Meeting report

OECD/EFSA Workshop on Developmental Neurotoxicity (DNT): The Use of Non-Animal Test Methods for Regulatory Purposes

https://doi.org/10.14573/altex.1701171

Scientists from 15 countries across the world, representing stakeholders from regulatory agencies, non-governmental organizations (NGOs), academia and industry, reached a consensus that current data requirements for in vivo developmental neurotoxicity (DNT) testing are not sufficient to screen and characterize potentially hazardous compounds. In addition, there was agreement on the need to develop a standardized in vitro testing battery to generate additional data on the effects of chemicals on the developing nervous system.

The need for more effective DNT screening is driven by the scientific fact that the developing nervous system might be more sensitive to exposures to some chemical classes of hazardous substances. In addition, recent societal concerns have been raised linking the rise in children’s neurodevelopmental impairments (e.g., learning disabilities) to chemical exposures. Despite a clear deficit in knowledge concerning DNT effects, only approximately 140 in vivo guideline studies (according to OECD 426 & EPA OPPTS 870.630) have been conducted to date, leaving a huge data gap on the DNT potential of chemicals within the universe of thousands of compounds present in industrial, agricultural and consumer products. This deficit is mainly due to the fact that currently accepted guideline studies are at present not mandatory data requirements and are extremely time- and cost-intensive. Additionally, they can result in methodological and scientific uncertainties. This includes the challenges in extrapolation of findings from rats to humans that result from timing differences in brain development, toxicokinetics, and inherent difficulties in the use of non-homologous functional tests (Tsuji and Crofton, 2012; Dorman et al., 2001; Kaufmann, 2003). For these reasons, DNT has been regarded as an area in need of the development of alternative methods in order to establish a time-efficient and cost-efficient predictive testing strategy.

A series of workshops held over the past decade (Lein et al., 2007; Crofton et al., 2011; Bal-Price et al., 2012, 2015a) have fostered the development of in vitro assays or methods using alternative model organisms that assess the impact of chemicals on cellular processes critical to normal brain development, including: neural proliferation, differentiation, migration, neurite outgrowth, synaptogenesis, myelin formation, and neural network formation and function. Many of these human cell-based assays have been used to study small numbers of chemicals (n < 15; e.g., Harrill et al., 2011; He et al., 2012; Rempel et al., 2015; Baumann et al., 2016; Brown et al., 2016) or to derive mechanistic information for limited numbers of chemicals (e.g., Gassmann et al., 2010; Balmer et al., 2012; Balmer and Leist, 2014; Barenys et al., 2016). Only a few have been utilized to screen larger numbers (n > 15) of compounds (e.g., Stiegler et al., 2011; Zimmer et al., 2012; Culbreth et al., 2012; McConnell et al., 2012; Krug et al., 2013; Valdivia et al., 2014; Mundy et al., 2015; Hoelting et al., 2016; Nyffeler et al., 2016).

On the scientific premise that alternative methods are available and can be assembled into a larger DNT screening battery, a joint OECD/EFSA workshop was held in Brussels on October 18 and 19, 2016 that aimed to facilitate the use of such methods in regulatory decision making. Specific objectives of this workshop were:

1. Development of a consensus that the proposed testing battery of alternative DNT methods is ready to be applied right now, and could be used in a fit-for-purpose manner for either screening and prioritization, or as a first starting point to conduct targeted testing in a tiered testing approach in the process of hazard identification and characterization for specific chemical risk assessment.
2. Identification of the next steps necessary to encourage the regulatory use of the alternative methods depending on their level of readiness.
3. Outline what could become an integrated approach to testing and assessment (IATA) for the purposes of screening and prioritization or hazard assessment.

The meeting was co-chaired by Ellen Fritsche (Leibniz Research Institute for Environmental Medicine, IUF) and Kevin Crofton (US Environmental Protection Agency, US EPA). Meeting participants and their affiliations are reported in the supplementary file at https://doi.org/10.14573/altex.1701171s.

Disclaimer: The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency (US-EPA), of the European Food Safety Authority (EFSA) and the German Federal Institute for Risk Assessment. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. The opinions expressed and arguments employed herein are those of the authors and do not necessarily reflect the official views of the Organization for Economic Cooperation and Development (OECD), European Commission (EC) or of the governments of its member countries.
The meeting started with a presentation by a speaker from the European Food Safety Authority (EFSA), summarizing the epidemiological evidence for research needs for DNT testing. Despite the evident complexity in the interpretation of epidemiological read-outs, there is sufficient evidence that early-life exposures to some chemicals may result in long-term adverse health consequences for the developing offspring, and that a multidisciplinary approach including experimental toxicological investigations for DNT endpoints is needed. This was followed by a speaker from the US EPA on the history and path forward concerning alternative test methods for DNT, concluding on the readiness to apply DNT in vitro assays, the availability of reference chemicals for demonstration of predictability and the availability of open databases for sharing methods and results. The regulatory perspective was introduced by speakers from the US EPA, Danish EPA, German Federal Institute for Risk Assessment (BfR) and European Chemicals Agency (ECHA), who presented current experiences with the US EPA/OECD DNT guideline testing. This provided a regulatory perspective for a fit-for-purpose DNT testing paradigm, including the use of alternative in vitro testing preceding targeted in vivo testing. There was consensus amongst the speakers from the different regulatory agencies in support of the implementation of a standardized DNT in vitro testing strategy, driven by problem formulation (i.e., screening and prioritization vs. chemical specific hazard identification/characterization).

Speakers from EU and US industries (representing European Crop Protection Association/Crop Life America) reiterated that there is a strong need for alternative methods for tiered DNT testing to support candidate selection, decision making and Mode of Action (MoA) exploration that identify DNT hazards in a more time- and cost-efficient manner. For regulatory applications, practical scientific issues like quality control, reproducibility, sensitivity, specificity, predictive capability and exposure considerations must be addressed with a test battery when using alternative approaches. There was also agreement that future efforts should include the development of a testing strategy guidance for DNT by OECD.

The value of development and use of adverse outcome pathways (AOPs) for DNT to understand key event (KE) relationships was pointed out by a speaker from the European Commission-Joint Research Centre (EC-JRC). One of the applications of the AOP concept is endpoint selection of DNT assays that increase regulatory confidence, since identifying the causative link between KEs and AOs for DNT provides a mechanistic understanding and increases the scientific confidence in the relevance of the in vitro testing battery. The KEs identified in the existing DNT AOPs (AOP-Wiki: https://aopwiki.org/; Bal-Price et al., 2015b) could serve as anchors for development of such in vitro assays. However, the development of a sufficient number of specific DNT AOPs will take time and should not delay development and implementation of a testing strategy. Therefore, it was suggested that neurodevelopmental processes be utilized as KEs, and thus chemical testing across a potential testing battery could inform AOP-building in the future.

The last talks, given by academic researchers, were geared to set the stage for breakout group discussions. They reviewed the scientific principles of alternative DNT methods to test the fundamental neurodevelopmental processes critical for normal brain development. This was referred to as a “process control” based testing strategy for DNT. In addition, they clearly underlined the regulatory benefit of testing biological processes directly linked to toxicity endophenotypes based on the assumption that nervous system development is impaired when key biological processes are disturbed (Lein et al., 2005; Smirnova et al., 2014). To put this principle into action, a case study was presented for the DNT compound methylmercury that was tested across a large variety of DNT assays covering different neurodevelopmental processes (KEs) and identifying the most sensitive endpoint from those. It was concluded that a complementary in vitro testing battery can be conducted in a relevant cell system using human-derived cells, and this would reduce some uncertainties in using an in vitro system for regulatory decision making. This statement was supported by the OECD-funded “Report on Integrated Testing Strategies for the identification and evaluation of chemical hazards associated with the developmental neurotoxicity (DNT)” (Fritsche, 2016).

The meeting continued with four breakout groups discussing the following topics that were summarized on day 2 of the workshop.

1. The regulatory need for alternative DNT testing (Chair: Roland Solecki, BfR; Rapporteur: Martin Wilks, University of Basel);
2. Proposing a draft DNT testing battery (Chair: Antonio Hernandez, University of Granada; Rapporteur: Anna Bal-Price, EC, JRC);
3. How can knowledge from new DNT tests contribute to epidemiology and vice versa (Chair: Stanley Barone Jr., US EPA; Rapporteur: Marcel Leist, University of Konstanz);
4. Implementing a draft DNT testing battery (Chair: Susanne Hougaard Bennekou, Danish EPA; Rapporteur: Elissa Reaves, US-EPA).

There was a clear overall consensus among the workshop participants that DNT is a highly relevant toxicological measure, and that the amount of data generated to date is not sufficient to provide confidence on the safety of the thousands of untested chemicals to which pregnant women, infants and children may be exposed, nor to be informative or supportive of epidemiological observations on neurodevelopmental disturbances.

Since there is no current a priori requirement for DNT testing in the EU, there was a consensus of an urgent need for a problem formulation-driven, fit-for-purpose testing paradigm to supply data for risk assessment to support management decisions. Such a testing strategy should be developed and implemented to achieve two aims, conducted simultaneously. The first aim is to begin using the battery of currently available alternative test methods to generate data that could be used to prioritize chemicals for further testing. The second aim is to generate data that informs risk management decisions. Examples would include data on mechanisms of action, or data allowing refinement of
assessment groups in cumulative risk assessment. For this second aim, a solid scientific validation, the generation of OECD guidance and eventually the development of standard data requirements were considered necessary.

Priority for the first aim should be given, where possible, to a human cell-based testing battery covering basic neurodevelopmental processes to be put into place immediately for screening and prioritization purposes. Moving forward, the testing strategy should be flexible, able to incorporate new technologies, and adapt to the different problem formulations. The testing battery should be part of an IATA strategy, and the process should result in an OECD guidance document on DNT. Achieving the second aim will require testing of larger numbers of compounds (e.g., 200-300) as quickly as possible using the process-control strategy in multiple labs, in order to characterize assay performance and build confidence that the battery is suitable for regulatory purposes beyond prioritization and screening. In vitro testing must be transparent and standardized following guidance on good cell culture practice (Coecke et al., 2005), including stem cells and stem cell-derived models (Pamies et al., 2017), sharing of protocols and raw data.

This proposal to begin chemical testing for both aims has economic implications, with the highest priority being budget and funding activities. Different scientific organizations, representing regulatory authorities, academia and industry were unanimous in supporting this conclusion. Funding for increased testing of large numbers of chemicals and interlaboratory comparisons of assays is the necessary first step to move forward towards a modern, ethical and predictive new paradigm for testing any potential chemical associated with DNT, which is representing a relevant socio-economic, toxicological measure.

The final question put to the attendees was “Are we there yet? Do we currently have assays that are ready for chemical screening?” The consensus, based on published literature, the EFSA DNT review (Fritsche et al., 2015) and OECD report (Fritsche, 2016), and data presented by the speakers, indicated that the response to this question depends on the problem formulation. Thus, the answer was “yes” for screening and prioritization, since we have already reached a high level of confidence in a number of process-control based assays (Fig. 1). Conversely, the answer for other regulatory needs, such as replacement of animal testing or deriving health-based exposure limits, was “not yet” as one requires more confidence and less uncertainty in the alternative assays (Fig. 1). The task now is to establish performance standards and a testing strategy guidance for an in vitro DNT testing battery (consisting of in vitro methods and an alternative organism like the zebrafish), followed by challenging not the single assays, but the whole in vitro testing battery by compound testing.

Overall, the meeting was successful in generating productive discussions between regulators, academic scientists and industry that led to a consensus on the need, procedure and content of an alternative DNT testing strategy. This was an important step taken towards a novel and efficient DNT testing paradigm for regulatory purposes using in vitro methods. Thus, the original ideas brought up by the National Research Council of the US on the future of toxicology (NRC, 2007; Leist et al., 2008) and as outlined in a European roadmap (Leist et al., 2014) are now finally about to be applied to the field of DNT. As confidence in results increases, the domains of regulatory uses will increase and may convince risk managers to implement additional data requirements for DNT testing. The priority now is to establish a concise roadmap that defines the procedures and milestones of this mission.

References
Balmer, N. V., Weng, M. K., Zimmer, B. et al. (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, 4104-4114. https://doi.org/10.1093/hmg/ddr239
Balmer, N. V. and Leist, M. (2014). Epigenetics and transcriptomics to detect adverse drug effects in model systems of human development. *Basic Clin Pharmacol Toxicol* 115, 59-68. https://doi.org/10.1111/bcpt.12203
Bal-Price, A., Coecke, S., Costa, L. et al. (2012). Advancing the science of developmental neurotoxicity (DNT): Testing for better safety evaluation. *ALTEx* 29, 202-215. https://doi.org/10.14573/altex.2012.2.202
Bal-Price, A., Crofton, K. M., Leist, M. et al. (2015a). International STakeholder NETwork (ISTNET): Creating a developmental neurotoxicity (DNT) testing road map for regulatory purposes. *Arch Toxicol* 89, 269-287. https://doi.org/10.1007/s00204-015-1464-2
Bal-Price, A., Crofton, K. M., Sachana, M. et al. (2015b). Pata-
tive adverse outcome pathways relevant to neurotoxicity. Crit
Rev Toxicol 45, 83-91. https://doi.org/10.3109/10408444.20
14.98133
Barenys, M., Gassmann, K., Baksmeier, C. et al. (2016). Epigal-
locatechin gallate (EGCG) inhibits adhesion and migration of
neural progenitor cells in vitro. Arch Toxicol, in press. https://
doi.org/10.1007/s00204-016-1709-8
Baumann, J., Gassmann, K., Masjosthusmann, S. et al. (2016).
Comparative human and rat neurospheres reveal species dif-
fferences in chemical effects on neurodevelopmental key
events. Arch Toxicol 90, 1415-1427. https://doi.org/10.1007/
s00204-015-1568-8
Brown, J. P., Hall, D., Frank, C. L. et al. (2016). Evaluation of
a microelectrode array-based assay for neural network ontog-
ey using training set chemicals. Toxicol Sci 154, 126-139.
https://doi.org/10.1093/toxsci/kfw147
Coecke, S., Balls, M., Bowe, G. et al. (2005). Guidance on good
cell culture practice. A report of the second ECM ACM task
force on good cell culture practice. Altern Lab Anim 33, 261-
287. https://doi.org/10.1077/1-4020-5476-1 49
Culbreth, M. E., Harill, J. A., Freudenrich, T. M. et al. (2012).
Comparison of chemical-induced changes in proliferation and
apoptosis in human and mouse neuroprogenitor cells. Neurotoxicology 33, 1499-1510. https://doi.org/10.1016/j.
neuro.2012.05.012
Dorman, D. C., Allen, S. L., Byczkowski, J. Z. et al. (2001).
Methods to identify and chararcterize developmental neurotoxi-
icty for human health risk assessment. III: Pharmacoki-
netic and pharmacodynamic considerations. Environ Health Perspect 109, Suppl 1, 101-11. https://doi.org/10.1289/
ehp.01109s1101
Fritsche, E., Alm, H., Baumann, J. et al. (2015). Literature re-
view on in vitro and alternative developmental neurotoxicity
(DNT) testing methods. EFSA Support Publications 12, 4. 
https://doi.org/10.2903/sp.efsa.2015.EN-778
Fritsche, E. (2016). Report on integrated testing strategies for
the identification and evaluation of chemical hazards associ-
ated with the developmental neurotoxicity (DNT). In Report of
the OECD/EFSA workshop on developmental neurotoxicity
(DNT): The use of non-animal test methods for regulatory
purposes. OECD Environment, Health and Safety Publi-
cations Series on Testing and Assessment 242. ENV/JM/
MONO 63. https://www.efsa.europa.eu/sites/default/files/12.
FRITSCHE.pdf
Gassmann, K., Abel, J., Bothe, H. et al. (2010). Species-specific
differential AhR expression protects human neural progenitor
cells against developmental neurotoxicity of PAHs. Environ Health Perspect 118, 1571-1577. https://doi.org/10.1289/
ehp.0901545
Harrill, J. A., Freudenrich, T. M., Robinette, B. L. and Mundy,
W. R. (2011). Comparative sensitivity of human and rat neu-
cultures to chemical-induced inhibition of neurite out-
growth. Toxicol Appl Pharmacol 256, 268-80. https://doi.
org/10.1016/j.taap.2011.02.013
He, X., Imanishi, S., Sone, H. et al. (2012). Effects of methyl-
mercury exposure on neuronal differentiation of mouse and
human embryonic stem cells. Toxicol Lett 212, 1-10. https://
doi.org/10.1016/j.toxlet.2012.04.011
Hoelting, L., Klima, S., Karreman, C. et al. (2016). Stem cell-
derived immature human dorsal root ganglia neurons to iden-
tify peripheral neurotoxicants. Stem Cells Transl Med 5, 476-
487. https://doi.org/10.5966/scm.2015-0108
Kauffmann, W. (2003). Current status of developmental neuro-
toxicity: An industry perspective. Toxicol Lett 140, 161-169.
https://doi.org/10.1016/S0378-4274(02)00503-9
Krug, A. K., Balmer, N. V., Matt, F. et al. (2013). Evaluation of
a human neurite growth assay as specific screen for develop-
mental neurotoxicants. Arch Toxicol 87, 2215-2231. https://
doi.org/10.1007/s00204-013-1072-y
Lein, P., Silbergeld, E., Locke, P. and Goldberg, A. M. (2005).
In vitro and other alternative approaches to developmental
neurotoxicity testing (DNT). Environ Toxicol Pharmacol 19, 
735-744. https://doi.org/10.1016/j.etap.2004.12.035
Lein, P., Locke, P. and Goldberg, A. (2007). Meeting report:
Alternatives for developmental neurotoxicity testing. Envi-
ron Health Perspect 115, 764-768. https://doi.org/10.1289/
ehp.9841
Leist, M., Hartung, T. and Nicotera, P. (2008). The dawning
of a new age of toxicology. ALTEX 25, 103-114. https://doi.
org/10.14573/altext.2008.2.103
Leist, M., Hasiwa, N., Rovida, C. et al. (2014). Consensus re-
port on the future of animal-free systemic toxicity testing. 
ALTEX 31, 341-356. https://doi.org/10.14573/altext.1406091
McConnell, E. R., McClain, M. A., Ross, J. et al. (2012). Evalu-
ation of multi-well microelectrode arrays for neurotoxicity
screening using a chemical training set. Neurotoxicology 33,
1048-1057. https://doi.org/10.1016/j.neuro.2012.05.001
Mundy, W. R., Padilla, S., Breier, J. M. et al. (2015). Expanding
the test set: Chemicals with potential to disrupt mammalian
brain development. Neurotoxicol Teratol 52(Pt A), 25-35.
https://doi.org/10.1016/j.ntt.2015.10.001
NRC – National Research Council (2007). Toxicity Testing in
the 21st Century: A Vision and a Strategy. London: The Na-
tional Academies Press. https://doi.org/10.17226/11970
Nyffeler, J., Karreman, C., Leisner, H. et al. (2016). Design of
a high-throughput human neural crest cell migration assay to
indicate potential developmental toxicants. ALTEX 34, 75-94.
https://doi.org/10.14573/altext.1605031
Pamies, D., Bal-Price, A., Simeonov, A. et al. (2017). Good cell
culture practice for stem cells and stem-cell-derived models. 
ALTEX 34, 95-132. https://doi.org/10.14573/altext.1607121
Rempel, E., Höting, L., Waldmann, T. et al. (2015). A transcrip-
tome-based classifier to identify developmental toxicants by
stem cell testing: Design, validation and optimization for
histone deacetylase inhibitors. *Arch Toxicol* 89, 1599-1618. https://doi.org/10.1007/s00204-015-1573-y

Smirnova, L., Hogberg, H. T., Leist, M. and Hartung, T. (2014). Developmental neurotoxicity – challenges in the 21st century and in vitro opportunities. *ALTEX 31*, 129-156. https://doi.org/10.14573/altex.1403271

Stiegler, N. V., Krug, A. K., Matt, F. and Leist, M. (2011). Assessment of chemical-induced impairment of human neurite outgrowth by multiparametric live cell imaging in high-density cultures. *Toxicol Sci 121*, 73-87. https://doi.org/10.1093/toxsci/kfr034

Tsuji, R. and Crofton, K. M. (2012). Developmental neurotoxicity guideline study: Issues with methodology, evaluation and regulation. *Congenit Anom 52*, 122-128. https://doi.org/10.1111/j.1741-4520.2012.00374.x

Valdivia, P., Martin, M., LeFew, W. R. et al. (2014). Multi-well microelectrode array recordings detect neuroactivity of ToxCast compounds. *Neurotoxicology 44*, 204-217. https://doi.org/10.1016/j.neuro.2014.06.012

Zimmer, B., Lee, G., Balmer, N. V. et al. (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*, 1116-1122. https://doi.org/10.1289/ehp.1104489

Ellen Fritsche1,2, Kevin M. Crofton3, Antonio F. Hernandez4, Susanne Hougaard Bennekou5, Marcel Leist6, Anna Bal-Price7, Elissa Reaves3, Martin F. Wilks8, Andrea Terron9, Roland Solecki10, Magdalini Sachana11 and Anne Gourmelon11

1IUF – Leibniz Research Institute for Environmental Medicine, Duesseldorf, Germany; 2Heinrich-Heine-University, Duesseldorf, Germany; 3U.S. Environmental Protection Agency (EPA), Durham, USA; 4University of Granada, Spain; 5The Danish Environmental Protection Agency (EPA), Copenhagen, Denmark; 6CAAT – Centre for Alternatives to Animal Testing, University of Konstanz, Konstanz, Germany; 7European Commission – DG Joint Research Centre (JRC), Bruessels, Belgium; 8SCAHT – Swiss Centre for Applied Human Toxicology, University of Basel, Basel, Switzerland; 9European Food Safety Authority (EFSA), Parma, Italy; 10Federal Institute for Risk Assessment (BfR), Berlin, Germany; 11Organisation for Economic Co-operation and Development (OECD), Paris, France