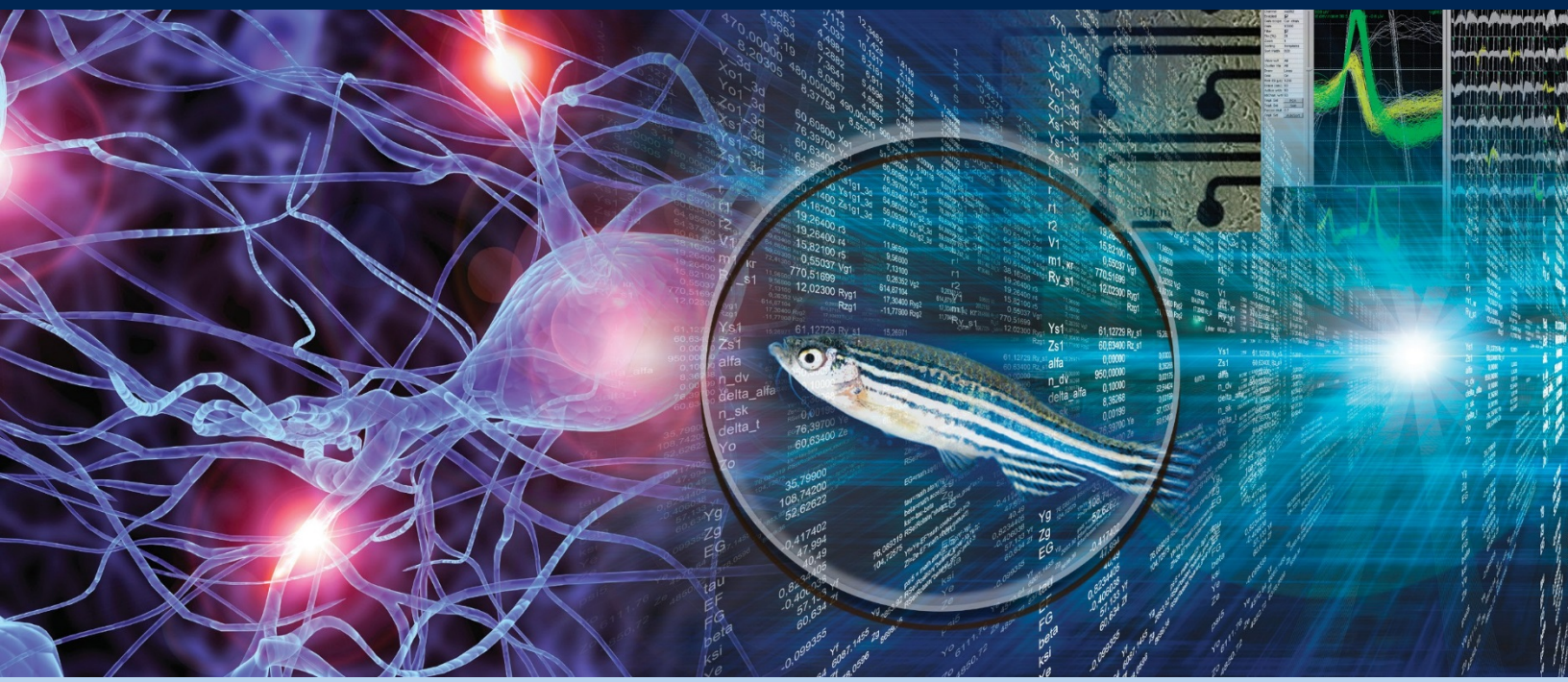
National Toxicology Program



Workshop Report on Integrated Testing Strategies for Developmental Neurotoxicity

TABLE OF CONTENTS

CONFIDENTIALITY

Materials made available to workshop participants as well as the discussions that take place during the workshop are confidential and may not be disclosed to or discussed with anyone who has not been officially designated to participate in the meeting. Participants are requested not to share the website username and password until notified otherwise by the NTP.

1. ABSTRACT

Over the past 5 years the NTP, through collaborations, has evaluated a set of medium throughput, high content, cell based assays and alternate animal models that capture critical neurodevelopmental processes, such as neuronal proliferation, differentiation/migration, functional network formation, cognitive behavior, and motor activity. The aim of this collaborative effort is to develop a test battery to evaluate the developmental neurotoxicity (DNT) potential of chemicals. As a culmination of the above efforts, the NTP held a closed workshop on *Integrated Testing Strategies for Developmental Neurotoxicity* from September 26-28, 2017, to promote and facilitate discussion and collaboration between workshop participants. This report, summarizes the discussions and outcomes of the workshop.

2. INTRODUCTION

Recent increases in the prevalence of neurological conditions such as autism spectrum disorder, attention deficit hyperactivity disorder, and cognitive deficits reflect a need to learn more about developmental neurotoxicity (DNT), and the potential role that environmental chemicals play in such disorders. There are approximately 10,000 inventoried chemicals in the US and less than 1% of these have data available on DNT (Judson et al., 2009; Makris et al., 2009). Currently, *in vivo* guideline DNT studies are usually triggered by evidence of neurotoxicity from other standard *in vivo* studies or structurally similar chemicals, and as a result many of the compounds in the environment with unknown DNT potential remain untested. Furthermore, guideline studies are time and resource intensive, and often do not identify subtle neurotoxic deficits (Tsuji & Crofton, 2012). Due to these reasons, there is growing consensus within the scientific, regulatory, and stakeholder community that there is a need to develop reliable and efficient screening approaches to prioritize chemicals with DNT hazards for further testing and to complement guideline studies. In parallel, the NTP was involved with the Tox21 program which was identifying the need for improving biological coverage and relevance (Phase III).

In response to these needs, and because the National Toxicology Program (NTP) receives a large number of nominations for toxicity testing, over the past 5 years the NTP evaluated a set of medium throughput high content cell based assays and alternate animal models through collaborations that capture critical neurodevelopmental processes, such as neuronal proliferation, differentiation/migration, functional network formation, cognitive behavior, and motor activity. If perturbed by exposure to developmental neurotoxicants, these processes are thought to be cause for concern for childhood neurodevelopmental disorders (Aschner et al., 2017).

As a culmination of the above efforts, the NTP held a closed workshop on *Integrated Testing Strategies for Developmental Neurotoxicity* from September 26-28, 2017, to promote and facilitate discussion and collaboration between workshop participants.

This workshop report summarizes the overall objectives, discussions, and outcomes of the workshop in the wider context of the neurotoxicity field.

3. SCOPE OF THE WORKSHOP

The NTP *Integrated Testing Strategies for Developmental Neurotoxicity* workshop took place from September 26-28, 2017, and brought together experts from academia, industry, government, and non-government agencies (see Appendix A for workshop agenda). The aim of the workshop was to discuss advancements and limitations of these models as screening tools, identify knowledge gaps, and examine data analysis approaches used to compare across different assays. Access to the workshop was limited so that participants could freely interact, and discuss their unpublished data in confidence.

The key objectives of the workshop were:

1. Solicit feedback on the utility of the battery approach to screen for potential DNTs
2. Determine the ability of individual assays to identify developmental toxicity or DNT
3. Identify knowledge gaps in the battery

4. Solicit feedback and discussion on NTP's data analysis strategy across assays in the battery
5. Discuss current challenges in comparing data across a battery of assays and potential ways to overcome them
6. Obtain regulatory perspective on the utility of a developmental/DNT battery approach for regulatory decision making
7. Provide an opportunity for researchers to freely exchange thoughts and ideas, and to discuss experiences with their data and how it compares with that of others
8. Form an ongoing "community" of scientists to move the frontier of alternatives to animal DNT/neurotoxicity testing

4. Chemical Library

Since few large sets of chemicals have been screened for functional DNT effects and data has not been analyzed consistently across assays, the NTP created and distributed a chemical library of 80 compounds (expanded to 91) to collaborators, who screened the compounds for activity in their respective assay. For more information on the chemical library see Appendix B. The chemical library consists of compounds where the chemical identity and purity is known. Included in the library are chemicals with known DNT or neurotoxic properties (38 compounds); duplicates to test the assay reproducibility (4 compounds); negative controls (i.e., compounds with no known neurotoxic effects, 5 compounds); and ~50% compounds with unknown toxic effects on the nervous system, some of which had concerns for DNT due to structural similarities with known DNTs or based on data on related chemicals within the same class. The chemicals were separated into categories as follows: drugs (19), flame retardants (15), industrial chemicals (15), PAHs (17), pesticides (18), and negative controls (5). Utilizing a standard library enables comparison of data across assays and chemical class. NTP pooled and analyzed screening data from the various *in vitro* and alternate animal model assays to evaluate the effects of chemicals and chemical classes across a variety of biological processes involved in neurodevelopment.

5. Participants

Invited participants attending the workshop included:

- Researchers from academia and the private sector
- Regulatory agencies from governmental and non-governmental organizations in North America and Europe, i.e., U.S. EPA, Health Canada, OECD, Danish EPA and the European Food Standards Agency
- Government researchers from the U.S. EPA and NIEHS/NTP

The full list of presenters and members of the NTP organizing committee is given in Appendix C.

6. Structure of the Workshop

The presentation and discussion sessions were grouped under the following themes:

1. ***In vitro* high content screens for neurotoxicity and developmental neurotoxicity**

2. **Comparative analysis and discussion of *in vitro* screens**
3. **Alternate animal models for developmental neurotoxicity**
4. **Comparative analysis and discussion of alternate animal models**
5. **Bringing it all together**
6. **Perspective from regulatory agencies on DNT evaluation**

During sessions one and three, which took place over the first two days of the workshop, researchers presented experimental methodologies and data on screening of the NTP chemical library in various *in vitro* and alternate animal models. *In vitro* cell-based assays covered certain aspects of neurodevelopment and neurodegeneration e.g., neuron outgrowth, neuron firing, cell migration, astrocyte senescence, and neuron protein aggregation. Assays in alternate animal models evaluated behavior and development in zebrafish and freshwater planarian. During sessions two and four, NTP staff presented their approach to managing, processing, and analyzing the screening data generated from the *in vitro* and alternate animal model assays, and applying *in vitro in vivo* extrapolation (IVIVE) techniques to the data. An NTP-led discussion session allowed for participants to ask questions and provide feedback on specific aspects of NTP's approach.

Presentations and discussions on day three focused on: 1) summarizing progress; 2) outlining the major outcomes and discussion points from the workshop; 3) placing the outcomes in the wider context of Tox21; 4) anticipating how a test battery may be used by regulatory agencies in decision making; and 5) discussing the experiences and activities happening internationally, e.g. at the EU and OECD level. A panel discussion with regulatory agencies concluded the workshop.

7. DISCUSSIONS AND CHALLENGES

7.1. *IN VITRO* AND ALTERNATE ANIMAL MODEL SCREENING ASSAYS

A. Experimental design

During both the discussion and question and answer session on *in vitro* and alternate animal model screening assays and comparative analysis, a number of points were raised with regards to experimental design and how it may have influenced the overall findings. In general, participants agreed on the importance of developing best practice guidelines for assays included in the test battery in order to improve reproducibility and reliability in the results.

Suggestions were made to include details of the interval between last dosing and testing (to distinguish between acute effects and DNT), data on overt toxicity (i.e., evaluating activity in visually normal animals rather than malformed animals), blinding during assessment, and statistical analysis. Another suggestion was made to zebrafish researchers to share images of characterized malformations to facilitate standardization. This will likely feed into other ongoing efforts by the NTP such as the Systematic Evaluation of the Application of Zebrafish in Toxicology (SEAZIT) project.

In vitro

Researchers used different concentration ranges and exposure times in their assays, which may have influenced the number of chemicals with positive responses. These elements of experimental design also have an influence on identifying neuro-specific effects and general toxicity (cell death), which the

participants agreed were important for screening. Also, different cell types (e.g., neurons and astrocytes) were found to respond differently to different chemicals and across different assays, resulting in variations in sensitivity and in the pattern of responses to chemicals. Different cell types may originate from various sources including rodent brain tissue (i.e., primary cultures), immortalized cell lines, or from the downstream differentiation of stem cells into specific neuronal cells. Hence, it is important to characterize the model. For stem cells, it was suggested that one may consider creating different cell lines from the same starting population to help reduce variability. It was also noted that the presence of more than one cell type in the system can give rise to different results; e.g., alterations in metabolism. As one potential solution, cell lines should be karyotyped to characterize the cell types growing in the system. Researchers also found variations in responses between male and female cell lines, allelic variability, and between species (e.g., cell lines derived from rat vs. human) and strain as is seen *in vivo* in rats. This discussion underscored the importance of diversity in models during screening.

Alternate animal models

Researchers shared the following observations on experimental design, which potentially influenced the measured outcomes:

- **Strain**: similar to studies in rodents, there was a discussion on the impact of strain of zebrafish on results.
- **Diet**: varying the diet affected the health and mortality of *Planaria*. Participants working with zebrafish also experienced this, and noted that work was ongoing to develop a fully defined diet for zebrafish to reduce variability and confounding.
- **Chorion**: depending on the protocol or assay, the chorion was either removed or kept intact. Whether this impacted chemical uptake was uncertain, but participants agreed that it was an important aspect to investigate.
- **Day and timing of the assay**: differences in locomotor activity and morphological effects were observed in assays with developing zebrafish, depending on the day and time of day on which the measurements were taken. One participant noted that in his experience, morphological evaluations taken after a longer time period following exposure (e.g., 120 hours-post-fertilization (hpf) rather than 96 hpf) increases sensitivity and reproducibility.
    - **Chemical removal**: To differentiate whether the compound had an acute neuroactive effect (e.g. sedation) or whether it was a developmental neurotoxicant, it was suggested that one may consider moving the animals to clean water (i.e., removing chemical exposure) prior to testing.
    - **Behavioral considerations**: There was a discussion on the number of days post fertilization at which behavior was measured and the implications on the results. Additionally, to reduce variability in locomotor activity, it was suggested to allow fish to "rest" for approximately 15 minutes prior to testing.

B. Biological coverage and relevance

In vitro

The *in vitro* assays presented at the workshop provide biological coverage of some of the key neurodevelopmental events; e.g., proliferation, outgrowth, migration, and formation of functional networks. While complete coverage is not necessary for an initial screening evaluation, a thorough assessment of how biologically representative the test battery is would help build confidence in the

outcomes. Some models to consider for future incorporation into the battery include early developmental effects on neural stem cells, models that capture 3-D structural changes such as neural tube closure (a critical defect in DNT), and incorporation of mixed cultures to include glia/astrocytes. While 3-D models for more complex developmental neurobiology processes are available, e.g., chick embryos and amphibian models, participants noted that adapting them to toxicity testing and high-throughput screening (HTS) would be a challenge. In addition to covering key events of neurodevelopment, the importance of defining the biological plausibility and relevance of the *in vitro* assays was discussed, since there are no direct links to endpoints *in vivo* and in humans.

Alternate animal models

The alternate animal models provide information on behavior, motor activity, and development, while being both rapid and cost effective. Many toxicological pathways seen in humans are conserved in zebrafish (e.g. ion channels and receptor sub-types), and their brains respond in a similar way to mammals. However, to use the zebrafish as a model to complement mammalian tests, other target organ toxicities expanding beyond teratology and locomotor activity should be incorporated. There was also discussion on time of exposure; and incorporating DNT studies where fish are exposed early (up to 5 days-post-fertilization (dpf)) and tested for effects later (during the juvenile period) in the absence of chemical.

C. Exposure and metabolism

In vitro

Participants agreed on the need to account for physico-chemical properties of the compound (e.g., volatility, lipophilicity), exposure, metabolism, and efflux in the *in vitro* assays. For example, in order to be able to relate *in vitro* concentrations to *in vivo* concentrations, the chemical concentration inside the cell versus how much sticks to the sides of the well/plastic should be considered. Incorporating models for the blood-brain and/or placental barrier in the DNT test battery was proposed to potentially screen out those compounds to which exposure of the developing nervous system is likely to be limited. Another participant informed the group that work to incorporate metabolism into HTS within the US EPA's HTS ToxCast program may influence the DNT field.

Alternate animal models

Zebrafish researchers acknowledged the need to relate internal doses to those in humans. Several researchers estimated chemical bioavailability and uptake in whole zebrafish embryos for a few compounds and noted that for the vast majority of compounds, little is known about how much chemical is getting into the fish or how much is transported out of cells. Participants agreed that more work needs to be done to evaluate chemical partitioning into the yolk sac to provide information on internal exposure. Similar to the *in vitro* assays, the material of the plate, the media used, whether the chorion was on or off, the log P of the compound, and exposure times were identified as important considerations. Uptake and metabolism in *Planaria* have not been investigated; however its importance was noted.

D. Utility of assays within a test battery

NTP presented an example of a NTP class nomination of about 10+ replacement flame retardants (FRs; organohalogens and aromatic phosphates) that are present in isomeric and chemical mixtures in commerce (resulting in over 50 individual isomers). This is a case example where all individual chemicals

cannot be tested by traditional DNT Guideline studies. Hence, a battery approach was used to evaluate the DNT potential of these FRs. The test battery included, among others, assays on *in vitro* neural crest migration, outgrowth and firing studies, and behavioral assays in zebrafish. In the battery, replacement FRs exhibited comparable activity to phased-out FRs, thereby suggesting a need for further in-depth hazard characterization for this class of compounds (Behl et al., 2015 and 2016; Jarema et al., 2015).

In vitro

Some of the DNT assays used to screen the NTP compound library are still in the assay development phase, require optimization, and/or are not amenable to HTS; however, participants agreed that these should not be discarded since they may be useful as a second-tier screen. Several participants noted that the most important criteria for an assay is to provide reliable and reproducible information that can be used to inform on the underlying biology and/or adverse outcome pathways (AOPs) of a chemical. Some examples include the incorporation of 2-D and 3-D mixed cultures, 'brain-on-a-chip', and models that incorporate metabolism.

Alternate animal models

It was noted that zebrafish are sensitive models for evaluating impacts on early development because this stage is highly conserved among vertebrate species. Hence, zebrafish assays are good tools for prioritization and prediction of developmental defects that occur relatively early in gestation. However, they may not be optimal if the toxicity resulting from a chemical exposure occurs late in gestation; for this purpose, other models need to be incorporated. There was also a discussion on the current use of ~ 5 dpf as the exposure duration, and the consideration to use different exposure windows. In response, it was mentioned that different exposure regimens could be used depending on the experimental questions that need to be addressed and that there is flexibility in how these assays may be conducted. Some participants suggested that to find alternate animal models and cell-based assays that evaluate common neurodevelopmental effects seen in mammals, the AOPs that lead to these effects first need to be identified. Several participants agreed that for risk assessment, alternate animal models could complement (but not yet replace) *in vivo* mammalian studies to predict adverse developmental and neurodevelopmental effects in humans. For example, alternate animal models have a role within an integrated testing strategy, and in the prioritization and selection of agents to be tested in mammals (including in the selection of endpoints such as time-windows for consideration in mammalian studies based on the nature of the toxicity noted).

7.2. DATA ANALYSIS

In advance of the workshop, NTP staff received datasets from nine labs invited to the meeting; researchers uploaded their raw and processed data in multiple different formats to NTPs database known as Chemical Effects in Biological Systems (CEBS) bins. NTP designed a database and pipeline to extract, transform and load the data into a single database for comparative analyses and discussions of the data across labs. Further, for some alternate animal models, aggregate endpoints were created to compare endpoints across labs in a similar format (for example, "fraction of embryos with developmental malformation" were aggregated from individual animal data). Data were then normalized to vehicle control variability, and analyzed using benchmark concentration (BMC) modelling approaches, which are already being utilized in in the HTS field and have been utilized in a regulatory setting (Ryan et al., 2016; Hsieh et al., 2015; Sirenko et al., 2013). In this workshop, the NTP used 3 x

standard deviation (SD) of the vehicle control response variation (after outlier removal) as the BMR (benchmark response). A website was created (https://sandbox.ntp.niehs.nih.gov/neurotox/, password protected) allowing workshop participants to analyze and compare their own data with that of others.

At the workshop, the NTP presented their approach to analyzing data from the *in vitro* assays, alternate animal model assays, and across the entire dataset. Discussions focused initially on the approach to data processing, i.e., whether outlier removal is appropriate and data normalization should be conducted.

Some of the key challenges raised during the discussion are highlighted below:

- Managing, formatting, and streamlining data for analysis
- Handling diversity in the experimental design, e.g., differences in concentrations tested, number of replicates and vehicle controls per plate, etc.
- Handling a large number of data points (approximately one million)
- Obtaining a BMR for alternate animal models, since this is the first time that a BMC has been applied to data from alternate animals
- Handling the higher variability in the alternate animal model data (specifically behavior) as compared to *in vitro* data when selecting a BMR
- Determining whether a statistically significant response is biologically relevant which can be defined by the individual research labs' historical experience and statistical methods that they apply based on their experience
    - More dialogue between statisticians and toxicologists is needed to better understand what is considered a biologically significant response
- Handling discrepancies in "active" (i.e., a positive response in a given assay) calls between NTP's and the laboratories' independent methods of analysis: in most cases, the results matched well, but there were some specific cases, when discrepancies were noted due to differences in the way that the data and the response thresholds were identified and analyzed.
- Identifying weak responses e.g., by analyzing multiple independent tests for a reproducible response

Participants identified a need for consensus on how to identify active compounds and on the statistical approaches used to evaluate data on an individual assay basis, and to compare results across the test battery. NTP presented the two statistical approaches for estimating BMCs: the parametric Hill model and non-parametric Curvep. These two models showed relatively good concordance in most cases, and diverged only in some cases at low BMC values. The NTP noted there might be a need to balance a streamlined approach with tailoring the analysis for each individual assay.

One of the considerations in future assays was the identification of tipping points, where cultured cells can no longer adapt or recover from chemical injury, and how this translates to *in vivo* concentrations and *in vivo* tipping points. A suggestion was made to evaluate metabolomics and transcriptomics data around the tipping point to inform on responses and pathways and how these relate to pathways in human disease. A critical component that would be required for this type of an analysis would be a time-course study.

There was a suggestion to check whether there is a batch effect in zebrafish behavior data (e.g., measurements are affected by experiment dates).

7.3. REGULATORY PERSPECTIVE

Among invited participants, there was an expert panel comprising representatives form national and international regulatory agencies that are working on DNT. The members of the panel provided some regulatory background to the workshop participants to consider how data from the DNT test battery could be used in decision making, and to discuss what further work needs to be done to make a DNT test battery acceptable. One panel member informed participants that currently, the guideline DNT study (OECD TG 426) is the gold standard for providing information on the potential effects of a compound on the developing brain. However, data quality and methodology vary according to the person conducting the study. One panel member remarked that data from a DNT test battery could be used as a larger weight of evidence to inform on a decision, but that it would not be used to generate a safety standard. The panel member from the OECD gave an overview of the work going on in this area at the OECD level. A scientific panel discussion organized by the European Food Safety Authority (EFSA) recommended developing a DNT battery of *in vitro* tests. EFSA released an external scientific report describing available *in vitro* methods, and held a workshop. A DNT expert group is now tasked with providing guidance on the integration of DNT *in vitro* tests into different regulatory decisions by the end of 2019. The OECD emphasized the incorporation of other aspects of DNT in addition to locomotor activity in zebrafish to include a measure for startle and learning & memory in future screens to more fully represent equivalent measures to rodent DNT guideline studies.

Participants were asked to consider the following questions in reference to the replacement FRs case study:

- If a compound/class is negative across most assays, what can we say about the compound/ class?
- If a compound/class of compounds shows positive effects across, what does it tell us?

The OECD panel member encouraged NTP to bring the FRs work to the OECD as a case example for DNT IATA to encourage dialogue on how the data may be used in decision making.

The panel discussed the importance of developing reliable, reproducible, and standardized assays with appropriate controls (identifying positive controls is a challenge) to improve utility and gain regulatory acceptance. Further, generating and evaluating data will build regulatory confidence in the battery by bringing about more familiarity and acceptance with these data streams. A panel member suggested consulting OECD guidance for describing non-guideline *in vitro* test methods to facilitate their consideration in regulatory applications (OECD, 2014) during the development of DNT assays. Also, integrating the test battery into a decision framework would enable parties to understand how the data can be used.

Panel members explained that a different level of confidence in the data is required for regulatory acceptance, and how the data could be used depends on which regulatory question is being asked. For example, test battery data on a new chemical is more likely to be acceptable than similar data on a data rich industrial chemical or on some pesticides. Also, if a compound is active in the test battery, that information could be used to trigger another targeted test at a dose relevant to humans. Regulatory agencies need quantifiable information that can be used in decision making. The emphasis was on generating high quality data independent of the throughput.

In terms of screening, regulatory agencies review the available data on a chemical, then if there are concerns or data gaps, the agency requests specific types of data/studies from the company/submitter. The burden of data generation is therefore on companies, which have a vested interest in the chemical. However, the burden of identifying which data needs to be generated to answer specific questions or fill gaps remains with the regulatory agency. For a DNT test battery to be used in this context, all the relevant parameters and metrics need to be defined.

Panel members identified the following questions that regulatory agencies would want to ask of a test battery:

- What does the data mean with regards to function?
- Does the battery contain all the potential AOPs?
- What are the limitations within the test battery i.e., what are the gaps in biological coverage?

Ultimately, data from a test battery would need to enable the regulatory agency to understand the underlying biology, AOPs and key events/target effect, so that it is then possible to move away from a one-size-fits-all approach to a targeted testing paradigm.

8. OUTCOMES AND NEXT STEPS

The NTP created a website (https://sandbox.ntp.niehs.nih.gov/neurotox/, password protected) where researchers can visualize their raw data, as well as the resulting NTP analysis of their data, and compare their data with other assays in the battery to get a better understanding of the range of effects shown by a diverse set of chemicals. The participants were delighted to see this resource, and wished to make it public after the primary publications are complete. Therefore, we plan on making the website publicly available without password protection at the same time the primary publications for the NTP workshop are released; the estimated timeline for the website to go public is 1 year.

There was interest expressed by some at the workshop regarding expanding the functionality of the website, including enabling the ability to upload new datasets to compare to existing datasets. Based on level of effort and resources required, the NTP will internally evaluate the scope of expansion.

Some of the main discussion themes and challenges are summarized as follows:

- Addressing volatility, exposure (blood/brain, placental barriers) and metabolism factors in the *in vitro* assay battery, and exposure and metabolism in the alternate animal models.
- Identifying which assays are the most reliable and/or sensitive.
- Identifying key experimental parameters in individual assays that influence reproducibility and developing best practice guidelines or standardized assays.
- Identifying the optimal approach to aggregate, compare, and analyze test battery results.
- *In vitro, in vivo* extrapolation (IVIVE) extrapolation
- Assessing whether the battery sufficiently covers the key events in neurodevelopment, and captures the complexity, for the purposes of screening.
- Understanding the biological relevance of outcomes in the test battery in terms of human disease.

- Understanding and integrating the needs and requirements of regulatory agencies into the development of the DNT test battery.
- Building regulatory confidence in the test battery e.g., by identifying AOPs within the data to better link the assay outcomes to biological relevance, validating assays, and participating in open dialogue and involving end users.

8.1. NEXT STEPS

Further work needs to be done to establish which assays are the most reliable and sensitive, and what questions we need to ask to be able to achieve this. For example, do we base sensitivity on which assays pick up the highest number of active chemicals or the most potent? Data on more chemicals will be needed to answer these questions. NTP is continuing to analyze the data and encourage collaboration between researchers, and one of the immediate next steps is to prepare the data and comparative analysis for publication. NTP will continue to explore IVIVE approaches as they become available. NTP has been in communication with the editor of *Toxicological Sciences*, who has shown a high level of interest and support for presenting output from this workshop in a special issue. All participants are encouraged to submit research papers based on their work. Following the primary publications, NTP will take the lead in putting together a comparative analysis manuscript. Concurrent with the publication of the *Toxicological Science* special issue, NTP plans on publicly releasing the NTP website for the neurotoxicity workshop, to share the data and methods used for the NTP integrated analysis presented at the workshop.

9. PUBLICATIONS

1. Aschner M, Ceccatelli S, Daneshian M, Fritsche E, Hasiwa N, Hartung T, Hogberg HT, Leist M, Li A, Mundi WR, Padilla S, Piersma AH, Bal-Price A, Seiler A, Westerink RH, Zimmer B, Lein PJ. (2017) Reference compounds for alternative test methods to indicate developmental neurotoxicity (DNT) potential of chemicals: example lists and criteria for their selection and use. ALTEX. 34(1):49-74.
2. Behl M, Rice JR, Smith MV, Co CA, Bridge MF, Hsieh JH, Freedman JH, Boyd WA. (2016). Editor's Highlight: Comparative Toxicity of Organophosphate Flame Retardants and Polybrominated Diphenyl Ethers to Caenorhabditis elegans. Toxicol. Sci. Dec;154(2):241-252.
3. Behl M, Hsieh JH, Shafer TJ, Mundy WR, Rice JR, Boyd WA, Freedman JH, Hunter ES 3rd, Jarema KA, Padilla S, Tice RR. (2015). Use of alternative assays to identify and prioritize organophosphorus flame retardants for potential developmental and neurotoxicity. Neurotoxicol. Teratol. Nov-Dec;52(Pt B):181-93.
4. Hsieh JH, Sedykh A, Huang R, Xia M, Tice RR. (2015). A Data Analysis Pipeline Accounting for Artifacts in Tox21 Quantitative High-Throughput Screening Assays. J. Biomol. Screen. Aug;20(7):887-97.
5. Jarema KA, Hunter DL, Shaffer RM, Behl M, Padilla S. Acute and developmental behavioral effects of flame retardants and related chemicals in zebrafish. (2015). Neurotoxicol. Teratol. Nov-Dec;52(Pt B):194-209.

6. Judson, RS, Richard, A, Dix, DJ, Houck, K, Martin, M, Kavlock, R, Dellarco, V, Henry, T, Holderman, T, Sayre, P. (2009).The toxicity data landscape for environmental chemicals. Environ. Health Perspect. 117, 685–695.
7. Makris, SL, Raffaele, K, Allen, S, Bowers, WJ, Haas, U, Alleva, E, Calamandrei, G, Sheets, L, Amcoff, P, Delrue, N, et al. (2009). A retrospective performance assessment of the developmental neurotoxicity study in support of OECD Test Guideline 426. Environ. Health Perspect. 117, 17–25.
8. NTP (2004). A National Toxicology Program for the 21st Century: A Roadmap for the Future. Available at: https://ntp.niehs.nih.gov/ntp/about_ntp/ntpvision/ntproadmap_508.pdf
9. OECD (2014). Guidance Document for Describing Non-Guideline in Vitro Test Methods. OECD Environment, Health and Safety Publications. Series on Testing and Assessment No. 211. No. 211ENV/JM/MONO(2014)35. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2014)35&doclanguage=en
10. Pei Y, Peng J, Behl M, Sipes NS, Shockley KR, Rao MS, Tice RR, Zeng X. (2016). Comparative neurotoxicity screening in human iPSC-derived neural stem cells, neurons and astrocytes. Brain Res. May 1;1638(Pt A):57-73.
11. Ryan KR, Sirenko O, Parham F, Hsieh JH, Cromwell EF, Tice RR, Behl M. (2016). Neurite outgrowth in human induced pluripotent stem cell-derived neurons as a high-throughput screen for developmental neurotoxicity or neurotoxicity. Neurotoxicology. Mar 53:271-281.
12. Sirenko O, Cromwell EF, Crittenden C, Wignall JA, Wright FA, Rusyn I. (2013). Assessment of beating parameters in human induced pluripotent stem cells enables quantitative in vitro screening for cardiotoxicity. Toxicol. Appl. Pharmacol. 273(3):500-507.
13. Tsuji R, Crofton KM. (2012). Developmental neurotoxicity guideline study: issues with methodology, evaluation and regulation. Congenit Anom (Kyoto). Sep;52(3):122-8

APPENDIX A: WORKSHOP AGENDA

Tuesday, September 26

8:00 AM **Meet shuttle at hotel for ride to NIEHS (For workshop presenters)**

8:30 AM **Arrive at NIEHS and complete registration**

In vitro high content screens for neurotoxicity

Moderators: Mamta Behl | *NIEHS/NTP*
Johanna Nyffeler | *University of Konstanz*

9:00 AM **Welcome**
Mamta Behl | *NIEHS/NTP*

9:05 AM **Introductory remarks**
Linda Birnbaum | *NIEHS/NTP, Director*

9:10 AM **Workshop: Historical perspective**
Raymond Tice | *NIEHS/NTP, Retired*

9:25 AM **Workshop overview**
Mamta Behl | *NIEHS/NTP*

9:45 AM ***In vitro* neurotoxicity assessment of environmental chemicals using an organotypic human induced pluripotent stem cell-derived model**
Oksana Sirenko | *Molecular Devices*

10:05 AM **Break**

10:30 AM **Assessment of neurite outgrowth in LUHMES and stem cell derived peripheral neurons**
Tanja Waldmann | *University of Konstanz*

10:50 AM **Migration of human neural crest cells as functional endpoint to screen for developmental neurotoxicity**
Johanna Nyffeler | *University of Konstanz*

11:10 AM **Using iPSC derived cells for toxicology assays**
Mahendra Rao | *XCell Science Inc.*

11:30 AM **A cell based screen of neurotoxicants for induction of ALS-linked TDP-43 pathology**
Benjamin Wolozin | *Boston University*

11:50 AM **Development of a screen for the identification of neurotoxins capable of inducing senescence**
Julie Andersen | *Buck Institute for Research on Aging*

12:10 PM	**Lunch in NIEHS cafeteria**
1:10 PM	**Screening compounds for potential developmental neurotoxicity using assays for proliferation, neurite outgrowth, synaptogenesis and network formation** Timothy Shafer | *U.S. EPA*

Comparative analysis and discussion of in vitro screens

Moderators: Timothy Shafer | *U.S. EPA*
Kristen Ryan | *NIEHS/NTP*

1:40 PM	**Introduction to the NTP's data analysis approach** Kristen Ryan | *NIEHS/NTP*
1:50 PM	**"Where's my data"? "What did you do with it?"** Andy Shapiro, Jui-Hua Hsieh, Frederick Parham | *NIEHS/NTP*
2:20 PM	**Discussion on NTP's comparative analysis approach**
3:00 PM	**Break**
3:20 PM	**Comparative analysis of *in vitro* assays** Andy Shapiro, Jui-Hua Hsieh, Frederick Parham | *NIEHS/NTP*
3:45 PM	**Discussion *on comparison between in vitro assays***
4:45 PM	**Group photo**
5:00 PM	**Adjourn Day 1**
5:00 PM	**Shuttle back to hotel (For workshop presenters)**
6:30 PM	***Optional* - Presenters to enjoy dinner and network at a local restaurant**

Wednesday, September 27

8:30 AM	**Meet shuttle at hotel for ride to NIEHS (For workshop presenters)**

Alternate animal models for developmental neurotoxicity

Moderators: Arantza Muriana | *Biobide*
Vicki Sutherland | *NIEHS/NTP*

9:00 AM	**Workshop overview and goals for Day 2** Mamta Behl | *NIEHS/NTP*
9:10 AM	**Alternative animal models in comparison to conventional mammalian approaches for the evaluation of developmental toxicity** Paul Foster | *NIEHS/NTP*

9:30 AM	**Multi-dimensional assessment of chemical activity using a high throughput early zebrafish system** Robert Tanguay | *Oregon State University*
9:50 AM	**Teratological and behavioral screening of the NTP compound library in zebrafish larvae** Katharina Dach | *University of California, Davis*
10:10 AM	**Break**
10:35 AM	**Screening the NTP library for neurotoxic potential using zebrafish** Arantza Muriana | *Biobide*
10:55 AM	**Evaluation of the freshwater planarian Dugesia japonica as an alternative animal model for medium-throughput developmental neurotoxicology studies** Eva-Maria Collins | *University of California, San Diego*
11:15 AM	**Survival and teratogenic evaluation of 91 compounds** Javier Terriente | *ZeClinics*
11:35 AM	**Prioritizing compounds for DNT based on an early developmental model for zebrafish** Stephanie Padilla | *U.S. EPA*
11:55 AM	**Lunch in NIEHS cafeteria and poster set-up**

Comparative analysis and discussion of alternate animal models

Moderators: Mamta Behl \| *NIEHS/NTP*
Robert Tanguay \| *Oregon State University*

1:00 PM	**Comparative analysis on alternate models** Andy Shapiro, Jui-Hua Hsieh, Frederick Parham | *NIEHS/NTP*
1:30 PM	**Discussion on the comparative analysis of alternate animals**
2:20 PM	**Break**
2:40 PM	**Discussion on comparing across *in vitro* and alternate animal models**
3:40 PM	**Applying *in vitro* in vivo extrapolation (IVIVE) methods to the data** Nisha Sipes | *NTP/NIEHS*
4:00 PM	**Break**
4:15 PM	**Poster session**
5:30 PM	**Adjourn Day 2**
5:30 PM	**Shuttle back to hotel (For workshop presenters)**

Thursday, September 28

8:30 AM **Meet shuttle at hotel for ride to NIEHS (For workshop presenters)**

Bringing it all together

Moderator: Elizabeth Maull | *NIEHS/NTP*

9:00 AM **How does this effort fit with Tox21?**
Richard Paules | *NIEHS/NTP*

9:20 AM **What did we hear so far? Summary and recap of discussions**
Kristen Ryan | *NIEHS/NTP*

9:35 AM **Utilizing a battery approach to prioritize compounds and complement guideline DNT testing: Are we there yet?**
Mamta Behl | *NIEHS/NTP*

9:55 AM **Break**

Perspective from Regulatory Agencies on DNT Evaluation

Moderators: Paul Foster | *NIEHS/NTP*
Nigel Walker | *NIEHS/NTP*

10:15 AM **Minding the gap: Ideas for better integration of alternative DNT testing and risk assessment.**
Francis Bailey | *Health Canada*

10:35 AM **New OECD expert group on DNT: Working towards the development of a guidance on interpretation of *in-vitro* DNT data for use in an integrated approach to testing and assessment**
Magdalini Sachana | *OECD, Environment Health and Safety Division*

10:55 AM **The DNT: A regulator's perspective**
Elizabeth Mendez | *U.S. EPA*

11:15 AM **Break**

11:30 PM **Discussion: What have we heard and what did we learn?**
Francis Bailey | *Health Canada*
Kevin Crofton | *U.S. EPA*
Susanne Hougaard Bennekou | *Danish EPA/EFSA*
Elizabeth Mendez | *U.S. EPA*
Magdalini Sachana | *OECD*

12:30 PM **Wrap-up and next steps**
Mamta Behl | *NIEHS/NTP*

12:45 PM **Closing Remarks**
Paul Foster | *NIEHS/NTP*

12:50 PM Adjourn Day 3

12:50 PM Shuttle to airport or hotel (For workshop presenters)

APPENDIX B: CHEMICAL LIBRARY

CASRN	Chemical name	Category
848641-69-0	1-Ethyl-3-methylimidazolium diethylphosphate	Industrial
36913-39-0	1-Methyl-4-phenylpyridinium iodide	Drug*
1241-94-7	2-Ethylhexyl diphenyl phosphate (EHDP)	Flame Retardant
183658-27-7	2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB)	Flame Retardant
109-86-4	2-Methoxyethanol	Industrial*
5436-43-1	2,2',4,4'-Tetrabromodiphenyl ether	Flame Retardant
60348-60-9	2,2',4,4',5-Pentabromodiphenyl ether (BDE-99)	Flame Retardant
68631-49-2	2,2',4,4',5,5'-Hexabromodiphenyl ether (BDE-153)	Flame Retardant
1746-01-6	2,3,7,8-Tetrachlorodibenzo-p-dioxin	Industrial
111-94-4	3,3'-Iminodipropionitrile	Industrial*
79-94-7	3,3’,5,5’-Tetrabromobisphenol A	Flame Retardant
203-64-5	4-H-Cyclopenta(d,e,f)phenanthrene	PAH
51-21-8	5-Fluorouracil	Drug*
28094-15-7	6-Hydroxydopamine hydrochloride	Drug*
51-52-5	6-Propyl-2-thiouracil	Drug*
83-32-9	Acenaphthene	PAH
208-96-8	Acenaphthylene	PAH
103-90-2	Acetaminophen (4-hydroxyacetanilide)	Negative
638-38-0	Acetic acid, manganese (2+) salt	Industrial*
50-78-2	Acetylsalicylic acid	Negative
79-06-1	Acrylamide	Industrial*
116-06-3	Aldicarb	Pesticide*
26787-78-0	Amoxicillin	Drug
120-12-7	Anthracene	PAH
2465-27-2	Auramine O	Industrial
56-55-3	Benz(a)anthracene	PAH
50-32-8	Benzo(a)pyrene	PAH
205-99-2	Benzo(b)fluoranthene	PAH
192-97-2	Benzo(e)pyrene	PAH
207-08-9	Benzo(k)fluoranthene	PAH
191-24-2	Benzo[g,h,i]perylene	PAH
633-65-8	Berberine chloride	Drug
26040-51-7	Bis(2-ethylhexyl) 3,4,5,6- tetrabromophthalate (TBPH)	Flame Retardant
56-35-9	Bis(tributyltin)oxide	Pesticide*
80-05-7	Bisphenol A	Industrial*
1478-61-1	Bisphenol AF	Industrial
80-09-1	Bisphenol S	Industrial
133-06-2	Captan	Pesticide
55406-53-6	Carbamic acid, butyl-, 3-iodo-2-propynyl ester	Pesticide
63-25-2	Carbaryl	Pesticide*

CASRN	Chemical name	Category
2921-88-2	Chlorpyrifos (Dursban)	Pesticide*
218-01-9	Chrysene	PAH
64-86-8	Colchicine	Drug*
50-70-4	D-Glucitol	Negative
52918-63-5	Deltamethrin	Pesticide*
117-81-7	Di(2-ethylhexyl) phthalate	Industrial*
439-14-5	Diazepam	Drug*
53-70-3	Dibenz(a,h)anthracene	PAH
215-58-7	Dibenz[a,c]anthracene	PAH
50-29-3	Dichlorodiphenyltrichloroethane (DDT)	Pesticide*
60-57-1	Dieldrin	Pesticide*
56-53-1	Diethylstilbestrol	Drug*
50-28-2	Estradiol	Drug
860302-33-6	Firemaster 550	Flame Retardant
86-73-7	Fluorene	PAH
76-44-8	Heptachlor	Pesticide*
70-30-4	Hexachlorophene	Drug*
127-07-1	Hydroxyurea	Drug*
29761-21-5	Isodecyl diphenyl phosphate	Flame Retardant
50-81-7	L-Ascorbic acid	Negative
6080-56-4	Lead (II) acetate trihydrate	Industrial*
58-89-9	Lindane	Pesticide*
12108-13-3	Manganese, tricarbonyl[(1,2,3,4,5-.eta.)-1-methyl-2,4-cyclopentadien-1-yl]-	Industrial*
115-09-3	Methyl mercuric (II) chloride	Pesticide*
110-54-3	n-Hexane	Industrial*
91-20-3	Naphthalene	PAH
56-38-2	Parathion	Pesticide*
52645-53-1	Permethrin	Pesticide*
85-01-8	Phenanthrene	PAH
50-06-6	Phenobarbital	Drug*
57-30-7	Phenobarbital sodium salt	Drug*
68937-41-7	Phenol, isopropylated, phosphate (3:1)	Flame Retardant
129-00-0	Pyrene	PAH
83-79-4	Rotenone	Pesticide*
82385-42-0	Saccharin Sodium Salt hydrate	Negative
107534-96-3	Tebuconazole	Pesticide*
56803-37-3	tert-Butylphenyl diphenyl phosphate	Flame Retardant
97-77-8	Tetraethylthiuram disulfide	Drug*
50-35-1	Thalidomide	Drug*
108-88-3	Toluene	Industrial*
1330-78-5	Tricresyl phosphate	Flame Retardant
115-86-6	Triphenyl phosphate	Flame Retardant
115-96-8	Tris(2-chloroethyl) phosphate	Flame Retardant

CASRN	Chemical name	Category
13674-84-5	tris(Chloropropyl) phosphate, TCPP	Flame Retardant
2001-95-8	Valinomycin	Drug
1069-66-5	Valproic acid sodium salt	Drug*

* Compound is a known developmental neurotoxicant from literature

† Compound was tested by specific labs and not provided by the NTP

APPENDIX C: CONTRIBUTORS

Ainhoa Alzualde
Biobide Bionaturis Group
alzualde@biobide.es

Julie Andersen
Buck Institute for Research on Aging
jandersen@buckinstitute.org

Peter Ash
Boston University
peterash@bu.edu

Francis Bailey
Health Canada
francis.bailey@canada.ca

Stanley Barone
U.S. EPA
barone.stan@epa.gov

Mamta Behl
NIEHS/NTP
mamta.behl@nih.gov

Linda Birnbaum
NIEHS/NTP
linda.birnbaum@nih.gov

Bradley Collins
NIEHS/NTP
bradley.collins@nih.gov

Eva-Maria Collins
University of California, San Diego
emszcollins@gmail.com

Kevin Crofton
U.S. EPA
crofton.kevin@epa.gov

Katharina Dach
University of California, Davis
kdach@ucdavis.edu

Paul Foster
NIEHS/NTP
foster2@niehs.nih.gov

Eduardo Gonzalez
University of California, Davis
azgonzalez@ucdavis.edu

Danielle Hagstrom
University of California, San Diego
dhagstrom@ucsd.edu

Susanne Hougaard Bennekou
Danish EPA/ EFSA
suhou@mst.dk

Jui-Hua Hsieh
NIEHS/NTP
jui-hua.hsieh@nih.gov

Pamela Lein
University of California, Davis
pjlein@ucdavis.edu

Elizabeth Mendez
U.S. EPA
mendez.elizabeth@epa.gov

Rafael Miñana Prieto
ZeClinics
rafael.minana@zeclinics.com

Arantza Muriana
Biobide Bionaturis Group
muriana@biobide.es

Johanna Nyffeler
University of Konstanz
johanna.nyffeler@uni-konstanz.de

Stephanie Padilla
U.S. EPA
padilla.stephanie@epa.gov

Frederick Parham
NIEHS/NTP
fred.parham@nih.gov

Richard Paules
NIEHS/NTP
paules@niehs.nih.gov

Ying Pei
Xcell Science Inc.
ypei@xcellscience.com

Mahendra Rao
Xcell Science Inc.
mrao@xcellscience.com

Elissa Reaves
U.S. EPA
reaves.elissa@epa.gov

Kristen Ryan
NIEHS/NTP
kristen.ryan@nih.gov

Magdalini Sachana
OECD
magdalini.sachana@oecd.org

Timothy Shafer
U.S. EPA
shafer.tim@epa.gov

Andy Shapiro
NIEHS/NTP
andy.shapiro@nih.gov

Nisha Sipes
NIEHS/NTP
nisha.sipes@nih.gov

Oksana Sirenko
Molecular Devices
oksana.sirenko@moldev.com

Robert Tanguay
Oregon State University
robert.tanguay@oregonstate.edu

Javier Terriente
ZeClinics
javier.terriente@zeclinics.com

Raymond Tice
NIEHS/NTP
tice@niehs.nih.gov

Lisa Truong
Oregon State University
lisa.truong@oregonstate.edu

Tanja Waldmann
University of Konstanz
tanja.waldmann@uni-konstanz.de

Nigel Walker
NIEHS/NTP
walker3@niehs.nih.gov

Benjamin Wolozin
Boston University
bwolozin@bu.edu

Georgia Woods
Buck Institute for Research on Aging
gwoods@buckinstitute.org

APPENDIX D: SPEAKER ABSTRACTS

Tuesday, September 26

Historical Perspective

Raymond Tice | *NIEHS/NTP, Retired*

In 2008, the National Institute of Environmental Health Sciences /National Toxicology Program (NTP) entered into an agreement with the Environmental Protection Agency (EPA)/National Center for Computational Toxicology and the National Human Genome Research Institute (NHGRI)/NCATS Chemical Genomics Center (NCGC) on "high-throughput screening, toxicity pathway profiling, and biological interpretation of findings." In 2010, the U.S. Food and Drug Administration joined the agreement, known informally as Tox21. In Phase I (Proof of Principle; 2005-2010), the NCGC screened 1408 compounds (1353 unique) from NTP and 1462 compounds (1384 unique) from EPA in 140 quantitative high-throughput screening (qHTS) assays representing 77 cell-based reporter gene endpoints. In Phase II "Expanded Compound Screening" (2011-current), a 10K compound library is being screened in qHTS assays focused on nuclear receptor activation/inhibition and induction of cellular stress response pathways. Recognizing the limitations of the approach, in 2013, we initiated Phase III "Improving on Biological Coverage and Relevance", to focus on more physiologically-relevant in vitro cell systems (e.g., induced pluripotent stem cells differentiated cell populations); cell types (e.g., HepaRG) that incorporate xenobiotic metabolism; alternative animal models (e.g., zebrafish); and the development of a high-throughput transcriptomics platform for human, rat, mouse, zebrafish. Supporting Phase III was a decision to make available a diverse set of compounds (e.g., neurotoxicants, flame retardants, polycyclic aromatic hydrocarbons) to distribute via material transfer agreements to investigators with platforms of interest. This workshop is the culmination of that effort.

In vitro neurotoxicity assessment of environmental chemicals using an organotypic human induced pluripotent stem cell-derived model

Oksana Sirenko | *Molecular Devices*

Due to the increasing prevalence of neurological disorders possibly related to exposure to environmental toxicants, there is a need to develop reliable and efficient screening tools to identify environmental chemicals that could potentially affect human health. There is great interest in using stem cell derived cell models for *in vitro* high-throughput quantitative assays that would allow for detecting the potential hazard of chemicals and prioritizing them for further testing. We developed phenotypic screening assay testing neuronal toxicity using imaging methods. A set of 80 compounds were screened in a high-throughput high-content neurite outgrowth assay using induced pluripotent stem cells-derived human neurons. The library contained a diverse set of compounds including chemicals associated with developmental neurotoxicity (DNT) and neurotoxicity, such as polycyclic aromatic hydrocarbons and flame retardants. Effects on neurite outgrowth and cytotoxicity were assessed by measuring total outgrowth, branches, processes, and viable cells per well. Concentration-response profiles were evaluated using a Hill model to derive effective concentration values. Compounds were then ranked by activity and selectivity. 38 compounds were found active, of which 16 selectively inhibited neurite outgrowth parameters. This strategy is useful for rapidly identifying, ranking, and prioritizing compounds with DNT potential for further *in vivo* characterization.

Assessment of neurite outgrowth in LUHMES and stem cell derived peripheral neurons

Tanja Waldmann | *University of Konstanz*

Neurite outgrowth is a major process involved in development and is affected in developmental neurotoxicity. We developed *in vitro* tests using human developing central nervous system (NeuriTox test) and peripheral nervous system (PNS) (PeriTox test) neurons and screened the National Toxicology Program's (NTP) 80-compound library to identify toxicants interfering with neurite outgrowth. LUHMES can be rapidly differentiated into dopaminergic neurons by switching off myc expression and are used in the NeuriTox test. The PeriTox neurons are differentiated from hESC via neural crest cells state into PNS neurons. In both test methods the cells are seeded in 96-well plates and treated for 24 hours. After treatment, viable cells and neurite mass were quantified by high content analysis. The NTP80-library was screened in three independent experiments starting with a highest concentration of 20 µM. Compounds were considered as a hit if the ratio EC50(viability)/EC50(neurite area) was > 4 (NeuriTox test) or > 3.3 (PeriTox test). After the hit-confirmation phase seven compounds were defined as specifically neurotoxic and nine were generally cytotoxic in the NeuriTox test. Additionally, the library was screened in the PeriTox test. Rotenone, colchicine, diethylstilbestrol, berberine and valinomycin were hits in both systems. The unique NeuriTox hit, MPP^+ is known from *in vivo* studies to affect only dopaminergic neurons. Conversely, the known peripheral neurotoxicant acrylamide was found in the PeriTox, but not in the NeuriTox assay. Testing of this library showed that the tests are suitable for screening purposes, that there is a high hit confirmation rate, and that the tests allow sorting of toxicants according to potency.

Migration of human neural crest cells as functional endpoint to screen for developmental neurotoxicity

Johanna Nyffeler | *University of Konstanz*

Introduction: Neural crest cells (NCCs) are a transient stem cell population arising at the time of neurulation. NCCs migrate to various body parts and differentiate into cells of the peripheral nervous system, melanocytes, craniofacial bones and other structures. Therefore, failure of NCC to migrate can lead to severe developmental defects, so called neurocristopathies.

Aim: We developed an in vitro test using human NCCs and screened an 80-compound library of compounds (assembled by the National Toxicology Program, (NTP)) to identify toxicants interfering with NCC migration.

Methods: NCCs were differentiated from pluripotent stem cells. For the cMINC migration assay, cells were seeded in plates containing silicon stoppers to create a cell-free circular area. Removal of the stoppers allowed the start of migration. After 48 hours, the number of viable cells in the circular zone was quantified by high-content image analysis. Compounds were present during the last 24 hours. The NTP80-library was screened in three independent experimental runs at the highest non-cytotoxic concentration (≤ 20 µM). Compounds causing > 20% migration inhibition were re-ordered for confirmation and concentration-response curves were obtained.

Results: Internal quality controls lead to consistent results and all negative controls were correctly classified. The screen yielded 26 hits, of which 23 were confirmed. The hits comprised 10 flame retardants, 7 pesticides and 6 drug-like compounds. Comparison of concentration-response curves for migration and viability showed that all hits were specific. The extent to which migration was inhibited was 25-90%, and two organochlorine pesticides (DDT, heptachlor) were most efficient. In the second part of this study, (i) the cMINC assay was repeated under conditions that prevent proliferation; (ii) a transwell migration assay was used as a different type of migration assay; (iii)

cells were traced to assess cell speed. Some toxicants had largely varying effects among those assays, but each hit was confirmed in at least one additional test.

Conclusions: Testing of this library showed that the test is suitable for screening purposes, that there is a high hit confirmation rate, and that the test allows sorting of toxicants according to potency. NCCs were found to be particularly sensitive to environmental contaminants. The organochlorine and organophosphorus compounds especially deserve detailed future investigation.

Using iPSC derived cells for toxicology assays

Mahendra Rao | *XCell Science Inc.*

xCell Science generates induced pluripotent stem cell (iPSC) lines, engineered iPSC lines and patient specific iPSC lines and provides them to the research community. XCell differentiates these lines to generate neurons, astrocytes and oligodendrocytes. These cells are widely used by the research community for screening assays including toxicology based screens. We have used two male and two female lines to assess the effects of several compounds at different doses and their effect on neural stem cells, the precursor cells that represent the stem cells of the developing brain. We provide data to show that there are measurable differences in response to different compounds based on cell type and stage of differentiation and sex. Our results suggest that developing a data base of responses in well characterized widely available lines will be important for the toxicology community, and making the cells available as reference material will be equally important.

A cell based screen of neurotoxicants for induction of ALS-linked TDP-43 pathology

Benjamin Wolozin | *Boston University*

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder of motor neurons. The majority (~90%) of the ALS cases are sporadic, with environmental toxicants implicated as risk factors. The hallmark pathology of ALS is cytoplasmic aggregates composed of TAR DNA binding protein (TDP-43). TDP-43 is an RNA-binding protein that forms cytoplasmic aggregates to control response of RNA metabolism to stress.

This study sought to identify environmental toxicants that increase aggregation of TDP-43. The assay involved the screening the National Toxicology Program's NeuroTox 91 panel in rat PC12 cells stably expressing doxycycline-inducible TDP-43::EGFP. Cells were treated with four different doses of each toxicant for 18 hours and followed by quantification of TDP-43::EGFP inclusions using the GE In Cell analyzer 2000.

Four toxicants (lead (II) acetate trihydrate, methyl mercuric (II) chloride, bis(tributyltin)oxide, and colchicine) increased TDP-43 aggregates. Repeat analysis of the toxicants also induced TDP-43 inclusions. The follow up studies are using cultured primary neurons.

These results indicate that environmental toxicants can induce TDP-43 aggregation in cell lines similar to that observed in ALS, suggesting an approach for identifying toxicants that are putative risk factors for ALS. Epidemiological studies provide support for the role of these compounds as risk factors for ALS.

Development of a screen for the identification of neurotoxins capable of inducing senescence

Julie Andersen | *Buck Institute for Research on Aging*

I will discuss a novel means by which neurotoxicants may contribute to neurodegeneration—neurotoxicant-induced astrocytic senescence. Cellular senescence is a potent tumor suppressor mechanism resulting in proliferative arrest. Recent evidence suggests that senescent cells

contribute to age-related disease, including Parkinson's disease (PD); our own unpublished data demonstrate that the environmental herbicide paraquat (PQ) can induce astrocyte senescence that contributes to PD-associated neuropathology. I will describe a screening method we developed for identifying compounds similar to PQ, capable of inducing senescence of induced pluripotent stem cell-derived astrocytes, (based on their ability to significantly increase the activity of the senescent marker, senescent-associated beta-gal). Preliminary results suggest 10 putative astrocytic senescence-inducing neurotoxicants identified from the 80 neurotoxicant compound library used for the initial proof-of-principle screen. Presentation of our findings at the developmental neurotoxicity workshop will allow the opportunity to learn from experts in the field in order to: (1) improve the practical implementation of our screen and 2) establish the best course for validating our preliminary hits.

Screening compounds for potential developmental neurotoxicity using assays for proliferation, neurite outgrowth, synaptogenesis and network formation

Timothy Shafer | *U.S. EPA*

The Shafer laboratory uses primary cultures of rat cortical neurons grown on 48-well microelectrode array plates to assess the ability of compounds to interfere with development of neural network function. We have tested the 87 compound set of National Toxicology Program compounds in this assay by exposing cells from 2 hours after plating through day *in vitro* 12. Compounds are tested at 7 different concentrations (typically 0.03-30 μM), plus vehicle treated controls. Development of activity is monitored by recording from the same network at days *in vitro* 5, 7, 9, and 12. The goal is to identify compounds that alter development of functional neural networks. Since neural network activity is complex, we typically extract 17 different parameters related to network activity, and use the area under the curve to determine IC50 values for effects of chemicals on each metric. Examples of these parameters include those related to overall activity (spike rate, burst rate, number of active electrodes), burst structure (burst duration, interburst interval, number of spikes in a burst) and network connectivity (network bursts, synchrony, correlation co-efficient). We also assess overall health of the cells in order to separate effects on network development vs cytotoxicity.

Wednesday, September 27

Alternative animal models in comparison to conventional mammalian approaches for the evaluation of developmental toxicity

Paul Foster | *NIEHS/NTP*

This presentation will provide a short review of the major elements of developmental toxicity (embryo-fetal death, structural malformations, growth retardation, and functional deficits) together with an overview of how the National Toxicology Program (NTP) routinely studies developmental toxicity with both pre- and post-natal end points in mammals. Comparisons will be made of the conventional mammalian approach to the potential role of alternative animal species and where they would fit in an overall testing strategy. Finally, mention will be made of other NTP efforts in the refinement of a testing list for developmental toxicants that could be used in the evaluation of alternative methods.

Multi-dimensional assessment of chemical activity using a high throughput early zebrafish system

Robert Tanguay | *Oregon State University*

The Tanguay lab has developed a tier one screening protocol using early life stage zebrafish to detect and classify developmentally toxic chemicals across broad test concentration ranges. The initial goal is to identify chemicals that perturb normal developmental processes including assessment of two motor responses and 22 individual morphological endpoints. The experimental protocol uses dechorinated zebrafish embryos statically exposed beginning at 6 hours-post-fertilization (hpf) in individual sealed 96-well plates. The exposed zebrafish are kept in the dark and all studies are completed by 120 hpf. This assay was used to evaluate the National Toxicology Program library containing 91 suspect developmental neurotoxicants. The chemicals were delivered using precise HP D300e digital dispensing, and normalized to 0.64% dimethyl sulfoxide. A total of eight concentrations (up to 67 μM) with 36 animals/concentration were evaluated on triplicate plates. Of the 91 tested chemicals, 42 produced a significant increase in the occurrence of malformations or mortality compared to controls. For the embryonic photomotor response, 31 of the test chemicals were classified as hits and in the larval photomotor response, 67 of the 91 compounds were statistically different from the controls. This assay and the summary of the results will be discussed.

Teratological and behavioral screening of the NTP compound library in zebrafish larvae

Katharina Dach | *University of California, Davis*

Zebrafish are an attractive model for addressing the paucity of information regarding the toxic risk posed by most of the chemicals to which the developing human brain may be exposed.

Here, we used dechorionated Tropical 5D wildtype zebrafish embryos to screen the National Toxicology Program's 91-compound library for developmental neurotoxicity. Embryos were exposed to 5 concentrations of each chemical up to a maximum of 30 μM or to an equal amount of vehicle (0.5% dimethyl sulfoxide) in embryo media from 6 hours-post-fertilization (hpf) to 5 days-post-fertilization (dpf). During this time period, embryos were kept at 28°C under a 14-hour light and 10-hour dark cycle. Embryos were examined daily for malformations and mortality until euthanized at 5 dpf. Developmental neurotoxicity was assessed at 4 and 5 dpf using a light-dark locomotor behavioral assay.

Of the 55 chemicals screened so far, 42% increased mortality/malformations and 47% caused behavioral deficits. The chemicals screened to date included 15 flame retardants, of which 9 caused mortality/malformations and 7 led to behavioral abnormalities. All 5 negative controls had no effect on these endpoints. Chemicals provided in duplicates produced similar outcomes.

In future studies, positive hits will be further tested using the currently available transgenic zebrafish lines in the Lein lab to look for effects on specific neurodevelopmental processes, such as apoptosis and ratio of excitatory/inhibitory neurons.

Screening the NTP library for neurotoxic potential using zebrafish

Arantza Muriana | *Biobide*

Teratogenic and neurotoxic potential of 91 test compounds from the National Toxicology Program were assessed by determining their toxic effects in zebrafish embryos. First, maximum tolerated concentration was assessed. For this purpose, zebrafish embryos at 3-5 hours-post-fertilization (hpf) were treated with test items at 5 concentrations and embryo viability was evaluated at days 2 and 4 post-fertilization. Afterwards, chemicals were tested at 8 or 5 concentrations. Evaluation of developmental defects was also performed at 2 and 4 days-post-fertilization (dpf). EC50 and LC50 were calculated applying a nonlinear regression and a teratogenic index was calculated as the ratio between LC50 and EC50. Larvae from this experiment treated at the highest concentration without effect and the first toxic concentration were also used for internal dosing analysis to determine the

real concentration at which toxic effects were induced. Moreover, concentration of chemicals in the medium was also evaluated in the same experimental groups. Finally, for neurotoxicity determination, zebrafish larvae at 3 dpf were treated with test items at five concentrations and the analysis of the locomotor activity was performed after 48 hours of treatment.

Evaluation of the freshwater planarian Dugesia japonica as an alternative animal model for medium-throughput developmental neurotoxicology studies

Eva-Maria Collins | *University of California, San Diego*

The Collins lab is using the asexual freshwater planarian Dugesia japonica to test the 87 compound library for effects on neurodevelopment. Brain development is induced through amputation, because head regeneration is the only mode of neurodevelopment in this asexual species. A unique feature of this system is that we can test adult (intact) and developing/regenerating animals in parallel, which we hope will enable us to identify toxic effects that are specific to (neuro)development. To this end, we evaluate survival, animal morphology (shape and number of eyes) and a variety of behavioral endpoints to test nervous system function. Behavioral endpoints include unstimulated behaviors (gliding locomotion and percent time resting) and stimulated behaviors (phototaxis, thermotaxis, response to noxious heat). Because these behaviors depend on the function of neuronal subpopulations, we hope that they will enable us to dissect different types of neurotoxicity. As use of the planarians for developmental neurotoxicology screening is fairly new, we are excited to use this workshop to compare our planarian screening platform to other, more established systems to evaluate its strengths and weaknesses.

Survival and teratogenic evaluation of 91 compounds

Javier Terriente | *ZeClinics*

ZeClinics is a contract research organization/biotech company interested in developing efficient and reliable zebrafish screening tests to predict compound toxicity (general and organ related). Our aim inside the National Toxicology Program (NTP) consortium is to define a universal set of rules (incubation time, timing and type of end phenotypes, analysis procedure, etc.) that can be applied to all the zebrafish toxicology community (standard operating procedure like) and, eventually, to become the base for applying towards regulatory approval for the standardized test. In this study, zebrafish embryos were exposed to the NTP 91-compound list at on a 5 Log3 concentration curve. Endpoints evaluated included survival and teratogenic phenotypes such as body deformity, scoliosis, pigmentation and heart edema. It is important to note that compounds were already provided in dimethyl sulfoxide, which limited the maximum concentration range tested. In that regard, 49/91 compounds did not show any toxic phenotype at the maximum evaluated concentration. On the other hand, 39 showed mortality and teratogenic phenotypes. Among them, the most toxic compounds were Saytex CP-2000, 4,4-hexafluoroisopropylidene diphenol, 3-lodo-2-propynyl n-butylcarbamate, diethylstilbestrol, hexachlorophene, methylmercury chloride, rotenone and tetraethylthiuram disulfide.

Prioritizing compounds for DNT based on an early developmental model for zebrafish

Stephanie Padilla | *U.S. EPA*

The U.S. Environmental Protection Agency is evaluating methods to screen and prioritize chemicals for developmental neurotoxicity. We are exploring behavioral methods using zebrafish by designing a behavioral testing paradigm capable of assessing the effects of sublethal and sub-teratogenic concentrations of developmental neurotoxicants. The behavioral paradigm simultaneously tests 96 individual 6 day old zebrafish under both light and dark conditions in a multiwell plate using a video tracking system. By controlling the duration and intensity of light, we

are able to assess changes in locomotion during light-dark transitions, and adaptation to both light and dark. This format allows evaluation of large numbers of larvae, chemicals and concentrations. Using this paradigm, we have tested a set of chemicals that are considered positive or negative controls for eliciting developmental neurotoxicity in mammals. We have found that many developmentally neurotoxic compounds perturb behavior at sub-teratogenic doses, while many developmentally non-neurotoxic compounds do not perturb behavior. Exposure to developmental neurotoxicants may alter the overall level of activity in light and dark conditions and/or the pattern of activity. Therefore, results showed that careful behavioral evaluation of zebrafish larvae may be able to identify some mammalian developmental neurotoxicants. This abstract may not necessarily reflect official Agency policy.

Thursday, September 28

How does this effort fit with Tox21?

Richard Paules | *NIEHS/NTP*

Significant advances in toxicology have been achieved through the initial efforts of the first 10 years of the U.S. Tox21 Federal Collaboration. The National Toxicology Program (NTP) is striving to incorporate Tox21 approaches into our toxicological characterizations of chemicals to better define the potential adverse effects of exposures on human health. The NTP is engaged in various Tox21 joint collaborations in the new plan for moving Tox21 forward and in NTP-specific Tox21 projects that include the following: 1) evaluating in vitro models designed to developmental, neuronal and developmental neurotoxicities in responses to chemical exposures, 2) evaluation of embryonic zebrafish models in understanding adverse effects of chemicals, 3) evaluating in vitro models designed to capture population variance in responses to chemical exposures due to genetic differences, 4) using the S1500+ gene set in high-throughput quantitative transcriptomic screens of human liver organoid models to evaluate the role of physiologically-relevant xenobiotic metabolism on responses to chemicals, and 5) developing and incorporating in vitro to in vivo extrapolation approaches to build better linkage between in vitro findings and actual risks to human health from chemical exposures. Important in the success of these projects is the development and use of sets of Reference Chemicals in order to benchmark the performance characteristics of these New Alternative Methods for use in regulatory and health care decisions.

Utilizing a battery approach to prioritize compounds for further DNT testing: Are we there yet?

Mamta Behl | *NIEHS/NTP*

The potential for neurotoxicity following exposure to environmental chemicals remains a high public priority due to concerns that recent increases in the prevalence of neurological disorders may in part be due to chemical effects. Thus, it is well-recognized that there is a need for reliable and efficient screening tools to identify, prioritize, and evaluate chemicals for their potential to induce acute neurotoxicity in adults or developmental neurotoxicity (DNT). To address this, the National Toxicology Program (NTP) created a 80/91-chemical library comprising a diverse set of compounds (e.g., neurotoxicants, flame retardants, polycyclic aromatic hydrocarbons) that was made available to researchers interested in evaluating high-throughput, high content cell-based, and alternate animal model assays for development, DNT, and neural activity. One of the major challenges in comparing results across different assay platforms is the choice of a common metric to assess chemical effects. Researchers typically use disparate methods of analysis thereby making it difficult to compare findings across assay platforms. To address this concern, the NTP adopted a benchmark concentration-like approach to be able to compare biological activities across the

various assays. This talk will discuss case-examples of how the NTP evaluated a battery of assays, highlighting successes and challenges we faced along the way.

Minding the gap: Ideas for better integration of alternative DNT testing and risk assessment

Francis Bailey | *Health Canada*

The rodent developmental neurotoxicity (DNT) study paradigm has evolved over time with the most recent test guidelines updated in 2007 with the introduction of Organization for Economic Co-operation and Development test guideline 426. Over the past 6 years, a joint United States Environmental Protection Agency (U.S. EPA)-Health Canada, Pest Management Regulatory Agency (HC PMRA) intergovernmental group has been working to develop a document to serve as internal guidance for regulatory reviewers in both countries.

From a HC PMRA perspective, this initiative has been undertaken to provide better context to key parameters necessary for the review of an in vivo DNT study, not only for the individual behavioral tests, but for their integration into the weight of evidence for the entire study and for the ultimate assessment of hazard and risk.

Although the focus of the guidance development has been on the in vivo assay, the H.C. PMRA has been monitoring international progress on in vitro testing strategies and has participated in dialogue with other regulators and researchers, to provide perspective on the potential impacts of such strategies on a data-rich risk assessment paradigm. In vitro DNT studies have not yet greatly impacted the pesticide risk assessments in Canada; this notwithstanding, there are challenges that remain in translating the results from in vitro DNT assays to meaningful points of departure for risk assessment. In order to increase confidence in integrating the data into the regulatory process, it is crucial that data is quantifiable, that the results from in vitro studies simulate or incorporate the impact of whole organism exposures on the compounds in question, and be validated against DNT compounds from in vivo scenarios.

New OECD expert group on DNT: Working towards the development of a guidance on interpretation of in-vitro DNT data for use in an integrated approach to testing and assessment

Magdalini Sachana | *OECD, Environment Health and Safety Division*

A new Organization for Economic Co-operation and Development (OECD) Expert Group on developmental neurotoxicity (DNT) has recently been formed and is currently seeking to expand and include more experts in *in vitro* DNT testing. A satisfactory geographical coverage has already been achieved, having experts from North America, Europe and Asia. The Expert Group will work towards the development of an OECD Guidance Document on interpretation of *in vitro* DNT data that potentially can be applied in an integrated approach to testing and assessment (IATA). This project was proposed by the European Food Safety Authority (EFSA) and was strongly supported by all the member countries and builds on the OECD/EFSA workshop on DNT that took place in October 2016. Additional background scientific documents for the development of the Guidance will come out after the completion of a new funded project supported by EFSA. The leads of the Expert Group are currently working to identify more resources to support the project and allocate drafting roles. The Guidance will focus on capturing available *in vitro* assays intended to explore nodal processes in neuronal development. A testing battery will be proposed that would rely on a set of complementary alternative assays intended to cover biological processes critical for normal neurological development. The document will provide fit-for-purpose guidance on the use of data from alternative test methods within the IATA driven by the regulatory problem formulation. At least two cases will be developed; the use of the testing battery for prioritization and screening, and

the use of the testing battery for single substance hazard identification, which will employ performance based criteria for validation. The project is expected to officially start in November 2017 and be finalized by the end of 2019.

The DNT: A regulator's perspective

Elizabeth Mendez | *U.S. EPA*

Historically, the U.S. Environmental Protection Agency's Office of Pesticide Programs (OPP) has used a variety of studies including the developmental neurotoxicity (DNT) to assess for the developmental neurotoxicity potential of pesticides. The DNT is a Tier 2 study and may be required using a weight-of-evidence (WOE) approach, if the pesticide (1) causes treatment-related neurological effects in adult animal studies, (2) causes treatment-related neurological effects in developing animals, following pre- and postnatal exposure, (3) the pesticide elicits a causative association between exposures and adverse neurological effects in human epidemiological studies, (4) the pesticide evokes a mechanism that is associated with adverse effects on the development of the nervous system, or (5) evidence of neurotoxicity occurs at dose levels close to the point of departure for the risk assessment.

These considerations are all based on the *in vivo* rodent model. However, the agency may request alternative studies (*in vivo*, in silico, high throughput) if it considers they would better address the regulatory needs. While no single assay will be sufficient to use for regulatory purposes (e.g., triggering an *in vivo* DNT study), alternative studies may be used as part of the WOE to triggering a guideline DNT, tailor a study design or; help elucidate a chemical's mode of action.