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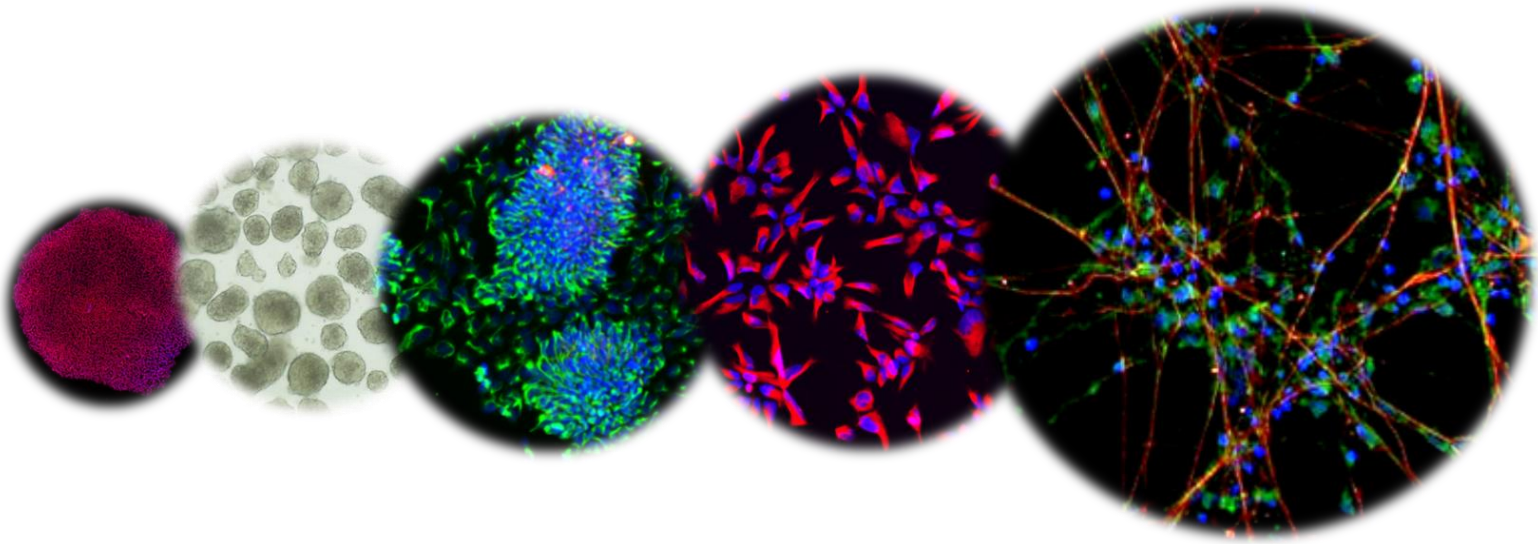

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Strategic aims for improving the regulatory assessment of Developmental Neurotoxicity (DNT) using non-animal methods

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Summary

Currently, the identification of chemicals that have the potential to induce developmental neurotoxicity (DNT) is based on animal testing, since there are no regulatory accepted alternative methods for this purpose. Since at the regulatory level, systematic testing of DNT is not a standard requirement within the EU legislation of chemical safety assessment, DNT testing is only performed in higher tiered tests triggered based on structure activity relationships or evidence of neurotoxicity in systemic adult studies. However, these triggers are rarely used and in addition do not always serve as reliable indicators of DNT as they are observed in an adult rodent animal. Consequently, to date only a limited amount of chemicals (Grandjean and Landrigan, 2006; Smirnova et al., 2014), mainly pesticides (Bjørling-Poulsen et al., 2008) have been tested under US EPA (OPPTS 870.630) or OECD DNT TG 426. Therefore, there is the pressing need for developing alternative methodologies that can more rapidly and cost-effectively screen large numbers of chemicals for their potential to cause DNT.

In this report we propose that *in vitro* studies could contribute to the identification of potential triggers for DNT evaluation since existing cellular models permit the evaluation of a chemical impact on key neurodevelopmental processes, mimicking different windows of human brain development, especially if human models derived from induced pluripotent stem cells are applied. Furthermore, the battery of currently available DNT alternative test methods anchored to critical neurodevelopmental processes and key events identified in DNT Adverse Outcome Pathways (AOPs) could be applied to generate *in vitro* data useful for various regulatory purposes. Incorporation of *in vitro* mechanistic information would increase scientific confidence in decision making, by decreasing uncertainty and leading to refinement of chemical grouping according to biological activity. We suggest development of Integrated Approaches to Testing and Assessment (IATA) based on key neurodevelopmental processes and existing AOPs relevant to DNT (Bal-Price and Meek, 2017b) as a tool for not only speeding up chemical screening, but also providing mechanistic data in support of hazard assessment and in the evaluation of chemical mixtures. Such mechanistically informed IATA for DNT evaluation could be developed integrating various sources of information (e.g., non-testing methods, in vitro approaches, as well as in vivo animal and human data), contributing to screening for prioritization, hazard identification and characterization, and possibly safety assessment of chemicals, speeding up the evaluation of thousands of compounds present in industrial, agricultural and consumer products that lack safety data on DNT potential. It is planned that the data and knowledge generated from such testing will be fed into the development of an OECD guidance document on alternative approaches to DNT testing.

1. Introduction

The developing nervous system is known to be more vulnerable to chemical exposure compared with the adult nervous system. The higher vulnerability of the developing brain results from the complex processes specific to development such as the commitment and differentiation of the neuronal progenitor cells followed by glial and neuronal cell proliferation, migration, differentiation into various neuronal and glial subtypes, synaptogenesis, pruning, myelination, networking and terminal functional neuronal and glial maturation (Rice and Barone 2000; Hogberg et al., 2009 and 2010; Stiles and Jernigan, 2010; Yang et al., 2014; Krug et al., 2013*)*. A challenge in evaluation of developmental neurotoxicity (DNT) induced by a chemical is that the neurodevelopmental outcome depends not only on the kind of exposure (dose, duration) but also on the developmental stage of the brain at the time of exposure (Rice and Barone, 2000). Additionally, the immature blood brain barrier (BBB) is not completely formed thus facilitating the entrance of a chemical into the foetal/neonatal brain (Adinolfi, 1985).

Currently, at the regulatory level, there is a recognized need for neurotoxicity evaluation (Bal-Price et al., 2012; 2015b), however systematic testing for DNT is not a mandatory requirement in the European Union for pesticides, biocides, pharmaceuticals or industrial chemicals. DNT testing is performed only as higher tiered tests that are triggered based on structure activity relationships or evidence of neurotoxicity in standard *in vivo* tests in adult animals, (Makris et al., 2009; Bal-Price et al., 2010 and 2012) either after acute exposure (OECD TGs 402, 403, 420, 423, 436 and 425), or repeated dose toxicity, sub-chronic (OECD TG 407 and 408) or chronic exposure (OECD TG 452). Additionally, DNT studies can also be triggered when data from extended one–generation reproductive toxicity study (TG 443) indicate a possible concern of neurotoxicity (Bal-Price et al., 2015b; ECETOC Document No 45).

At the same time, for regulatory purposes the identification of chemicals with neurotoxic potential is entirely based on the use of *in vivo* animal tests since there are no officially accepted alternative methods for this purpose. Two regulatory guidelines for rodent *in vivo* test methods used for DNT evaluation (OECD DNT TG 426 and TG443: Extended one-generation reproductive toxicity study) are based on neurobehavioral evaluation of cognitive, sensory and motor functions accompanied by morphometric and histopathological studies. Additional testing, specifically of offspring that have been exposed *in utero* and during early lactation, includes also evaluation of sexual maturation, behavioural ontogeny and learning and memory (OECD DNT TG 426). However, this test is not accompanied by detailed guidance on its use, leaving large flexibility in the study design and in the interpretation of the results.

These *in vivo* based guidelines are very resource intensive in terms of animals, time and overall cost (Rovida and Hartung 2009; Tsuji and Crofton 2012) and have been used only for a limited number of pesticides and industrial chemicals. Therefore there is only a small amount of DNT data available. Indeed DNT testing has been performed for less than 200 chemicals globally (Grandjean and Landrigan, 2006; Evans et al., 2016; Fritsche et al., 2017) mostly pesticides (Bjørling-Poulsen et al., 2008) and only a few of these studies contributed to risk assessment (Smirnova et al., 2014). Although the

majority of risk assessments can be considered protective for positive *in vivo* DNT effects, according to the authors animal DNT testing is not sufficient for covering hazards associated with DNT as animal DNT studies do not always identify human DNT toxicants (Aschner et al., 2017). This could be due to different pharmacokinetics in different species, including metabolic activity or placental transfer in animals compared to humans.

These issues highlight the pressing need for developing alternative methodologies that can more rapidly and cost-effectively screen large numbers of chemicals for their potential to cause DNT.

Decades of *in vitro* work using rodent and human neuronal models have delivered a range of reliable *in vitro* assays that currently permit quantitative evaluation (via concentration-response relationships) of the impact of a compound on key developmental processes and pathways critical for brain development. For instance, human neural stem cells can be used to assess several DNT-related endpoints, such as commitment and proliferation, apoptosis, cell migration, neuronal and glial differentiation, neurite outgrowth, myelination, axonal and dendritic elongation, synapse formation, synapse pruning, neurotransmitter receptor profiling, development of neuronal connectivity, spontaneous electrical activity, etc. (Fritsche et al., 2015; Coecke et al., 2007). Some of these assays are already at the High Throughput Screening (HTS) level, permitting a quantitative evaluation using a range of different *in vitro* cell models, including human induced pluripotent stem cell (hiPSC)-derived neuronal cultures. For instance, high-content analysis were performed by the U.S. EPA for neurite outgrowth (testing approximately 300 chemicals) (Mundy et al., 2010), including human iPSC-derived neurons (80 chemicals) (Ryan et al., 2016; Druwe et al., 2016), neural proliferation (Breier et al., 2008; Mundy et al., 2010) and synaptogenesis (Harrill et al., 2011). Biomarkers of neuronal and glial cell differentiation processes have been also identified using primary rodent cultures and human neural stem cells (Kuegler et al., 2010) based on gene (Hogberg et al., 2010, 2011) and protein expression (Mundy et al., 2008) as well as metabolomics and proteomics analysis (Schultz et al., 2015) and measurements of neuronal electrical activity under the exposure to different classes of chemicals including pesticides (Vassallo et al., 2016; Brown et al., 2016).

The data produced from such *in vitro* DNT studies could be used to support chemical screening and prioritization, as well as hazard and risk assessment by delivering information on mechanisms of toxicity (mode of action), including interspecies differences.

In this report, it is proposed to use the data derived from *in vitro* DNT studies within the context of Integrated Approaches to Testing and Assessment (IATA), for the following regulatory needs:

- Providing supplementary mechanistic information on chemically-induced DNT to support their hazard assessment
- Providing mechanistic data derived from alternative methods to support grouping and assessment of combined exposures to multiple chemicals (mixture risk assessment; MRA) with potential to induce DNT

- Providing supplementary information supporting triggering criteria for DNT testing

- Introducing in vitro screening approaches to prioritize chemicals for further in vivo testing according to DNT TG 426

To achieve these aims multiple sources of information need to be combined within IATA including data obtained from DNT *in vitro* assays, *in silico* modelling (such as QSARs) and read across as well as data obtained from non-mammalian species (for instance zebrafish model). At the same time the existing methods should be further optimized to be amenable for High Throughput Screening (HTS) and missing methods should be developed to expand the current set of available assays to ensure that critical key neurodevelopmental pathways and processes specific for DNT can be assessed using alternative approaches.

2. Key considerations on IATA development for DNT

A framework for the development of mechanistically-informed IATA for identification of chemicals with DNT potential should be based on various sources of information (non-testing methods, *in vitro* approaches, *in vivo* animal and human data), delivering data for different regulatory purposes. The increasing availability of Adverse Outcome Pathways (AOPs) will facilitate use of mechanistic knowledge (including that coming from *in vitro* studies) in DNT regulatory decision making processes (OECD GD 260, 2016).

Since an IATA should be customised for the specific regulatory need, the proposed IATA framework for DNT should consider a set of *in vitro* test methods for generation of missing data that can be used in a flexible combination (fit-for-purpose), anchoring the assays against molecular initiating events (MIEs) and a selected set of key events (KEs) at the cellular or tissue level described in the existing DNT-relevant AOPs (*https://aopwiki.org;* Bal-Price et al., 2015a; Bal-Price and Meek, 2017b) and those other putative AOPs identified in the literature. The assays that allow an evaluation of the key biological processes specific for brain development such as cell proliferation, migration, differentiation etc., may also be combined, where appropriate, with non-mammalian models (e.g. zebrafish) suitable for behavioural observations, some of which are available at HTS level. The HTS methods and those assays that are easily adaptable to an HTS platform should be used as first choice permitting the screening of a large number of chemicals, over a wide range of concentrations, in a time and cost-efficient manner.

Understanding the likelihood of the triggered events described in the AOPs as MIEs or KEs at lower levels of biological organisation (e.g. *in vitro* testing at the cellular level) or information from structure-activity relationships, can help to inform whether testing at higher levels of biological organisation (i.e., *in vivo*) is warranted (ENV/JM/MONO(2016)29).

Mechanistic information on pathways of toxicity specific for DNT could guide a design of IATA, composed of fit-for-purpose tools, including *in vitro* assays that are consistent with *in vivo* human biology, permitting evaluation of KEs identified in the relevant AOPs.

However, currently, only a few DNT AOPs are available (Bal-Price and Meek, 2017b) and the development of a sufficient number of specific DNT AOPs will take time. Therefore, so as not to delay development and implementation of a testing strategy, it was suggested during the OECD/EFSA DNT workshop (October 2016) that neurodevelopmental processes, whether or not incorporated into existing AOPs, can also be utilized as KEs, and thus chemical testing across a potential testing battery could feed back to inform further AOP development in the future (Fritsche et al., 2017; EFSA Workshop Report, 2017).

The preferential use of human *in vitro* models is advisable since it will decrease the need of cross-species extrapolation from animal-based findings. Data produced from IATA will require different levels of scientific confidence and different levels of acceptable uncertainty depending on the regulatory purpose. For instance in the case of screening and prioritization purposes a greater level of uncertainty could be tolerated in comparison to hazard identification or risk assessment where higher levels of reliability, certainty and validation will be required.

The final IATA design should be fit-for-purpose. Depending on the purpose and substance / mixture to be evaluated, it may require a different combination of DNT *in vitro* assays used together with other alternative tools such as QSAR, *in silico* modelling and possibly non-mammalian models. Therefore, different IATA solutions may be possible depending on the chemical(s) under investigation and the regulatory purpose and context (e.g., refining of *in vivo* testing, chemical screening, prioritization, grouping, hazard characterization or risk assessment). An *in vitro* battery of DNT tests supporting IATA development for different aims is discussed below.

3. How data derived from *in vitro* DNT testing could contribute to regulatory decision-making

3.1 Providing supplementary mechanistic information on chemically-induced DNT to support hazard assessment

Problem formulation: *Many different classes of pesticides are designed to target the nervous system of insect pests. Because of the similarity of neurochemical processes across taxa these compounds are likely to be neurotoxic to humans. This concern is of particular relevance to the developing human brain which, as mentioned above, is inherently much more vulnerable to a chemically-induced damage than the adult brain. Therefore, this class of regulated chemicals should be recognized as a priority for evaluating DNT potential.*

The EFSA Panel on Plant Protection Products and their Residues (PPR Panel) delivered a scientific opinion on the DNT potential of the neonicotinoid insecticides acetamiprid and imidacloprid (EFSA Scientific Opinion, 2013) recommending that "*in vitro assays may be regarded as complementary to animal testing because they may provide better understanding of the cellular/molecular mechanisms involved in developmental*

neurotoxicity. As such, in vitro tests could be incorporated into a DNT testing strategy to obtain mechanistic information or for purposes of screening/prioritisation."

Following this recommendation and focussing on the aspect of obtaining further mechanistic support, incorporation of supplementary information delivered from DNT *in vitro* testing and other alternative approaches (e.g. QSARs, computational modelling, read across) would increase weight of evidence and scientific confidence, provided by DNT *in vivo* studies (where results may often be equivocal/open to different interpretation) with respect to whether or not a chemical has the capacity to cause DNT effects and if so, by what mechanisms.

This can be achieved by using a battery of *in vitro* assays which permit evaluation of a range of key pathways and processes specific for brain development of humans at different developmental time points (exposure windows) using not only rodent but also human models, where possible, due to known interspecies differences (Fritsche et al., 2015).

The mechanistic information derived from the *in vitro* DNT assays could be used as a basis for biological groupings of chemicals according to the common mechanisms of toxicity or modes of action. Currently, some existing chemicals, including pesticides are already grouped according to their mode of action for instance pyrethroids (binding to voltage-gated sodium channels), rotenoids (inhibition of electron transfer from iron-sulphur centres in complex I to ubiquinone), and nicotinoids (binding to nicotinic acetylcholine receptors (nAChRs) mimicking the action of acetylcholine by opening the ion channels which allow the entry of Na^+ and Ca^{2+} into cells). This type of pesticide classification could be further refined based on *in vitro* mechanistic data. QSAR analysis would permit further grouping of the chemicals according to their structure as it has been done for instance for organochlorines, organophosphates or carbamates.

To facilitate biological grouping of chemicals, data could be generated by investigating effects at the molecular and cellular level using *in vitro* assays anchored to the key events of the relevant DNT AOPs (Bal-Price at al., 2015a), preferably those amenable to HTS, permitting testing of a larger number of chemicals, at the concentrations relevant to human exposure.

In vitro mechanistic studies will build knowledge on toxicity pathways involved in a chemically-induced impairment of brain-specific processes, supporting chemical, including pesticide, hazard assessment by increasing weight of evidence and thus reducing uncertainties in human health risk assessment.

3.2 Providing mechanistic data derived from alternative methods to support grouping and assessment of combined exposures to multiple chemicals (mixture risk assessment (MRA)) with potential to induce DNT

Problem formulation: *Humans (including children) are indisputably co-exposed to more than one chemical at a time. Chemicals causing similar effects and adverse outcomes can contribute to combined effects even when present individually at safe concentration levels. Grouping and assessment of chemicals in mixtures for specific effects such as DNT can be supported using mechanistic data from alternative methods in AOP-based IATA.*

Chemicals that are known to trigger specific DNT effects belong to different chemical types such as organic solvents or metals, or to different use categories such as pharmaceuticals, industrial chemicals, biocides or pesticides. Approximately 218 chemicals are identified as neurotoxicants of which 27 are metals or inorganic compounds, 41 are organic solvents, 48 are other organic substances and 102 are pesticides (Grandjean and Landrigan, 2014). In the most recent studies by Maffini and Neltner (2015) more than 300 chemicals were identified as potential DNT chemicals. These compounds belong to various regulatory use categories related to food, such as pesticides, food contact materials and food additives including flavourings, colourings and preservatives. These examples illustrate that common, similar or related toxic effects triggered by various chemicals may be differently regulated according to their use category and that combined effects of these chemicals across different regulatory domains are not currently considered (Evans et al., 2016). At the same time it is well documented in the existing literature that "mixture effects" can be greater than effects triggered by the most potent single chemical in a mixture, and the mixture effects may be additive or in some cases even synergistic (Kortenkamp et al., 2009; 2012; Kienzler et al., 2016).

MRA is very relevant to DNT evaluation as, for example, breast milk has been found to contain chemicals regulated as pesticides, along with those regulated as cosmetics (including UV filters, parabens, phthalates), together with persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs) (Schlumpf et al., 2010) confirming the co-exposure of infants simultaneously to multiple chemicals.

In the development and evaluation of IATA for the risk assessment of groups of chemicals associated with DNT, relevant mixtures should be defined based on realistic co-exposures or based on commonalities in chemical structure, key events, or adverse outcomes, depending on the purpose of the risk assessment and the problem formulation defined at the outset. DNT *in vitro* testing will facilitate the grouping of chemicals based on identification of impacts on shared key neurodevelopmental processes.

3.3 Providing supplementary information supporting triggering criteria for DNT testing under different regulations

Problem formulation*: The existing criteria for triggering DNT testing are based on systemic in vivo studies (short or long term) in adult rodents. However, adult animals cannot mimic processes that are specific to the brain during development. The current triggers are thus likely to be in some cases too insensitive to be able to decide when such studies are required. Therefore, there is a need to define in a more informed manner the criteria that should trigger DNT testing based not only on in vivo observations but also including triggers from in vitro studies that permit evaluation of pathways and processes specific for brain at different developmental stages.*

Currently, developmental neurotoxicity evaluation is triggered under REACH, PPP and BP regulations based on the effects observed in the systemic studies where, e.g. under REACH, depending on the tonnage level, a 28-day and/or a 90-day repeated-dose toxicity study is performed (e.g. OECD TG 407, 408/EU B.7, B.26). In case of chemicals produced or imported with volumes of over10 t/y, a repeated dose 28-day oral toxicity testing (OECD TG 407/EU B.7) together with a screening study for reproductive/developmental toxicity (TG 421) is required or a combination of a repeated dose toxicity study with the reproductive/developmental toxicity screening study (OECD TG 422) is recommended. At volumes over 100 t/y a sub-chronic (90-day) toxicity study (OECD TG 408/EU B.26) is required which can be waived under certain circumstances.

Testing for DNT effects is considered when the following triggers, viewed as predictive of possible neurotoxic activity, are met:

(1) structural and morphological brain abnormalities
(2) clear signs of behavioural or functional adverse effects
(3) structure-activity relationships (a compound similar in structure to a known neurotoxic chemical)
(4) mode of action of a chemical that has been closely linked to neurotoxic or developmental neurotoxic effects (e.g. cholinesterase inhibition or thyroid effects)

However, these triggers are not specific for brain development and furthermore they are mostly observed in adult animals that are not always a relevant model, at least for evaluation of certain processes and pathways that take place only during brain development. As mentioned before complexity and vulnerability of the developing brain is very different from adult (mature) brain. More reliable triggers for DNT studies can be observed when both a repeated dose toxicity study (TG 407) and a reproductive/developmental toxicity screening study following TG 422 or 421 are available.

Therefore, it is proposed that also *in vitro* studies could contribute to the identification of potential triggers for DNT testing since cell culture models (human and in some cases rodent) permit to study a chemical impact on the specific, key developmental pathways and processes relevant to humans, applying different exposure scenarios focussed on different windows of brain development that cannot be studied in the adult brain.

Including triggers based on *in vitro* DNT assays would increase the probability of flagging chemicals with potential to cause specifically DNT as it would be based on experiments designed for DNT evaluation, using animal or human neuronal models that mimic key

processes specific for brain development including cell proliferation, migration, neuronal and glial differentiation, synaptogenesis, neuronal network formation and function, etc. A similar approach could be applied to the evaluation of critical signalling pathways that are fundamental for brain development such as BDNF, ERK, CREB, RTK-P13K-AKT, mTOR, PLCg1, NCAM-FGFR, GDNF-RET, Wnt, Shh, Notch, TGFβ-BMP and others (OECD/EFSA Workshop Report, 2017). If these signalling pathways or key neurodevelopmental processes would be affected by a chemical at the concentrations relevant to possible human exposure such information could serve as a potential trigger for further follow up *in vitro* or if necessary *in vivo* DNT studies. This approach could be of a particular importance in the process of 'substance evaluation' under REACH in a first step relevant to the identification of a SVHC (Substance of Very High Concern). Incorporation of supplementary *in vitro* data to support triggering criteria for DNT testing would be in compliance with the REACH regulation which encourages use of *in vitro* approaches where possible. Use of such data could be introduced into ECHA guidance and may also be considered for inclusion in the REACH annexes on information requirements.

3.4 Introduction of in vitro screening approach to prioritize chemicals for further in vivo testing according to DNT TG 426

Problem formulation: *the current in vivo OECD TG 426 is very resource intensive in terms of animals, time and overall cost. It is therefore rarely used resulting in a small number of chemicals tested for their DNT potential. Hence, there is an urgent need to develop non-animal methods that will speed up the process of chemical screening to identify those chemicals with DNT potential.*

The EFSA PPR Panel delivered a scientific opinion on the DNT potential of the neonicotinoid insecticides acetamiprid and imidacloprid (EFSA Scientific Opinion, 2013). To evaluate the DNT potential of these insecticides the PPR Panel commented on the current OECD DNT TG 426 stating that "*DNT guidelines are complex, time consuming, costly and not suitable for routine testing of high numbers of chemicals. Some concerns in terms of feasibility and animal welfare have been raised in the scientific literature. Although the protocol of the guidelines is well designed and covers a broad window of exposure, the critical phase for some effects might be missed and not all effects would be found. Furthermore, the interpretation of results is difficult because of knowledge gaps concerning normal brain development on the functional, structural and molecular levels, thus complicating risk assessment of compounds (Beronius et al., 2013). A number of issues related to the interpretation of DNT studies have been raised such as excessive variability that may mask treatment-related effects.*"

Additional review of the performance of developmental neurotoxicity (DNT) testing following DNT test guideline 426 (TG 426) was performed by Makris et al., (2009) stating that the OECD DNT guideline represents the best available science for assessing the potential for DNT in human health risk assessment, and data generated with this protocol are relevant and reliable for the assessment of these endpoints. The reproducibility, reliability, and sensitivity of these methods have been demonstrated, using a wide variety of test substances, in accordance with OECD guidance on the validation and international acceptance of new or updated test methods for hazard

characterization. Multiple independent, expert scientific peer reviews affirm these conclusions.

However, another review of the performance of DNT evaluations (Claudio et al., 2000) according to the US EPA DNT guideline (OPPTS 870.6300 - a prototype of the OECD TG 426) concluded that current regulatory practice does not adequately require the conduct of developmental neurotoxicity tests and is insufficient to determine with certainty that no harm will occur to exposed infants and children.

Summing up, based on the existing reviews (EFSA Scientific Opinion, 2013; Claudio et al., 2000; Makris et al.,2009) of both US EPA (OPPTS 870.6300) and the OECD (426) DNT TGs, in addition to the fact that they are not always triggered appropriately within the current tiered system for testing, the current DNT TG presents a number of challenges and limitations:

- It does not expose developing animals during all critical periods of vulnerability
- It does not assess effects that may become evident later in life
- It does not include methodology for consideration of different pharmacokinetics in different animals and humans, including metabolic activity or placental transfer in animals compared to humans
- Methodology for assessment of neurobehavioral, neuropathological, and morphometry is highly variable, flexible and guidance is not provided on data interpretation (consequently it is difficult to compare data between studies)
- Testing of neurochemical changes is limited
- Required tests provide little mechanistic understanding of the underlying pathways involved
- The recommended methods are low throughput, time-and resource-consuming

Deficiencies in the testing methodology for developmental neurotoxicants represent a significant gap and increase the uncertainty in the establishment of safe levels of exposure to developing individuals. This highlights the urgent need for developing new methodologies that can more rapidly and cost-effectively screen large numbers of chemicals for their potential to cause DNT (Bal-Price et al., 2015b; Fritsche et al., 2017).

Considering the current information requirements for DNT evaluation within the existing regulations in the EU it is clearly impossible at this time to replace animal testing with alternative *in vitro* methods. Therefore, the efforts should be directed towards the overall improvement of the current *in vivo* testing following TG 426 by incorporating *in vitro* methods as a first step in a tiered approach. Similar consensus has been reached by the participants of the recent OECD/EFSA DNT Workshops (Fritsche et al., 2017, Bal-Price at al., 2017a) during which it was suggested that *in vitro* DNT assays are ready to be used for chemical screening and prioritisation. However, for other regulatory needs, such as replacement of animal testing or deriving health-based exposure limits, these assays require further standardization to reach higher scientific confidence.

4. Conclusion

This JRC report describes possible applications of *in vitro* approaches for developmental neurotoxicity evaluation for various regulatory purposes.

The EFSA PPR Panel, OECD and US EPA support the development of an integrated *in vitro* developmental neurotoxicity testing strategy complementary to the rodent *in vivo* methods (OECD TG 426, US EPA TG OPPTS 870.6300, TG 443) in order to refine and speed up the evaluation of a high number of chemicals for their DNT potential. The battery of methods should be composed of robust, reliable and standardized *in vitro* assays, relevant for the assessment of human toxicity to support tiered, cost-effective chemical screening, hazard identification and characterisation as well as risk assessment. Therefore IATA has been proposed as a practical solution to provide testing strategies composed of *in vitro* assays anchored to key events identified in the DNT AOPs and other key neurodevelopmental processes. In parallel, further development of AOPs relevant to DNT should take place as they will provide mechanistic information on the causal links between MIEs, KEs and AO of regulatory concern, providing the biological context for the *in vitro* assays anchored to AOP(s) key events and facilitating development of AOP-informed IATA for various regulatory purposes.

Currently, the main task is to establish performance standards and readiness criteria for evaluation of individual *in vitro* DNT assays which can be fed into the development of a guidance on building testing strategies (consisting of *in vitro* methods and alternative organisms like the zebrafish) (Bal-Price et al., 2017a). Such a testing strategy (e.g. DNT AOP-informed IATA) should be challenged with a range of chemicals across the full battery of *in vitro* test methods to support validation and build confidence. These efforts should eventually support the development of an OECD Guidance Document (GD) on available *in vitro* DNT test methods used alone or in combination within the context of an IATA addressing various regulatory needs based on the principle of 'fit-for-purpose'. A proposal for such an OECD Guidance Document has been recently included in the OECD work programme and will be developed in collaboration with EFSA, European (including EC JRC) and USA DNT experts.

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List of abbreviations and definitions

AO	Adverse Outcome
AOP	Adverse Outcome Pathway
CNS	Central Nervous System
CRA	Cumulative Risk Assessment
DNT	Developmental neurotoxicity
EFSA	European Food Safety Authority
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
GD	Guidance Document
HCA	High-Content Analysis
hESCs	Human Embryonic stem cells
hiPSCs	Human Induced Pluripotent Stem Cells
HTS	High Throughput Screening
IATA	Integrated Approaches to Testing and Assessment
iPSCs	Induced Pluripotent Stem Cells
ISTNET	International Stakeholder Network for Creating a (Developmental) Neurotoxicity Testing Roadmap
KE	Key Event
KER	Key Event Relationship
MIE	Molecular Initiating Event
NT	Neurotoxicity
OECD	Organisation for Economic Co-operation and Development
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SAR	Structure-Activity Relationship
TG	Test Guideline
WoE	Weight of Evidence
WNT	Working Group of National Coordinators for the Test Guideline Programme

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