLDEHIWET, Z. 2011. Fipronil interferes with the differentiation of mouse N2a neuroblastoma cells. *Toxicol Lett,* 201**,** 86-91.
SINGH, A. K. & KASHYAP, M. P. 2015. An Overview on Human Umbilical Cord Blood Stem Cell-Based Alternative In Vitro Models for Developmental Neurotoxicity Assessment. *Mol Neurobiol*.
SINGH, A. K., KASHYAP, M. P., JAHAN, S., KUMAR, V., TRIPATHI, V. K., SIDDIQUI, M. A., YADAV, S., KHANNA, V. K., DAS, V., JAIN, S. K. & PANT, A. B. 2012. Expression and inducibility of cytochrome P450s (CYP1A1, 2B6, 2E1, 3A4) in human cord blood CD34(+) stem cell-derived differentiating neuronal cells. *Toxicol Sci,* 129**,** 392-410.
SINGH, A. K., KASHYAP, M. P., KUMAR, V., TRIPATHI, V. K., YADAV, D. K., KHAN, F., JAHAN, S., KHANNA, V. K., YADAV, S. & PANT, A. B. 2013. 3-Methylcholanthrene Induces Neurotoxicity in Developing Neurons Derived from Human CD34+Thy1+Stem Cells by Activation of Aryl Hydrocarbon Receptor. *Neuromolecular Medicine,* 15**,** 570-592.
SLOTKIN, T. A., MACKILLOP, E. A., RYDE, I. T. & SEIDLER, F. J. 2007. Ameliorating the developmental neurotoxicity of chlorpyrifos: a mechanisms-based approach in PC12 cells. *Environ Health Perspect,* 115**,** 1306-13.
STACPOOLE, S. R., SPITZER, S., BILICAN, B., COMPSTON, A., KARADOTTIR, R., CHANDRAN, S. & FRANKLIN, R. J. 2013. High yields of oligodendrocyte lineage cells from human embryonic stem cells at physiological oxygen tensions for evaluation of translational biology. *Stem Cell Reports,* 1**,** 437-50.
STANSFIELD, K. H., PILSNER, J. R., LU, Q., WRIGHT, R. O. & GUILARTE, T. R. 2012. Dysregulation of BDNF-TrkB signaling in developing hippocampal neurons by Pb(2+): implications for an environmental basis of neurodevelopmental disorders. *Toxicol Sci,* 127**,** 277-95.
STIEGLER, N. V., KRUG, A. K., MATT, F. & LEIST, M. 2011. Assessment of Chemical-Induced Impairment of Human Neurite Outgrowth by Multiparametric Live Cell Imaging in High-Density Cultures. *Toxicological Sciences,* 121**,** 73-87.
STUMMANN, T. C., HARENG, L. & BREMER, S. 2009. Hazard assessment of methylmercury toxicity to neuronal induction in embryogenesis using human embryonic stem cells. *Toxicology,* 257**,** 117-26.
SUN, H., SHEN, O. X., XU, X. L., SONG, L. & WANG, X. R. 2008. Carbaryl, 1-naphthol and 2-naphthol inhibit the beta-1 thyroid hormone receptor-mediated transcription in vitro. *Toxicology,* 249**,** 238-42.
TAKAHASHI, K., KAMIYA, K., URASE, K., SUGA, M., TAKIZAWA, T., MORI, H., YOSHIKAWA, Y., ICHIMURA, K., KUIDA, K. & MOMOI, T. 2001. Caspase-3-deficiency induces

hyperplasia of supporting cells and degeneration of sensory cells resulting in the hearing loss. *Brain Res,* 894**,** 359-67.
TALENS-VISCONTI, R., SANCHEZ-VERA, I., KOSTIC, J., AMPARO PEREZ-ARAGO, M., ERCEG, S., STOJKOVIC, M. & GUERRI, C. 2011. Neural Differentiation from Human Embryonic Stem Cells as a Tool to Study Early Brain Development and the Neuroteratogenic Effects of Ethanol. *Stem Cells and Development,* 20**,** 327-339.
TAMM, C., SABRI, F. & CECCATELLI, S. 2008. Mitochondrial-mediated apoptosis in neural stem cells exposed to manganese. *Toxicol Sci,* 101**,** 310-20.
TOFIGHI, R., MOORS, M., BOSE, R., IBRAHIM, W. N. & CECCATELLI, S. 2011. Neural stem cells for developmental neurotoxicity studies. *Methods Mol Biol,* 758**,** 67-80.
TONG, W. & SUN, G. Y. 1996. Effects of ethanol on phosphorylation of lipids in rat synaptic plasma membranes. *Alcohol Clin Exp Res,* 20**,** 1335-9.
TOSCANO, C. D. & GUILARTE, T. R. 2005. Lead neurotoxicity: from exposure to molecular effects. *Brain Res Brain Res Rev,* 49**,** 529-54.
TRAN, V., HOFFMAN, N., MOFUNANAYA, A., PRYOR, S. C., OJUGBELE, O., MCLAUGHLIN, A., GIBSON, L., BONVENTRE, J. A., FLYNN, K. & WEEKS, B. S. 2006. Bifenthrin inhibits neurite outgrowth in differentiating PC12 cells. *Med Sci Monit,* 12**,** Br57-62.
TYLER, W. J., ALONSO, M., BRAMHAM, C. R. & POZZO-MILLER, L. D. 2002. From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. *Learn Mem,* 9**,** 224-37.
VASSALLO, A., CHIAPPALONE, M., DE CAMARGOS LOPES, R., SCELFO, B., NOVELLINO, A., DEFRANCHI, E., PALOSAARI, T., WEISSCHU, T., RAMIREZ, T., MARTINOIA, S., JOHNSTONE, A. F., MACK, C. M., LANDSIEDEL, R., WHELAN, M., BAL-PRICE, A. & SHAFER, T. J. 2016. A multi-laboratory evaluation of microelectrode array-based measurements of neural network activity for acute neurotoxicity testing. *Neurotoxicology*.
VERMA, S. K., RAHEJA, G. & GILL, K. D. 2009. Role of muscarinic signal transduction and CREB phosphorylation in dichlorvos-induced memory deficits in rats: an acetylcholine independent mechanism. *Toxicology,* 256**,** 175-82.
VON STACKELBERG, K., GUZY, E., CHU, T. & CLAUS HENN, B. 2015. Exposure to Mixtures of Metals and Neurodevelopmental Outcomes: A Multidisciplinary Review Using an Adverse Outcome Pathway Framework. *Risk Anal,* 35**,** 971-1016.
WALDMANN, T., REMPEL, E., BALMER, N. V., KONIG, A., KOLDE, R., GASPAR, J. A., HENRY, M., HESCHELER, J., SACHINIDIS, A., RAHNENFUHRER, J., HENGSTLER, J. G. & LEIST, M. 2014. Design principles of concentration-dependent transcriptome deviations in drug-exposed differentiating stem cells. *Chem Res Toxicol,* 27**,** 408-20.
WANG, S., BATES, J., LI, X., SCHANZ, S., CHANDLER-MILITELLO, D., LEVINE, C., MAHERALI, N., STUDER, L., HOCHEDLINGER, K., WINDREM, M. & GOLDMAN, S. A. 2013. Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. *Cell Stem Cell,* 12**,** 252-64.
WESTERHOLZ, S., DE LIMA, A. D. & VOIGT, T. 2010. Regulation of early spontaneous network activity and GABAergic neurons development by thyroid hormone. *Neuroscience,* 168**,** 573-89.
WILLIAMS, G. R. 2008. Neurodevelopmental and neurophysiological actions of thyroid hormone. *J Neuroendocrinol,* 20**,** 784-94.
WIN-SHWE, T. T., YOSHIDA, Y., KUNUGITA, N., TSUKAHARA, S. & FUJIMAKI, H. 2010. Does early life toluene exposure alter the expression of NMDA receptor subunits

and signal transduction pathway in infant mouse hippocampus? *Neurotoxicology,* 31**,** 647-53.
WNUK, A., RZEMIENIEC, J., LITWA, E., LASON, W., KRZEPTOWSKI, W., WOJTOWICZ, A. K. & KAJTA, M. 2016. The Crucial Involvement of Retinoid X Receptors in DDE Neurotoxicity. *Neurotox Res,* 29**,** 155-72.
WONG, C. T., AHMAD, E., LI, H. & CRAWFORD, D. A. 2014. Prostaglandin E2 alters Wnt-dependent migration and proliferation in neuroectodermal stem cells: implications for autism spectrum disorders. *Cell Commun Signal,* 12**,** 19.
YANG, D., HOWARD, A., BRUUN, D., AJUA-ALEMANJ, M., PICKART, C. & LEIN, P. J. 2008. Chlorpyrifos and chlorpyrifos-oxon inhibit axonal growth by interfering with the morphogenic activity of acetylcholinesterase. *Toxicology and Applied Pharmacology,* 228**,** 32-41.
YOCHUM, C. L., BHATTACHARYA, P., PATTI, L., MIROCHNITCHENKO, O. & WAGNER, G. C. 2010. Animal model of autism using GSTM1 knockout mice and early post-natal sodium valproate treatment. *Behav Brain Res,* 210**,** 202-10.
ZARAZUA, S., RIOS, R., DELGADO, J. M., SANTOYO, M. E., ORTIZ-PEREZ, D. & JIMENEZ-CAPDEVILLE, M. E. 2010. Decreased arginine methylation and myelin alterations in arsenic exposed rats. *Neurotoxicology,* 31**,** 94-100.
ZENG, X., CHEN, J., DENG, X., LIU, Y., RAO, M. S., CADET, J. L. & FREED, W. J. 2006. An in vitro model of human dopaminergic neurons derived from embryonic stem cells: MPP+ toxicity and GDNF neuroprotection. *Neuropsychopharmacology,* 31**,** 2708-15.
ZHANG, J., DAI, H., DENG, Y., TIAN, J., ZHANG, C., HU, Z., BING, G. & ZHAO, L. 2015. Neonatal chlorpyrifos exposure induces loss of dopaminergic neurons in young adult rats. *Toxicology,* 336**,** 17-25.
ZIMMER, B., KUEGLER, P. B., BAUDIS, B., GENEWSKY, A., TANAVDE, V., KOH, W., TAN, B., WALDMANN, T., KADEREIT, S. & LEIST, M. 2011a. Coordinated waves of gene expression during neuronal differentiation of embryonic stem cells as basis for novel approaches to developmental neurotoxicity testing. *Cell Death Differ,* 18**,** 383-95.
ZIMMER, B., LEE, G., BALMER, N. V., MEGANATHAN, K., SACHINIDIS, A., STUDER, L. & LEIST, M. 2012. Evaluation of developmental toxicants and signaling pathways in a functional test based on the migration of human neural crest cells. *Environ Health Perspect,* 120**,** 1116-22.
ZIMMER, B., PALLOCCA, G., DRESER, N., FOERSTER, S., WALDMANN, T., WESTERHOUT, J., JULIEN, S., KRAUSE, K. H., VAN THRIEL, C., HENGSTLER, J. G., SACHINIDIS, A., BOSGRA, S. & LEIST, M. 2014. Profiling of drugs and environmental chemicals for functional impairment of neural crest migration in a novel stem cell-based test battery. *Arch Toxicol,* 88**,** 1109-26.
ZIMMER, B., SCHILDKNECHT, S., KUEGLER, P. B., TANAVDE, V., KADEREIT, S. & LEIST, M. 2011b. Sensitivity of Dopaminergic Neuron Differentiation from Stem Cells to Chronic Low-Dose Methylmercury Exposure. *Toxicological Sciences,* 121**,** 357-367.
ZOELLER, R. T. & CROFTON, K. M. 2005. Mode of action: developmental thyroid hormone insufficiency--neurological abnormalities resulting from exposure to propylthiouracil. *Crit Rev Toxicol,* 35**,** 771-81.
ZYCHOWICZ, M., DZIEDZICKA, D., MEHN, D., KOZLOWSKA, H., KINSNER-OVASKAINEN, A., STEPIEN, P. P., ROSSI, F. & BUZANSKA, L. 2014. Developmental stage dependent neural stem cells sensitivity to methylmercury chloride on different biofunctional surfaces. *Toxicology in Vitro,* 28**,** 76-87.