Conference Report

# Developmental Neurotoxicity (DNT): A Call for Implementation of New Approach Methodologies for Regulatory Purposes: Summary of the 5th International Conference on DNT Testing

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Received March 19, 2025;


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ALTEX 42(2), 323-349.
doi:10.14573/altex.2503191

## Abstract

The 5th International Conference on Developmental Neurotoxicity (DNT) Testing (DNT5) took place in April 2024 in Konstanz, Germany, organized by CAAT-Europe, the University of Konstanz, and scientists from the US EPA, SCAHT, and CAAT at Johns Hopkins University Bloomberg School of Public Health. The conference convened experts from regulatory agencies, industry, and academia to explore the latest advancements in DNT testing and the integration of animal-free new approach methodologies (NAMs) into next-generation risk assessment (NGRA). The key topic was the application and further development of the recently established DNT *in vitro* test battery (DNT-IVB). To support this, OECD held a satellite meeting to discuss necessary next steps for further implementation of the DNT-IVB in regulatory contexts. Validation of new DNT test methods and use of their data for *in-vitro-to-in-vivo* extrapolations in physiologically based kinetic models were also important themes of the main meeting. In this context, the question was raised when a comprehensive biological and chemical coverage by the DNT-IVB would be reached. A need for additional testing data was recognized. Context-specific validation approaches for the entire DNT-IVB and the potential for intelligent combinations of assays to enhance the predictive power of the test battery were also

addressed. Many presentations demonstrated the field's embrace of novel developments, including the use of multi-endpoint embryonic zebrafish tests, the development of artificial intelligence-driven computational approaches, and the establishment of complex, electrically active brain organoids and other self-organizing structures. Through its highly interactive format, DNT5 promoted extensive collaborative efforts in advancing the field toward more human-relevant, scientifically reliable, and ethical toxicological assessments.

**Plain language summary**

The 5th International Conference on Developmental Neurotoxicity (DNT) Testing (DNT5) took place in April 2024 in Konstanz, Germany. Experts from regulatory agencies, industry, and academia convened to discuss how best to integrate animal-free new approach methodologies (NAMs) into next-generation risk assessment. The key topic was the application and further development of the recently established DNT *in vitro* test battery (DNT-IVB). The use of data from the DNT-IVB was a central theme. For instance, it was discussed how data from cell culture methods could be used to predict safe human exposures. Moreover, the conference addressed the need for comprehensive biological and chemical coverage by the DNT-IVB. Many presentations demonstrated the field's embrace of novel developments, including the use of multi-endpoint embryonic zebrafish tests, the development of artificial intelligence-driven computational approaches, and the establishment of complex, electrically active brain organoids and other self-organizing structures.

## 1 Setting the stage and introductory keynote

### 1.1 Background

The DNT5 conference[1] was held in Konstanz, Germany, from April 7-10, 2024[2], to define and discuss actions needed to improve the development of time-efficient, human-relevant predictive *in vitro* developmental neurotoxicity (DNT) methods and to boost their use for risk assessment and regulatory decision making[3]. This followed a tradition of pivotal workshops organized in Konstanz to support DNT testing. For instance, outcomes of previous meetings have included a repository of control compounds and criteria to judge assay readiness (Aschner et al., 2017; Bal-Price et al., 2018).

The DNT5 conference convened diverse stakeholders from around the globe, including research scientists, regulators, industry representatives, academics, and representatives of non-governmental organizations (Fig. 1). At symposia, interactive workshops, keynote speakers, and poster sessions, participants discussed several relevant topics, including:

- Recent applications of new approach methodologies (NAMs)
- Refinement, exemplification, and implementation of the DNT *in vitro* test battery (DNT-IVB)
- Novel screening strategies and projects
- Current understanding of human DNT mechanisms
- Linking epidemiology to testing and regulations
- Regulatory risk-assessment strategies

Human brain development involves an intricate and dynamic series of events that begins in early embryogenesis with the formation of the neural tube. Neural stem cells differentiate into progenitor cells, which give rise to neurons and glial cells. Key neurodevelopmental processes (KNDPs), such as neuronal proliferation, migration, synaptogenesis, and myelination occur during this period. These processes are tightly regulated by genetic factors and a complex signaling network. However, these processes are also particularly sensitive to environmental perturbations, including chemical exposures, which can lead to alterations in brain development (Consortium for Children's Environmental Health, 2025). DNT refers to adverse effects on the developing nervous system from toxic substances during prenatal and early postnatal periods, potentially leading to cognitive deficits, behavioral disorders, and increased risks of neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) (Grandjean and Landrigan, 2006; Kuehn, 2010; Landrigan, 2010; Sagiv et al., 2010; Rossignol et al., 2014). The increasing prevalence of some neurodevelopmental disorders has heightened concerns about the role of early chemical exposures, emphasizing the need for effective DNT testing strategies to identify and mitigate potential risks (Eriksson, 1997; Olney, 2002; King and Bearman, 2009; Grandjean and Landrigan, 2014; Hansen et al., 2015; Abbasi et al., 2016; Kwan, 2020; Maenner et al., 2023).

Historically, DNT testing has relied heavily on animal models, particularly rodents. However, in addition to raising ethical concerns, traditional *in vivo* DNT tests are expensive, resource intensive, and time consuming (Smirnova et al., 2014). As a result,

[1] **Disclaimer:** This article reports on a multi-stakeholder meeting and its individual presentations. It is not a consensus statement, and opinions of individual presenters are not necessarily shared by all authors. Moreover, the presentation summaries compiled in this article should not be understood as views of the institution of employment of the presenters. For instance, there were significant differences in opinion regarding the use of the zebrafish embryo test. Some industries, particularly cosmetics, are unable to use animal testing and moreover do not support testing on vertebrates at any life stage.

[2] https://www.uni-konstanz.de/dnt5/about-the-conference

[3] Conference program: https://tinyurl.com/4tz7ks4a

## 5th International Conference on Developmental Neurotoxicity Testing (DNT5)

*7-10th April 2024, Konstanz, Germany*

www.uni-konstanz.de/dnt5/

**Fig. 1: Group photo of conference participants**

DNT assessment according to regulatory guideline studies (e.g., OECD TG 426) has been performed for less than 1% of the thousands of chemicals – including industrial chemicals, pesticides, and consumer products – in use today (Grandjean and Landrigan, 2006, 2014; Aschner et al., 2017). Furthermore, animal models do not always accurately predict human outcomes, leading to potential gaps in safety assessments. In response to these limitations, the scientific community has increasingly turned to NAMs, particularly *in vitro*, *in silico*, and non-mammalian models.

In parallel to bottom-up academic projects to change DNT safety evaluations, a new NAM-based testing strategy has also been promoted by, for example, the European Food Safety Authority (EFSA) in Europe, and the Environmental Protection Agency (EPA)[4,5] and the National Institute of Environmental Health Sciences (NIEHS) in the USA. Moreover, the Organisation for Economic Co-operation and Development (OECD)[6] held a satellite meeting to the DNT5 conference in Konstanz to discuss further implementation of the DNT-IVB. Another key player in the process is industry: several large companies use NAMs for internal prioritization and/or investigative toxicology. In addition, several small/medium enterprises (SMEs) are emerging that offer DNT testing, for instance in assays of the original or extended DNT-IVB (e.g., DNTOX[7], Steinbeis CAAT-Europe[8], Neurosetta[9]), in zebrafish embryos[10], using artificial intelligence (AI)-driven EmbryoNet analysis[11] (Čapek et al., 2023), or as part of integrated NAMs/modelling packages (SaferWorldbyDesign[12]).

4 https://www.epa.gov/chemical-research/epa-new-approach-methods-work-plan-reducing-use-vertebrate-animals-chemical
5 https://www.epa.gov/system/files/documents/2021-11/nams-work-plan_11_15_21_508-tagged.pdf
6 https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/in-vitro-assays-for-developmental-neurotoxicity.html
7 https://dntox.de/
8 bit.ly/406YoHq
9 https://neurosetta.com/
10 https://biobide.com/
11 http://embryonet.de/
12 https://saferworldbydesign.com/

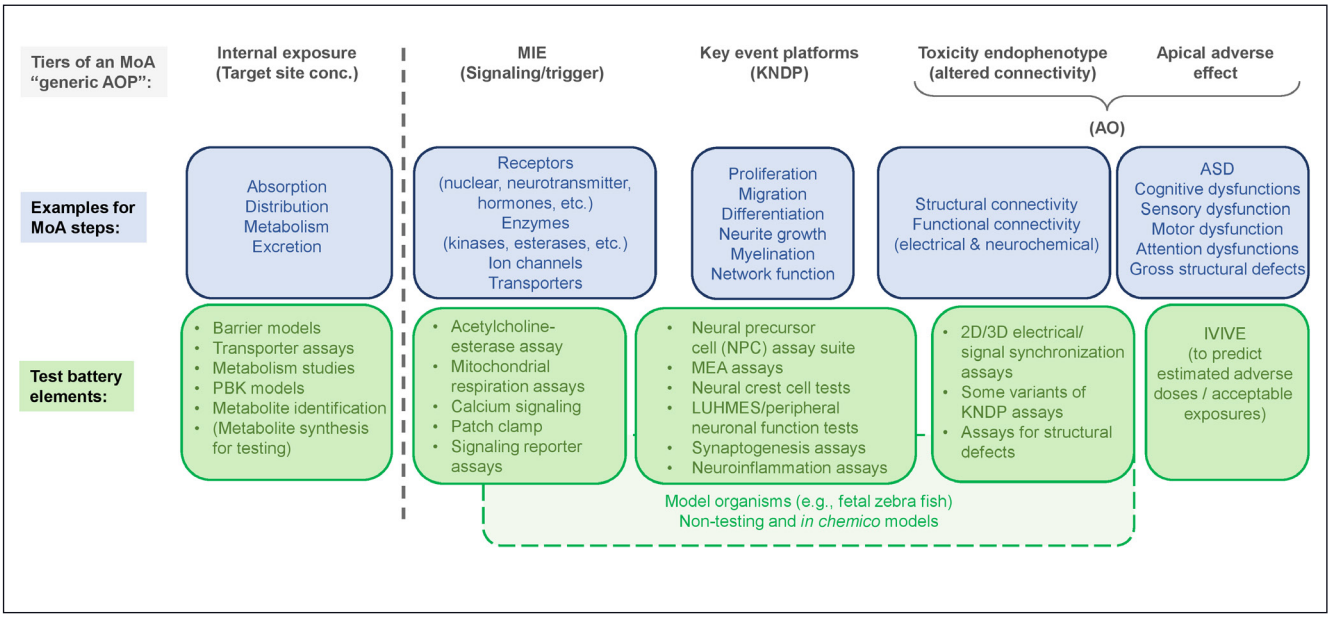


**Fig. 2: Overview of the sequence of events leading to DNT and potential DNT assessment methodologies**
Headings (top) indicate steps within a putative mode of action (MoA) for a DNT toxicant. The structure roughly resembles that of an AOP, in that it includes hierarchical levels of biological organization. However, the MoA exemplifies the activity of chemicals, while AOPs are compound-agnostic and do not include a toxicokinetic module. The scheme does not represent a given sequence of biological processes, but rather an assembly of most steps that may lead to various adverse outcomes (AOs). The second row gives examples of the steps in a MoA. The first step (separated by the dashed line) comprises all processes that determine how a chemical is "handled" by the body. It results in a time-concentration profile of the test chemical in various parts of the nervous system at a given dose/exposure. Key neurodevelopmental processes (KNDPs) have a central position in the scheme: in AOP terminology, they may be seen as superordinate KEs (or as an assembly of KEs). All disturbances in KNDPs may lead to altered structural or functional connectivity. This would represent an internal AO or toxicity endophenotype (TEP), which differs from the external (apical) AO by two major characteristics: (i) it may be directly linked to KNDP dysfunctions and therefore be predicted by KNDP assays, and (ii) it may not be easily observed by classical approaches (i.e., behavioral/functional testing of individual human subjects). Nevertheless, the TEP is always assumed to underlie such external symptoms. The third line exemplifies how NAMs and other methodologies contribute to the detection and quantification of chemical actions/effects. Strictly speaking, the first box mainly focuses on methods that describe the behavior of chemicals in the body (including entry and exit). The other boxes give an overview of the many methods discussed at the DNT5 meeting. Notably, the methods are not always perfectly aligned with MoA steps. For instance, some KNDP assays may also provide information on MIEs or on the TEP.

### 1.2 Introductory keynote: *In vitro* DNT testing – from concept to use

In his keynote address, **Kevin Crofton** (retired from US EPA/R3Fellows LLC) described the history of DNT NAMs, from concept to practical application. When efforts began in the early 2000s, skepticism surrounded *in vitro* DNT testing, given the complexity of brain biology and the limitations of early models. Since then, significant progress has been achieved through a series of influential workshops and publications, as detailed in a recent publication by Smirnova et al. (2024a).

Among these advances, the development of the DNT-IVB represents a significant milestone. The DNT-IVB is a suite of more than a dozen high-throughput, cell-based assays designed to assess the effects of chemicals on KNDPs (Fig. 2) such as proliferation, apoptosis, cellular migration, differentiation, neurite outgrowth, synaptogenesis, myelination, and neural network formation and function – all of which are critical for understanding how chemicals might interfere with nervous system development and function (Bal-Price et al., 2018; Carstens et al., 2022; Blum et al., 2023). In addition to reducing reliance on animal models, the DNT-IVB and similar *in vitro* approaches allow for the rapid screening of large numbers of chemicals at a lower cost than guideline studies in animals. In 2023, OECD issued a guidance document outlining the use of the DNT-IVB and explaining how to interpret its data, as part of integrated approaches to testing and assessment (IATA) (OECD, 2023). At present, the DNT-IVB is ready for use in screening and prioritization of chemicals as well as weight-of-evidence assessments[13,14]. Hazard characterization

[13] https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0054
[14] https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0057

in regulatory settings and weight-of-evidence evaluations should follow (Fritsche et al., 2017; Bal-Price et al., 2018; Sachana et al., 2019; Carstens et al., 2022; Blum et al., 2023; OECD, 2023). While the DNT-IVB has been critical in providing a more comprehensive and mechanistically informed assessment of potential neurotoxicants and there has been consistent support for its use in conjunction with other lines of evidence in weight of evidence assessments, results from the battery in isolation are at present considered insufficient to categorize compounds for DNT (Juberg et al., 2023; Cöllen et al., 2025).

## 2 Keynote 1: Charting the path from brain organoid modeling to *in vitro* epidemiology through a hormonal signaling atlas

Understanding the complex interplay between genetic and environmental influences on neurodevelopmental disorders is extremely challenging (Cheroni et al., 2020). **Giuseppe Testa** (Università degli Studi di Milano) emphasized the need to develop new "maps" to make sense of how genetic and environmental factors converge to influence neurodevelopment. For example, advances in genomic studies have revealed a highly fragmented picture of ASD, characterized by both rare genetic variants of high penetrance and extreme polygenicity (Bougeron, 2015; Kelley and Pașca, 2022). This complexity is further compounded by the diversity of environmental factors at play during brain development, necessitating new approaches that can capture the full scope of these interactions.

Brain organoids were suggested as a powerful tool for modeling human neurodevelopment *in vitro*. These organoids have been demonstrated, through transcriptomic analysis, to develop in a way that closely mimics the temporal and spatial dynamics of the human brain (Cheroni et al., 2022). Thus, organoids are a useful system for studying the effects of environmental disruptions, such as those caused by endocrine-disrupting chemicals (EDCs), on KNDPs. To bridge the gap between epidemiological data and experimental models, Testa's team is integrating data from the SELMA cohort[15] (which tracks prenatal exposure to EDCs and subsequent neurodevelopmental outcomes) with organoid-based models to unravel the molecular mechanisms underlying observed correlations (Caporale et al., 2022). As detailed in this publication, brain organoids were used to investigate the effects of EDC mixtures on gene expression and neurodevelopmental processes, ultimately linking these findings to known genetic risk factors for ASD. This approach offers a new paradigm for integrating human experimental evidence with epidemiological data to inform risk assessment and public health policy.

With the ultimate aim of developing a multi-modal hormonal signaling atlas for human neurodevelopment, Testa's team aims to combine bulk and single-cell gene expression data with mass spectrometry-based steroid quantification to create a comprehensive resource for understanding how endocrine signals influence brain development and how these signals are disrupted by environmental exposures. Ongoing efforts to automate and industrialize brain organoid production for large-scale studies will support the scalability and reproducibility of these models.

The data, published studies, and strategies presented by Testa exemplified how insights from large-scale epidemiological studies may be integrated with information from novel model systems of neural tissue development to both advance neurodevelopmental research and inform effective, human-relevant approaches to toxicology and public health.

## 3 Bridging DNT4 to DNT5: Historical perspective

### 3.1 Achievements and challenges since the 2014 DNT4 conference

Acknowledging the transatlantic effort and collaborative spirit that has driven the field forward, **Marcel Leist**'s (University of Konstanz) keynote address reflected on the evolution of DNT testing, emphasizing that, since 2014, the field has progressed from optimizing assays for specific niches to focusing on implementation for risk assessment. This shift represents a significant step towards integrating DNT NAMs into regulatory frameworks. Key milestones since 2014 include: (i) the assembly of an initial DNT testing battery during the Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNATS) project[16] (Kadereit et al., 2012; Zimmer et al., 2014), (ii) the development of many human stem cell-based NAMs, and (iii) integration of individual NAMs into IATAs.

Reflecting on the growth of the DNT field, Leist used the metaphor of a "DNT giant" to describe the expanding body of knowledge and tools now available. He cautioned that while standing on the shoulders of this giant allows for greater insight, it also presents challenges for the next generation, who must navigate an increasingly complex field. In this context, historical records and thorough documentation are increasingly important to allow building upon existing knowledge and ensure continued progress without redundant efforts. While increasingly complex test systems such as brain organoids are providing insight into the genetic and functional aspects of neurodevelopmental toxicology, a balance must be established between expanding the testing repertoire and maintaining a manageable, functional testing battery. In conclusion, Leist used the analogy of a Black Forest cake and its multiple ingredients to illustrate that successful DNT testing requires the proper combination of screening, method development, mechanistic understanding, validation, and implementation. Looking to the future, he encouraged those in the field to continue building on recent achievements while addressing the challenges of implementing these tools in regulatory contexts, including scaling up testing efforts, improving descriptions of testing methods, and establishing an agreed-upon set of reference compounds.

15 https://selma.hotell.kau.se/en/selma-studien/

16 https://cordis.europa.eu/project/id/201619/reporting

To provide further historical context, the remaining talks in the session addressed recent advances and challenges faced in the implementation of NAMs for DNT testing from the perspective of stakeholders from the United States, Japan, the European Union, and industry.

### 3.2 United States

**Timothy Shafer** (US EPA ORD) focused on the implementation of DNT NAMs within the context of EPA's regulatory framework. Both the Toxic Substances Control Act and the Federal Insecticide, Fungicide, and Rodenticide Act allow for the use of NAMs in decision making, provided data are considered scientifically valid and of equivalent or better value to traditional methods.

Shafer highlighted three case studies demonstrating how DNT NAM data have been recently utilized by EPA.

- *Per- and polyfluoroalkyl substances (PFAS):* PFAS are a large category of chemicals with limited toxicological data. EPA screened 160 PFAS in various *in vitro* assays, including DNT assays, and found that 27% of these compounds were active. This information is now being used to prioritize further testing of PFAS (Carstens et al., 2023).
- *L-glufosinate:* DNT NAMs were used to evaluate a waiver request for L-glufosinate, a specific isomer of glufosinate, which is already registered as DL-glufosinate. The US EPA Office of Pesticide Programs sought to determine whether the L-isomer required a new guideline DNT study. By comparing the bioactivity of the L- and DL-isomers using neurite outgrowth and multielectrode array assays, EPA found no significant differences between the isomers. This data, combined with existing *in vivo* evidence, led the EPA to grant a waiver for the guideline DNT study, saving significant time, money, and animal use (Dobreniecki et al., 2022).
- *Organophosphates (OPs):* In 2023, EPA decided to evaluate the DNT potential for organophosphates individually, rather than as a class, using a weight-of-evidence approach that includes epidemiological, animal, and *in vitro* data[17]. This was based on new data that had become available since the reassessment of these compounds in 2016. This new data included epidemiological data as well as data from the DNT-IVB that demonstrated differences in the bioactivity of organophosphates, rather than consistent actions across the class.

Shafer emphasized that EPA is not relying solely on DNT NAMs for these decisions but is considering them in a weight-of-evidence approach with data from epidemiology and available *in vivo* studies. DNT NAMs can provide faster decision making, reduced costs, and decreased animal use, he concluded, stressing that EPA's experience in applying these methods could serve as a model for their broader implementation in regulatory processes.

### 3.3 Japan

**Yasunari Kanda** (National Institute of Health Sciences, Japan) described an increasing interest in NAMs within Asia, including ongoing discussions among Japan, China, and South Korea on integrating these methods into regulatory frameworks.

Japan is particularly interested in the multi-electrode array (MEA) system, a functional part of the DNT-IVB. Kanda summarized Japan's progress in applying the MEA system for both acute and chronic safety assessments, including drug-induced cardiac toxicity and seizure risk assessment using human induced pluripotent stem cell (iPSC)-derived neurons (Ishibashi et al., 2023). MEA technology could be extended to assess pesticides and other chemicals, Kanda noted, although vendor differences in iPSCs remain a challenge. Japan is also exploring the integration of MEA data with RNA sequencing data and the potential role of AI/machine learning (ML) in regulatory contexts.

Kanda outlined remaining challenges for DNT NAMs in Japan, including improved integration with traditional risk assessment methods, agreement on a framework for *in-vitro*-to-*in-vivo* extrapolation (IVIVE; at present under construction under the auspices of the OECD), and continued pursuit of emerging technologies like microglial models and thyroid hormone signaling assays. Ongoing international collaborations can help Japan and other countries effectively integrate NAMs into regulatory considerations, he concluded.

### 3.4 European Union

**Iris Mangas** (EFSA) discussed the use of NAMs for single-chemical DNT hazard characterization in the EU, focusing on the use of the DNT-IVB for pesticide risk assessment. Recent changes in both EU policy and EFSA's strategy have emphasized the integration of NAMs in the shift toward next-generation risk assessment (NGRA). EFSA has been a frontrunner in implementing the DNT-IVB, including through the development of IATA case studies for pesticide risk assessment, which have been crucial for developing interpretative guidance and mapping uncertainties (Masjosthusmann et al., 2020; Crofton and Mundy, 2021; EFSA et al., 2021; Escher et al., 2022; European Commission et al., 2024). Three such IATA case studies (deltamethrin, flufenacet, and acetamiprid) have informed final decisions by the European Commission (EC), with DNT-IVB data playing a key role in these assessments, particularly by filling gaps in the understanding of neurodevelopmental processes and linking mechanistic data to adverse outcomes.

Noting that only a small percentage of approved chemical active substances have been tested with the full DNT-IVB, Mangas outlined EFSA's ongoing program for assessing pesticide neurotoxicity, which includes generating data on 100 European-approved pesticides using the full DNT-IVB and addressing scientific uncertainties through projects like the BrainHealth Partnership[18]. Another important EFSA project supports a collection of all data on animal-based DNT studies that can be identified (added as a post-meeting note). Altogether 153 DNT studies, conducted on 144 chemicals and one stressor, were found to contain both extensive data summaries and regulatory reviews (Crofton and Mundy,

[17] https://www.regulations.gov/document/EPA-HQ-OPP-2008-0915-0056

[18] https://www.braincouncil.eu/projects/csa/

2024). These data were used to update Appendix A of the *Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery* (OECD, 2023), i.e., to provide a "*recommended DNT reference chemical test set for use in* in vitro *DNT assay development and evaluation*" (Mundy and Crofton, 2024).

In conclusion, Mangas emphasized the success of the DNT-IVB in providing relevant, high-quality data for DNT hazard identification. While advocating for the incorporation of DNT *in vitro* data into regulatory pesticide risk assessments, she also stressed the need for further work on the transferability and standardization of the DNT-IVB to fully realize its potential in regulatory contexts.

### 3.5 Industry

**Sue Marty** (Dow) provided an industry perspective on application of the DNT-IVB, noting that industry adoption has been slow due to several main concerns:

- *Domain of applicability:* The DNT-IVB has been tested on 81 chemicals, including reference chemicals, agrochemicals, and others – a relatively small number compared to other *in vitro* batteries, such as those for estrogen receptor pathways. This illustrates the need for increased testing across a broader chemical space, including challenging substances like surfactants, chelators, volatiles, and dimethyl sulfoxide (DMSO)-insoluble compounds.
- *Reliability:* As classic validation of the DNT-IVB has not been conducted, developing performance criteria for DNT-IVB assays will help to enhance reproducibility and robustness, as will interlaboratory testing using a standardized list of positive and negative controls to verify assay reproducibility. Clear expectations for control data, replicates, and acceptable variability could build confidence in the methods, as could a decision framework to guide the interpretation of results.
- *Assay coverage:* The 17 assays of the core DNT-IVB cover several critical neurodevelopmental processes. Some new elements are under active development, e.g., coverage of microglia (neuroinflammation) and of hormone-mediated effects on neurodevelopment. While they are not part of the DNT-IVB, there remains uncertainty concerning the interpretation of negative results.
- *Remaining uncertainties:* Reaching agreement on positive and negative reference chemicals has been difficult, which precludes a definitive understanding of DNT-IVB sensitivity and specificity. Further, the quantitative relationship of assay endpoints to adverse outcomes remains poorly understood.
- *Lack of a decision framework:* Uncertainty also stems from the lack of a regulatory "decision framework" dependent on context of use. Establishment of a tiered testing framework could clarify various questions, such as: What is the optimal number of assays for different problem formulations? Which data (e.g., on potency) are required to prioritize a compound for follow up? What is a sufficient margin of exposure (e.g., after use of IVIVE)?
- *Economic considerations:* The cost of screening chemicals across all 17 assays is substantial, necessitating a thoughtful approach to prioritizing chemicals for testing.

In conclusion, Marty emphasized that further improvements to the scientific confidence and economic feasibility of the DNT-IVB are necessary to make the battery more suitable for industry use.

## 4 Regulatory implementation of *in vitro* tests

### 4.1 Overview

As the DNT field advances, regulatory implementation of *in vitro* tests has emerged as a critical focus in the transition toward more human-relevant, ethically sound toxicological assessments. Traditional animal-based methods, while foundational in the field, face significant limitations in terms of human relevance, scalability, and the ethical implications of animal use (Smirnova et al., 2014). The scientific community has increasingly turned to NAMs to address these challenges. NAMs, ranging from simple cell cultures to complex multicellular systems, replicate key aspects of human development and offer the potential for high-throughput screening and the ability to study specific developmental processes with greater precision. Regulatory agencies are now beginning to incorporate these *in vitro* approaches into their frameworks, but challenges remain. Workshop presentations delved into the progress made in the regulatory adoption of *in vitro* DNT tests, highlighting key projects, challenges, and future directions as the field continues to evolve.

### 4.2 The DNT-IVB project

The DNT-IVB was assembled to represent key physiological processes necessary for proper neurodevelopment, explained **Ellen Fritsche** (Swiss Center for Applied Human Toxicology (SCAHT) & DNTOX) (Fig. 3). Her talk focused on three important aspects of the DNT-IVB: relevance, reproducibility, and predictivity.

- *Biological relevance*: Fritsche focused on the human neurosphere assay as an example to illustrate biological relevance. This assay suite, which covers 7 of the 17 individual DNT-IVB NAMs and 5 of the 8 endpoints, is based on human fetal neural progenitor cells (hNPCs) cultivated as proliferative neurospheres that can differentiate into brain effector cells including neurons, astrocytes, and oligodendrocytes (Koch et al., 2022). For instance, oligodendrocytes generated in the assay exhibit appropriate markers, morphology, and physiologically relevant signaling, demonstrating the assay's biological relevance. Similar types of assays to assess biological relevance established endpoint-specific assay controls for each of the 17 DNT-IVB assays (OECD, 2023).
- *Toxicological relevance:* The 7 neurosphere assays in the DNT-IVB were shown to respond to modulation of signaling pathways linked to human neurodevelopmental disorders (e.g., microcephaly, schizophrenia, autism), indicating that the assays can identify chemicals acting on these pathways. Further, Csb-deficient BrainSpheres were demonstrated to recapitulate clinical pathological findings in Cockayne syndrome B (Kapr et al., 2024).
- *Reproducibility:* Three types of variabilities must be assessed: intra-assay, intra-laboratory, and inter-laboratory. Assay control reproducibility has been assessed across the European assays,

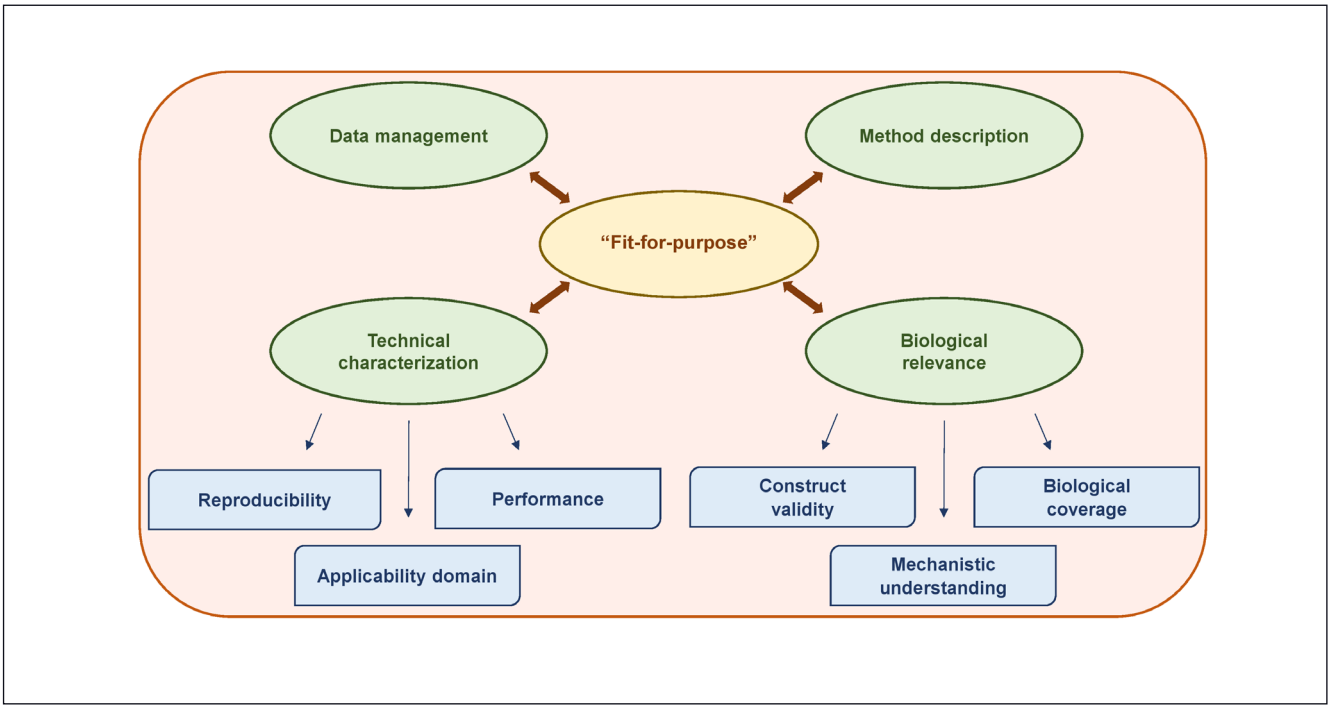


**Fig. 3: Major assessment elements to define the readiness of new approach methodologies (NAMs)**
Many NAMs, e.g., in the context of DNT assessment, especially when combinations or batteries of NAMs are considered (e.g., DNT-IVB), require modern, flexible approaches to establish their validity. Major elements of this process include a comprehensive method description, a clear and transparent data-management concept, technical characterization of the NAM, and assessment of its biological relevance. Requirements in these areas and their methods of assessment are not fixed but are adapted to and driven by the need to be fit-for-purpose. Each of the four main features may contain several elements and processes. For instance, technical characterization evaluates reproducibility, performance, and the (chemical) applicability domain of the NAM. Biological relevance means that (i) the NAM covers biological regulations and pathways relevant for toxicity *in vivo*, (ii) the phenotypic effects of many test items can be mechanistically explained in the NAM, and (iii) there is construct validity (with its various subdomains), meaning essentially that the test system contains elements resembling those of the *in vivo* situation, which react to disturbances similarly to the human brain.

and the data are publicly available, e.g., on the coefficients of variation for positive and negative controls (Masjosthusmann et al., 2020; OECD, 2023). Data are currently being consolidated to assess intra-lab reproducibility, which appears robust. Work on lab-to-lab transfer of all 17 assays, sponsored by EFSA, started in May 2024 (added as a post-meeting note). Reproducibility is crucial in regulatory settings, where consistent results across locations and operators must be demonstrated. In this context, discussions on harmonization of data processing pipelines used for the DNT-IVB data are ongoing (Blum et al., 2025).

- *Predictivity:* Predictivity of all 17 DNT-IVB assays was assessed using a classic validation approach with positive and negative compounds. Data indicate a sensitivity of 68% and a specificity of 100% for the EU component of the DNT-IVB (Blum et al., 2023), i.e., an approximate accuracy of 90% (OECD, 2023). Additional validation approaches have been suggested (Cöllen et al., 2025). In this context, it is important that the positive activity calls from the NAM of the DNT-IVB (here also called "hits") may require some follow-up for confirmation and toxicological validation (Smirnova et al., 2024a; Magel et al., 2024)

Multiple mechanisms of action are likely to converge on oligodendrocyte toxicity (Klose et al., 2023), and early DNT-IVB data indicate that oligodendrocyte and network formation assays currently account for most of the "hits". The DNT-IVB NPC5 assay, and similar assays that cover several stages of oligodendrocyte development, have been used to assess compounds like biocides and flame retardants (Klose et al., 2022; Cohn et al., 2024). In summary, Fritsche concluded that the strong biological and pathological relevance, reproducibility, and predictivity of the DNT-IVB demonstrate it is fit-for-purpose in regulatory settings.

### 4.3 Regulatory application of the DNT-IVB: Completing the picture

In 2023, OECD published the *Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery*, which refers to 5 IATA case studies illustrating how DNT-IVB data can be used. **Magdalini Sachana** (OECD) discussed important achievements of this project and the short- and medium-term plans to enhance regulatory implementation (OECD, 2023).

The OECD DNT-IVB project facilitates confidence-building in the battery through rigorous assay review and case studies. Standardized reporting, including the use of ToxTemp forms[19] for all 17 assays and an IATA template for data integration, familiarize regulators with the methods, allow rapid assessment of information adequacy, permit information sharing, link information to existing chemical databases, and identify aspects applicable to various problem formulations. Significant progress has been made in testing chemicals across the 17 DNT-IVB assays. More than 80 chemicals have been tested in all assays, with over 400 chemicals tested in some, and all data were analyzed using the ToxCast analysis pipeline.

Despite the initial success of the DNT-IVB, several elements that would boost regulatory uptake have been identified. Questions remain around how to use DNT-IVB data, IVIVE, and method reproducibility. Assay transferability efforts to naïve laboratories, establishment of an IATA framework template, and development of principles for quantitative IVIVE considering kinetic studies are underway to address these challenges. OECD's ongoing work includes evolving validation practices to make them less resource-intensive. OECD is currently developing an updated guidance document on validation, which will address evaluation of readiness, transferability, and peer review of new *in vitro* assays such as the DNT-IVB.

In terms of regulatory implementation, remaining challenges surround the use of DNT-IVB data (positive or negative) in diverse decisions or to address existing data requirements. Sachana discussed the development of a modular and adaptable IATA framework template that could embed a tiered testing strategy, allowing integration of DNT-IVB data with other information sources to address multiple regulatory problem formulations. The DNT-IVB will be one of the first endpoints to pilot this new framework, which aims to make IATA case studies applicable to more than one chemical and less labor-intensive. Sachana also touched on the complexities of applying toxicokinetic approaches to DNT testing, particularly in cases involving indirect exposure from mother to fetus or during lactation. OECD is developing tiered principles for IVIVE using physiologically based kinetic (PBK) modeling, which will be included as a module in the new IATA template. To conclude, she noted that a workshop reflecting on achievements of the DNT-IVB project and leveraging the lessons learned from previous case studies was planned for October 2024. It is intended to prepare an outline of the next steps that will allow the evolution of the *Initial Recommendations* (OECD, 2023) into a full guidance document.

### 4.4 Per- and polyfluoroalkyl substances and their mixtures as risk factors for neurodevelopmental disorders based on NAMs and epidemiological studies

Per- and polyfluoroalkyl substances (PFAS), a diverse group of over 10,000 chemicals used in industry for more than 70 years, are highly persistent in the environment, can cross the placenta, and accumulate in the brain, raising concerns about the potential contribution of some PFAS to neurodevelopmental disorders, explained **Oddvar Myhre** (Norwegian Institute of Public Health). While chemical risk assessment mainly relies on a chemical-by-chemical approach, the general population is exposed to complex mixtures, so understanding mixture toxicity is critical for assessing DNT risks associated with real-life PFAS exposures.

The NeuroTox sub-study of the Norwegian Mother, Father, and Child Cohort Study (MoBa)[20] explored the association between PFAS exposure and neurodevelopmental outcomes (e.g., ADHD, autism, and cognitive functions like working memory and language skills) in 3,600 mother-child pairs. While no significant associations were found between PFAS exposure and ADHD symptoms, language skills, or IQ, associations were found with non-verbal and verbal working memory (Skogheim et al., 2020, 2021). Further analysis revealed non-linear associations between two PFAS (PFOA and PFOS) and an increased risk of autism and ADHD, characterized by an inverted U-shaped dose-response curve, suggesting that mid-level exposures are associated with higher disease incidence, while higher exposures are unexpectedly associated with decreased disease incidence.

To complement epidemiological data, NAM-based studies (i.e., human synaptogenesis assays, neurosphere assays, and zebrafish larvae assays) were performed using human-relevant concentrations of six PFAS assessed in the MoBa study (Berntsen et al., 2017). While gene expression studies on approximately 160 genes in human NPCs showed no significant effects, preliminary results from synaptogenesis assays indicated potential morphological changes at higher PFAS concentrations. Mixture studies are ongoing. Zebrafish larvae exposed to PFOS and PFHxS showed hyperactivity in the visual motor response and acoustic startle response, indicating potential neurobehavioral effects. However, no significant effects were observed in proliferation or differentiation endpoints in the neurosphere assay for single PFAS or mixtures.

### 4.5 Exploiting avenues to increase regulatory use of non-animal data

While the European Commission (EC) has invested more than €1 billion into over 300 NAM projects over the past two decades, and numerous non-animal approaches are scientifically mature, integration of NAMs into regulatory testing remains slow, said **Maurice Whelan** (EC Joint Research Centre, JRC). Surveys and reports undertaken to identify evidence needs and barriers related to the integration of NAMs into regulatory contexts identified several challenges: lack of clear consensus on the use of NAMs or even on their readiness for regulatory use; lack of clarity regarding their applications; a perceived emphasis on demonstration (i.e., case studies) rather than validation; lack of standardization of methods and approaches; mistrust among stakeholders; and public concerns over "contentious" chemicals, which amplifies the need for rigor and transparency in the regulatory process. Such challenges suggest the need for a systemic change in the regulato-

[19] ToxTemp forms (Krebs et al., 2019) provide detailed information on the technical aspects of the assays, which can be accessed and evaluated by regulators or other users.

[20] https://www.fhi.no/moba-en

ry framework used to onboard new types of data, like NAMs, into decision making (Krieger et al., 2022; JRC et al., 2023; Schmeisser et al., 2023).

To assist in regulatory acceptance of NAMs, developers must understand the EU's complex regulatory landscape – not just the processes, but the underlying principles driving those processes and the key people behind them. For example, regulators require a comprehensive explanation of context of use, including an awareness of the influence of NAM data on the decision-making process relative to other available evidence, as well as the consequences of (possibly incorrect) decisions on health or environmental outcomes. The greater the influence of NAM data in decision making and the more significant the potential consequences, the more scientific credibility is required to convince regulatory authorities to use NAMs (Schruben, 1980; Patterson et al., 2021).

Innovation is needed in validation, Whelan said, to ensure that NAMs are reliable and relevant for regulatory use. While international guidelines are an important goal, they are difficult to establish. As intermediate steps, defining technical standards can help characterize NAM performance to inform users, while implementing good *in vitro* method practices can help make better use of research data in chemical assessments. Mechanistic knowledge frameworks like adverse outcome pathways (AOPs) are key to redefining regulatory toxicology endpoints, Whelan noted, as long as they strike a balance between being scientifically sound but practically useful in a regulatory context.

### 4.6 DNT assessment of contaminants of emerging concern in food

Unlike medical products, which undergo pre-market approval, many cosmetics or food-related products (e.g., dietary supplements and botanicals) are regulated after they are already on the market, necessitating efficient assessment methods, explained **Suzanne Fitzpatrick** (US Food and Drug Administration, FDA). NAMs are a priority for FDA's Center for Food Safety and Nutrition, particularly for assessing the DNT potential of metals like arsenic, cadmium, lead, and methyl mercury in foods commonly eaten by babies and young children[21].

FDA's Alternative Methods Working Group works to advance NAMs across the agency, facilitating cross-agency collaboration, opportunities for external organizations to present NAMs to FDA, and the adoption of new approaches through various types of partnerships. For example, the working group has adopted the BrainMixTox NAM – human brain organoids for assessing mixture neurotoxicity and understanding individual susceptibilities. This NAM will be studied for its ability to assess DNT of metals to determine whether it can be used as a test case for FDA's developing Drug Development Tool Qualification Program[22]. Scientific and analytical criteria for accepting NAMs for food chemicals fall under two main topics: (1) biological relevance (i.e., NAM identifies, measures, or predicts the *in vivo* event); and (2) technical characterization (i.e., NAM's performance characteristics increase confidence in allowing/removing products from the market).

FDA is also involved in broader federal and international efforts to address contaminants of emerging concern, such as 6PPD-quinone, a chemical from worn tires that may result in neurotoxicity and negative environmental impact (Babaei et al., 2024; Ma et al., 2024). Similarly, FDA collaborated with EFSA to create the International Liaison Group on Methods for Risk Assessment of Chemicals (ILMERAC), with the aim of harmonizing risk assessment methods globally. This collaboration focuses on areas such as chemical mixtures, AI, and organ-on-chip technologies, aiming to improve assessment and regulation of food safety worldwide.

## 5 DNT case studies and projects

### 5.1 Background

In the effort to refine DNT testing, significant emphasis has been placed on developing case studies and projects to demonstrate the utility and limitations of emerging NAMs. Such work can provide valuable insights into how NAMs can be integrated into NGRA workflows, offering more human-relevant and efficient alternatives to traditional *in vivo* methods. By leveraging *in vitro* and *in silico* tools, alongside computational modeling and high-throughput screening, researchers aim to improve the detection, understanding, and regulation of potential neurotoxicants. This section highlights a range of innovative projects and case studies that exemplify the progress and challenges in implementing these advanced methodologies, underscoring their potential to enhance DNT risk assessment and ultimately protect public health.

### 5.2 DNT case studies in the RISK-HUNT3*R* project

**Nadine Dreser** (University of Konstanz) described the RISK-HUNT*3R* project (Pallocca et al., 2022)[23], which aims to incorporate *in vitro* NAMs and *in silico* tools to provide NGRA via decision rules in a sequential, hierarchical workflow (Blum et al., 2023). The ASPIS[24]-initiated Alternative Safety Profiler Algorithm (ASPA) allows for flexible problem formulation, guiding users through executable steps and decision points to determine whether sufficient information has been gathered or whether further testing is required. A detailed description of ASPA can be found on the project website[24]. A graphic overview is given in Figure 4. ASPA is supported by a user-friendly dashboard (NAMASTOX) that facilitates documentation, reporting, and access to *in silico* methods linked to the workflow. Dreser presented three DNT case studies developed within RISK-HUNT*3R* to demonstrate how ASPA detects DNT hazards, as well as to define gaps and develop improvements:

- *Strobilurin pesticides:* Initial hazard characterization was performed using the neural crest cell migration assay (Nyffeler et al., 2017, 2018; Dolde et al., 2021), revealing a hazard alert

21 Closer to Zero initiative: https://www.fda.gov/food/environmental-contaminants-food/closer-zero-reducing-childhood-exposure-contaminants-foods

22 https://www.fda.gov/drugs/development-approval-process-drugs/drug-development-tool-ddt-qualification-programs

23 RISK assessment of chemicals integrating HUman centric Next generation Testing strategies promoting the 3Rs; https://www.risk-hunt3r.eu/

24 Animal-free Safety assessment of chemicals: Project cluster for Implementation of novel Strategies (ASPIS); https://aspis-cluster.eu/

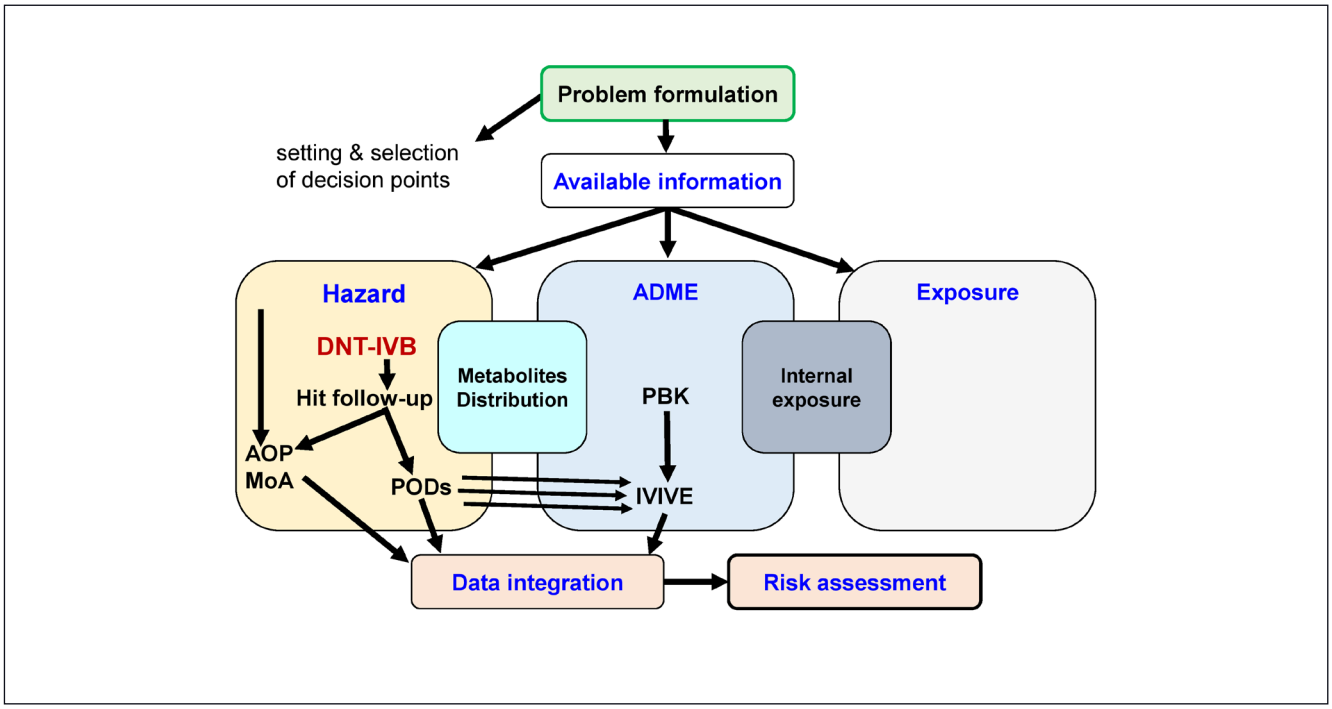


**Fig. 4: The alternative safety profiling algorithm (ASPA)**

ASPA is an integrated, new approach methodology (NAM)-based, next generation risk assessment (NGRA) approach that gives context to the DNT-IVB. ASPA starts with problem formulation, which is important for parametrizing decision points in the workflow. ASPA consists of modules (containing various executable steps), with several decision points between modules. The different parts of the algorithmic process can be subdivided into hazard identification and characterization, toxicokinetics (ADME), exposure assessment, and data integration/risk assessment. In the ADME part, a physiologically-based kinetics (PBK) model is established and used for *in-vitro*-to-*in-vivo* extrapolation (IVIVE). The DNT-IVB is part of hazard assessment. After a hit follow-up procedure, it yields points of departure (PoDs) for risk assessment and may inform a relevant adverse outcome pathway (AOP) or modes of action (MoAs). Information from the DNT-IVB and mechanistic information (e.g., from known AOPs/MoAs) are integrated with ADME information for risk assessment.

with a benchmark concentration (BMC) in the two-digit micromolar range. However, repeating the assay with a glucose-free medium (i.e., forcing cells to rely on mitochondrial respiration) reduced the BMC. Pre-exposure of cells to toxicant to allow bioaccumulation further decreased the toxicity threshold to submicromolar levels, highlighting the importance of considering intracellular concentrations and biokinetics to derive relevant points of departure (PODs) for risk assessment. Modeling approaches confirmed significant intracellular enrichment (Magel et al., 2024). This case study showed that a single screen hit can result in several PODs (even within the same test system) and thus an array of margins of exposure to be considered. This highlights the need for an uncertainty assessment and for tools to make weight-of-evidence approaches easily understandable and transparent.

- *Neonicotinoid pesticides:* This study explored the AOP for neonicotinoids, focusing on the link between molecular initiating events (MIEs) and adverse outcomes (e.g., altered neuronal connectivity and cognitive performance) (Grillberger et al., 2023). Calcium influx assays were used as an indirect measure (i.e., as biomarker (Hartung et al., 2024) of receptor binding), and *in silico* docking models predicted interactions with various receptor subtypes. To account for maternal and fetal metabolism, hepatocyte metabolism was modelled, and bioactive metabolites were predicted. Then metabolites were tested in NAMs, following the workflow of ASPA. The study emphasized the need to assess each metabolite for its potency and impact on key events (KEs) in the AOP.
- *Mixed compound set (regulatory relevance):* The final case study aimed to apply ASPA to the Classification, Labeling, and Packaging (CLP) process[25] for DNT assessment. This study highlighted the challenges of assessing DNT in a system that also considers other reproductive toxicity endpoints. Basic criteria for classification were proposed, such as labeling a compound reprotoxic if a DNT effect is detected or introducing a new "NAM-based health hazard" label. An example using bexarotene demonstrated how ASPA could classify compounds based on retinoic acid signaling and DNT effects.

[25] https://environment.ec.europa.eu/topics/chemicals/classification-labelling-and-packaging-chemicals_en

In conclusion, Dreser emphasized the importance of moving from the DNT-IVB to an integrated testing strategy using ASPA. This approach aims to avoid false positives that could be generated when all DNT-IVB assays are run in parallel, improve POD prediction, confirm AOP relevance, and explore the application of ASPA to regulatory frameworks like the CLP system.

### 5.3 Towards a virtual embryo: Computational modeling of neural tube closure defects

During embryogenesis, neural tube closure is a critical early developmental process that, when disrupted, can lead to severe congenital defects such as spina bifida. Currently, there is no high-throughput, non-animal test to assess chemicals' effects on neural tube closure.

**Harm Heusinkveld** (Utrecht University) described the generation of a computational model to simulate cellular behavior during neural tube closure, based on a detailed physiological map of the human-relevant KEs and processes involved (Heusinkveld et al., 2021). The agent-based model, in which cells are the "agents", includes detailed descriptions of cell behavior, interactions, and key molecular gradients crucial for proper neural tube formation.

Work on this model is ongoing, with the goal to make it able to simulate and visualize the outcomes of various perturbations. For example, the team demonstrated how hyperactivation of specific factors (e.g., BMP or the transcription factor Pax-3) can lead to defects in neural tube closure, effectively simulating spina bifida in a virtual environment. The model also allows investigation of the impact of genetic disruptions by including knock-out experiments.

The model's accuracy at its present (pre-publication) stage was validated by comparing its outputs to real-life *in situ* hybridization data, demonstrating that the model closely mimics biological processes. The ultimate aim is to integrate the model into NGRA strategies, where it can be used to assess the impact of chemical exposures on neural tube closure. One of the modelers' visions is to feed the model with transcriptomic fingerprints of a compound to obtain a prediction of the probability of neural tube closure defects based on specific gene expression changes. To further enhance the model's predictive power, the team is working on anchoring it to real-life biological data, including stem cell-based rosette formation assays and zebrafish models (Werner et al., 2021).

### 5.4 Prioritization of compounds for DNT testing using screening information from NAMs

The DNT Health Effects Innovation (DNT HEI) program aims to generate screening-level information using NAMs to evaluate DNT hazard (using a modified DNT-IVB, to be published in 2025) and prioritize compounds for further evaluation[26], explained **Helena Hogberg** (NIEHS). A stakeholder-nominated chemical library is being screened in three phases. Phase one is complete and includes 115 chemicals; phase two is currently underway with 108 chemicals, including defined mixtures and a larger number of DNT-negative compounds. Results are analyzed using a benchmark concentration approach to identify active compounds and assess their selectivity for DNT endpoints.

Phase one screening revealed the network formation assay to be the most sensitive across the tested compounds, with fungicides and insecticides as the most active classes. However, numerous hits in the network formation assay were not selective for DNT, demonstrating the importance of integrating cytotoxicity data to refine data interpretation. In the zebrafish embryo behavior assay, 27 compounds had the lowest activity concentration in at least one endpoint, and ToxPi analysis showed that, for 10 compounds, the mean slice score was higher than the mean of other DNT-IVB assays. Further, 25 compounds, including 10 of the most potent in the zebrafish assay, were shown to accumulate in the fish, which could explain the sensitivity results. Data will be used in an IATA for prioritizing compounds and could contribute to weight-of-evidence for regulatory decision making or to inform further testing.

Hogberg described an IATA case study involving mifepristone, a drug initially considered negative for DNT. A systematic literature review indicated that mifepristone can exhibit *in vitro* effects on NPC proliferation, differentiation, and dendritic spine density; *in vivo* data indicate effects on dopaminergic neurons, behavior changes, and memory impairment. IVIVE was performed to calculate the adverse estimated dose (AED) from the *in vitro* active concentrations, which were found to overlap with therapeutic doses in humans and doses observed to cause DNT effects *in vivo*, indicating a potential DNT concern.

To further reduce DNT-IVB-related uncertainty, Hogberg's team is working to enhance IVIVE and PBK modeling. Other ongoing projects involve reference compound selection to assist with assay transferability, development of additional IATA case studies, exploration of other approaches for prioritizing compounds based on DNT-IVB data, identification of supplementary assays to reduce biological uncertainties, and the development of a tiered or defined approach for DNT testing.

### 5.5 Practical application of NAMs for developmental and reproductive toxicity

Offering insights from a consumer product perspective, **Iris Müller** (Unilever) explained Unilever's use of a tiered, exposure-led, hypothesis-driven risk assessment approach that integrates NAMs to assess developmental and reproductive toxicity (DART) in the absence of animal testing. This approach involves a measure called the bioactivity-exposure ratio (BER)[27], which is central to their tiered approach (Baltazar et al., 2020; Middleton et al., 2022) and guides decisions on whether a substance poses a high or low risk. Unilever's framework involves collecting existing data from the literature, estimating exposure, and using a toolbox of human cell-based *in vitro* assays tailored to various DART endpoints (e.g.,

[26] DNT HEI uses the DNT-IVB described by OECD (OECD, 2023) except for the two neurite outgrowth assays from the University of Konstanz. The Division of Translational Toxicology also includes a zebrafish behavior assay in their testing battery.

[27] BER is calculated by dividing the lowest bioactivity POD by the internal *in vivo* exposure Cmax. https://seac.unilever.com/files/evaluating-a-systemic-safety-toolbox-for-use-in-next-generation-risk-assessment.pdf

gametogenesis, fertilization, implantation, embryonic development, and post-natal and multi-generational development) (Rajagopal et al., 2022). Assays include broad screening tools, such as high-throughput transcriptomics, a cell stress panel, and *in vitro* pharmacological profiling, along with targeted assays (e.g., a steroidogenicity assay, devTOX quickPredict[28], and ReproTracker[29]).

To evaluate the framework, Unilever tested 37 compounds (e.g., pharmaceuticals, cosmetics, plant protection products, food additives) with known DART outcomes, including varying chemical properties and modes of action. Comparison of risk predictions with existing human and animal toxicology data showed the framework to be a good starting point for building a fit-for-purpose, protective NGRA approach for DART.

To conclude, Müller summed up the uncertainties that should be addressed, which include expanding the number of tested compounds, improving the understanding of NAMs' biological relevance to avoid missing critical (neuro-)developmental effects that might not be captured by broader assays, and evaluating the need to refine exposure models for pregnancy and fetal development. Development of uncertainty calculations and decision-making models is also crucial for advancing the application of NAMs in DART testing.

## 6 Human translation and impact

### 6.1 Background

For DNT research findings to be impactful, it is essential to translate insights from *in vitro* models and animal studies into meaningful human health outcomes. Participant presentations explored cutting-edge methods and projects that are bridging the gap between experimental data and real-world human health impacts. By exploring species-specific differences, the integration of omics technologies, and innovative approaches like stem cell-based 3D models, presentations highlighted ongoing efforts to improve the human relevance of DNT testing.

### 6.2 Unraveling species specificities of neurotoxicity to improve risk assessment of endocrine-disrupting chemicals

Although the effects of some hormones in the developing brain are fairly well understood, many remain understudied, leading to uncertainties regarding potential toxicity, explained **Katharina Koch** (IUF & DNTOX). The ENDpoiNTs project[30] is a Horizon 2020 initiative aimed at unraveling the hormonal dependencies of brain development and developing test methods for endocrine disruption-mediated DNT. ENDpoiNTs has three primary tasks: i) improving the understanding of hormone involvement in brain development, ii) developing a testing battery using *in vitro* and *in silico* tools to detect endocrine-disrupting DNT, and iii) integrating these test methods into regulatory frameworks. To achieve these goals, agonists and antagonists of 14 hormone receptors were assessed for effects on neurodevelopmental processes modeled in the DNT-IVB neurosphere assay (i.e., proliferation, radial glia migration, differentiation into neurons and oligodendrocytes) (Masjosthusmann et al., 2020; Koch et al., 2022). Oligodendrocyte differentiation emerged as the most sensitive endpoint across several hormone receptors.

To assess biological relevance, benchmark concentrations were compared with fetal cord blood concentrations of natural hormones. All but two were within the range of fetal exposure, indicating potential relevance to human neurodevelopment. Koch presented data on two receptors: the glucocorticoid receptor and liver X receptor (LXR). Sex-specific effects were discovered for the glucocorticoid receptor – namely, male neural progenitor cells were more sensitive to receptor activation than female cells. Effects were confirmed as receptor-specific, and RNA sequencing analysis suggested that glucocorticoids might suppress proliferative responses by interfering with transcriptional processes. For LXR, oligodendrocyte differentiation was significantly reduced, likely due to disrupted cholesterol and lipid metabolism, critical for myelin production in the brain.

To assess species-specific differences, human and rat neurospheres were compared. While some characteristics were conserved, notable differences emerged, particularly in the influence of retinoic acid and glucocorticoid receptors on neurodevelopmental processes, underscoring the need to consider species-related specificities when extrapolating data from animal models to humans.

### 6.3 Linking DNT-IVB endpoints to AOPs using omics approaches

AOPs serve as a bridge between mechanistic data generated from *in vitro* assays and the prediction of adverse outcomes relevant to human health or environmental safety (OECD, 2016; Leist et al., 2017). The AOP KEs are measurable biological changes that occur sequentially and are critical for understanding how a chemical exposure leads to a harmful effect. Despite assay endpoints that could represent KEs in AOPs, there has been limited progress effectively linking MIEs to adverse outcomes in neurodevelopment using DNT-IVB data, said **Tim Shafer** (US EPA ORD).

An ongoing project at EPA aims to utilize omics technologies with DNT-IVB assays to establish a larger number of human-relevant AOPs for DNT, particularly beyond those related to thyroid hormone disruption (Spinu et al., 2019; Pitzer et al., 2023). The project started with the network formation assay, which uses primary rat neurons cultured on MEAs to measure neuronal network activity over time. To gain insight into mechanistic processes, transcriptomic and metabolomic analyses of cells harvested at identified toxicological "tipping points" have been conducted. "Tipping points" are the concentrations at which a biological system transitions from an adaptive, homeostasis-restoring response

[28] https://stemina.com/products-and-services/devtox-quickpredict/

[29] https://toxys.com/reprotracker/

[30] https://endpoints.eu/

to a maladaptive response, resulting in toxicity (Shah et al., 2016; Frank et al., 2018). Across tested chemicals in a proof-of-concept study, omics analyses revealed both commonalities and unique changes in categories including cellular and molecular functions and diseases and disorders, as well as in the canonical pathways and upstream regulators involved in the observed changes (Marable et al., 2022). Findings were used to build a putative AOP for cytosine arabinoside, linking its interaction with DNA synthesis to downstream effects such as decreased proliferation, increased apoptosis, and ultimately altered neurophysiology. When an additional 18 chemicals were tested at concentrations closer to their respective tipping points, only a few showed clear concentration-responsive changes in gene expression, while numerous changes in metabolite levels were seen. Focusing on four chemicals that showed consistent transcriptomic and metabolomic responses (i.e., tebuconazole, tributyltin, lead acetate, and fipronil) a mix of common upstream regulators and chemical-specific responses was again observed, linked to various DNT-relevant endpoints such as apoptosis, axonogenesis, and dendritic branching.

Although further data generation and time-course studies are needed to refine these putative AOPs, Shafer emphasized the potential of omics approaches to provide greater understanding of chemical mechanisms of action associated with DNT. By identifying common molecular pathways and their perturbations, omics could strengthen the link between *in vitro* assays and *in vivo* outcomes, ultimately aiding in the development of targeted, efficient testing strategies and their integration into regulatory frameworks.

### 6.4 Leveraging uncertainty knowledge to transform regulatory concepts for DNT assessment

Noting the numerous limitations of traditional rodent-based DNT testing, **Martin Paparella** (Medical University of Innsbruck) proposed a shift in regulatory thinking, suggesting that DNT NAM results be viewed as risk factors for organism-level toxicity and disease rather than direct predictors of specific adverse outcomes. This approach emphasizes identifying molecular and cellular effects as early indicators of potential toxicity, which could serve as the foundation for a new classification system.

He referenced a recent "Designathon" initiative by the JRC and the European Partnership for Alternatives to Animal Testing, which challenged the scientific community to develop a classification system for chemicals based solely on NAMs without relying on traditional animal data. Paparella's Designathon proposal included translating the existing Globally Harmonized System category boundaries, typically expressed in milligrams per kilogram for repeated dose and acute toxicity, into microgram-based boundaries using high-throughput kinetic models. These models would consider long-term exposure scenarios, such as a nine-month exposure period for DNT, to estimate chemical-specific risk categories. By matching benchmark concentrations derived from *in vitro* and *in silico* data with these new category boundaries, chemicals could be classified into high, medium, or low concern categories. This classification would not only incorporate the benchmark concentrations but would also account for human variability – a major uncertainty in translating rodent data to humans.

In summary, Paparella argued that such an approach could be a practical and effective strategy for classifying many chemicals with a few well-characterized and mature NAMs, evolving and improving over time with scientific progress.

### 6.5 Stem cell-based 3D models for disease states and DNT

Brain organoids are three-dimensional, miniaturized, and simplified versions of the brain, created from human iPSCs. They represent a test system that may form the basis for new DNT-related NAMs. Their complex biology and the many functional cell interactions within organoids can enhance the human relevance of DNT testing, suggested **Lena Smirnova** (Johns Hopkins University). Brain organoids include various types of neurons and glial cells, such as oligodendrocytes and astrocytes (Pamies et al., 2017; Morales Pantoja et al., 2024). They are designed for reproducibility, standardization, and scalability – crucial factors for toxicological studies. The physiological relevance of the organoid model can be further enhanced by incorporating microglia and increasing organoid size by exploring vascularization and perfusion methods. Brain organoid microglia were demonstrated to exhibit phagocytic ability and to respond to inflammatory stimuli, and preliminary data indicate that inclusion of microglia might increase the electrical activity of brain organoids.

Brain organoids can be used for several purposes:

- *Biomarker discovery:* Extracellular vesicles (EVs) derived from organoids can reflect brain-specific gene expression, which can be correlated with blood samples to identify potential biomarkers for neurodevelopmental disorders (Smirnova et al., 2024b). This method was validated by studying the effects of lead exposure, demonstrating dose-dependent regulation of specific genes within EVs from lead-treated organoids.
- *Chemical testing:* All the KEs assessed in the DNT-IVB are present within brain organoids. A mini-brainbow system is under development, using CRISPR/Cas9 to label various cell types with specific fluorescent markers. This system will allow real-time tracking of cell differentiation and response to chemicals. Preliminary data demonstrate differential effects of cuprizone, a demyelinating agent, on mature versus immature oligodendrocytes (Romero et al., 2023) – an effect previously seen only by examining morphology.
- *Disease modeling:* Brain organoids can be used to study gene-environment interactions, particularly in the context of autism. By exposing brain organoids derived from stem cells with autism-related mutations to environmental toxicants (e.g., chlorpyrifos), Smirnova's team observed alterations in key neurotransmitter levels and disrupted metabolic pathways, which are also observed in individuals with autism. These changes were more pronounced in mutation-containing organoids compared to controls, suggesting a heightened sensitivity to environmental insults in a genetically susceptible background. Furthermore, incorporating sex differences revealed that male- and female-derived organoids showed unique response patterns.

Finally, Smirnova introduced the concept of "organoid intelligence", integrating brain organoids with advanced sensors and machine learning to explore cognitive functions like learning and

memory *in vitro* (Smirnova et al., 2023; Alam El Din et al., 2024). Using high-density MEAs, early efforts to model learning and memory induced long-term potentiation in organoids by stimulating specific neurons. Sustained activation was seen in distant neurons over several hours, demonstrating that the organoids could maintain synaptic changes typical of the plasticity thought to underlie learning processes. Blocking NMDA and AMPA receptors, critical components of synaptic plasticity, inhibited these effects, confirming that the observed changes were indeed due to synaptic activity. The goal of this research is to develop sophisticated *in vitro* systems capable of modeling complex cognitive behaviors, potentially replacing animal cognition tests and providing a more human-relevant platform for studying processes underlying learning, memory, and neurodegenerative disorders.

## 7 Highlights on current developments

### 7.1 A forum for discussion and exchange

During the conference, some time was devoted to parallel workshops in which conference participants discussed issues of high importance for future implementation of the DNT-IVB in small groups. The five topics addressed by the breakout groups were:

1. How to contextualize DNT hazard with information on maternal toxicity or non-DNT toxicity domains (non-DNT)
2. Opportunities for an immediate or near-future application of the DNT-IVB, e.g., as a prioritization step or add-on to other information
3. How to establish comprehensive and integrative data-interpretation procedures for multi-dimensional endpoints
4. How to incorporate a battery of MIE tests for DNT into the DNT-IVB
5. How to achieve scientific validation of DNT NAMs

The discussions allowed an intensive exchange of detailed technical knowledge from the diverse group of participants. Discussions also allowed identification and alignment of differing perspectives. Many ideas on further refinement and implementation of the DNT-IVB were collected; the outcome is covered in a separate publication (Cöllen et al., 2025).

### 7.2 Selected presentations

As a second interactive part of the conference, six presenters were selected from submitted abstracts. Their studies showcased new methods and models that aim to improve detection and understanding of DNT. In addition, this session presented a further opportunity for discussion of all data presented at the conference thus far.

### 7.3 Assessing endocrine disruption (ED)-induced developmental neurotoxicity using C17.2 cells

**Andrea Cediel-Ulloa** (Uppsala University) introduced a novel *in vitro* test method, based on C17.2 immortalized murine neural progenitor cells. A 10-day differentiation and exposure protocol was employed to evaluate the effects of agonists and antagonists of various hormonal receptors, including thyroid hormone, glucocorticoid, and retinoic acid receptors, on neuronal differentiation and morphology. Results demonstrated that exposure to certain agonists, such as those targeting retinoic acid receptors and peroxisome proliferator-activated receptor delta, decreased neurite outgrowth and branching, while glucocorticoid receptor agonists increased neuronal differentiation. Notably, these effects were rescued by co-exposure with the corresponding antagonists. Further testing of 25 endocrine-disrupting chemicals revealed that compounds like endosulfan and cypermethrin negatively impacted neuronal morphology, effects that were mitigated by RAR and RXR antagonists. This study highlighted the potential of C17.2 cells as an *in vitro* model for identifying ED-induced DNT mechanisms (Cediel-Ulloa et al., 2025).

### 7.4 A new assay to detect endocrine disrupting chemicals with relevance to DNT

In the context of the ENDpoiNTs project, **Kevin Schlüppmann** (IUF-Leibniz & DNTOX) observed that endocrine-active compounds affected one of the assays of the DNT-IVB, the NPC1 neurosphere assay, measuring precursor cell proliferation. Upon closer investigation, agonists of the glucocorticoid receptor (GR) and the retinoic acid receptor (RAR) were found to specifically inhibit proliferation. On this basis, a new assay (NPC1_RAR_GR) was developed and advanced to the pre-validation stage (performed with PEPPER, a public-private platform for the pre-validation of testing methods on endocrine disruptors). The assay can be run in an antagonist mode or in an agonist mode to identify ligands of RAR and GR that potentially impact neurodevelopment. The assay could potentially be incorporated into a second tier of the DNT-IVB, for mechanistic follow-up and more detailed characterization of compounds or to eliminate false negatives.

### 7.5 Single-cell $Ca^{2+}$ imaging assay designed to assess disruptions in neuronal signaling

**Eike Cöllen** (University of Konstanz) showed how the disturbance of neuronal signaling may be captured by a $Ca^{2+}$ imaging assay. His test method captures the time-dependent changes of the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in individual cells. This change plays a key role in neurotransmitter release, synaptic transmission, and neuronal differentiation. He highlighted that transient disruptions in $[Ca^{2+}]_i$, particularly during sensitive developmental periods, may have lasting effects on brain connectivity. The $Ca^{2+}$ imaging assay was effective in identifying neurotoxic effects of certain neonicotinoid pesticides that were not detected by other *in vitro* tests. The assay is applicable to various human neuronal cell types, including dopaminergic LUHMES cells and astrocytes, and it also provided evidence of nicotine's neurotoxic impact on glutamate signaling. The assay has the potential to offer additional information to the DNT-IVB for improving mechanistic understanding of hits identified elsewhere or for measuring a molecular initiating event linked to some DNT-relevant AOPs.

### 7.6 Novel high-throughput screening tool for neuroepithelial cell organization

**Gavin Knight** (University of Wisconsin Madison) introduced the RosetteArray® assay, which models early neural tube formation, a critical process in neurodevelopment, using human pluripotent

stem cells to replicate the self-organization of neural rosettes. Knight demonstrated the assay's scalability and reproducibility, emphasizing its potential as a valuable addition to DNT testing. The RosetteArray's sensitivity to known neurotoxicants and its ability to model specific regions of the neural axis make it a promising tool for identifying compounds that may disrupt the neurodevelopmental processes.

### 7.7 Embryonic zebrafish behavior assay

**Jui-Hua Hsieh** (NIEHS) discussed the potential inclusion of zebrafish functional tests into the DNT-IVB. The model offers whole-organism testing capabilities, different from most other DNT NAMs. Hsieh detailed efforts by an OECD consortium to establish a standardized zebrafish behavior testing protocol, focusing on the light-dark transition test. This assay assesses zebrafish activity patterns as indicators of neurobehavioral disruption. The research showed that zebrafish assays detect some chemicals thought to trigger DNT with greater sensitivity than some existing DNT-IVB assays. This suggests that there are scenarios in which zebrafish larvae can be considered a Tier 1 screening tool for potential DNT toxicants (Fig. 1).

### 7.8 Neurodevelopmental effects of PFAS

**Sebastian Gutsfeld** (University of Leipzig) presented research using larval zebrafish as a model. His findings revealed that exposure to PFOS and PFHxS, two structurally similar PFAS, led to hyperactivity in zebrafish larvae during the dark phase of a light-dark transition test. Further investigation identified peroxisome proliferator-activated receptor delta (PPARδ) as a key mediator of this effect, demonstrating a novel mechanism by which PFAS may disrupt neurodevelopment. Gutsfeld's work highlights the utility of zebrafish models in exploring mechanisms of chemical toxicity and supports the use of gene editing for rapid hypothesis testing in DNT risk assessment.

## 8 Keynote 2: DNT as a pioneer discipline for NGRA

Modern NGRA is predominantly based on data generated using NAMs (Dent et al., 2018). The NGRA concept typically integrates cutting-edge technologies and data sources that go beyond traditional risk assessment practices. **Marcel Leist** (University of Konstanz) proposed that DNT testing is uniquely positioned to spearhead NGRA and to act as a pioneering discipline that blazes a trail for other toxicological disciplines.

Leist outlined some key defining principles of NGRA (Pallocca and Leist, 2022), including:

(i) a tiered approach,
(ii) iterative processes,
(iii) thorough documentation,
(iv) being exposure-led,
(v) being hypothesis-driven, and
(vi) being safety-focused.

A tiered approach is a resource-efficient strategy in which initial screenings are conducted using high-throughput, cost-effective methods. More detailed and complex testing is conducted in subsequent tiers if potential risks are identified. Iterative processes allow for continuous refinement of information as new data emerge, ensuring that the evaluation becomes increasingly reliable and accurate over time. Comprehensive documentation provides for the transparency and traceability of all steps, decisions, and uncertainties – essential for ensuring that the risk assessment process is scientifically robust and can be scrutinized or reproduced by others.

In many cases, current *in vitro* DNT testing is also exposure-led, hypothesis-driven, and safety-focused, aligning with three further principles of NGRA. These principles are actively applied through advanced models and integrated testing strategies, which "assemble all necessary elements to form complex structures and functions". Exposure-led thinking, which focuses on internal exposure at toxicity targets (accounting, for instance, for the blood-brain barrier and the placental barrier), has a long-standing tradition and acceptance in DNT testing (Kadereit et al., 2012; van Thriel et al., 2012), adding further proof of its pioneering role for advancing NGRA principles.

Importantly, Leist reminded the audience that toxicology is a highly model-dependent discipline, necessitating the discussion of strategies for developing and validating models, defining models' applicability domains, and translating model data into real world-relevant information. Just as maps are not the real landscape, models are not exact replicas of the real world, noted Leist. Models cannot be classified as "good" or "bad" – it is more appropriate to consider them as being useful (or not useful) for answering specific scientific questions. A given model may be useful for some questions but not for others, implying that models can only be evaluated in the context of a particular question or hypothesis.

Leist explained that the term "model" has many different meanings in colloquial language and in various scientific disciplines. For DNT testing, the term "test method" might be more appropriate than "model". Each test method (NAM) is a "model" in the above sense, and in this function its major building blocks are the exposure scheme, the test endpoint(s), the data-interpretation procedure (e.g., prediction model), the test system (e.g., selected cell type), and a defined purpose (Aschner et al., 2017; Bal-Price et al., 2018; Pamies et al., 2018; Holzer et al., 2023; Cöllen et al., 2024). Defining the purpose of a DNT test method is critical, as many possible objectives exist. For example, one might aim to predict whether a chemical triggers a specific impairment (e.g., sensory dysfunctions or delayed language acquisition), the minimum or maximum concentration of a chemical that triggers brain function impairment, or the likelihood of impairment at a given exposure level – each goal requires a different approach (Fig. 2).

A common criticism of *in vitro* models is that cells neither talk nor feel, and thereby they lack the complexity of whole organisms. Leist provided a two-pronged counterargument. On the one hand, animal models lack many such capacities as well (e.g., rats cannot display chemical-induced disturbances of language capacities; they are also extremely poor models for pain research, e.g., for headaches; and it is challenging to define an autistic rat). On the other hand, cell-based models may accurately reflect the changes occurring within the brain after chemical exposure: many DNT effects are due to an altered connectivity between certain brain parts, and this is measurable in cell cultures. Connectivity disruptions – also

termed a toxicity endophenotype (Kadereit et al., 2012; Smirnova et al., 2014; Bal-Price et al., 2015) – can result from a disturbance of several KNDPs such as neurite loss, failed differentiation, or altered synaptic function – all of which are crucial indicators of DNT.

To shift the focus from endpoint assessments such as behavioral changes, Leist introduced the concept of "process control" rather than "end-stage control", based on the idea that KNDPs are essential for a fully functional nervous system. Structural or functional disturbances in KNDPs can lead to significant deficits (Bal-Price et al., 2015; Aschner et al., 2017). NAMs and mechanistic assays can evaluate KNDPs to detect potential hazards. Assays that probe KNDP disturbances focus on adversity, as cell function disturbances have a higher chance of indicating adversities than do MIE and signaling assays.

Leist proposed DNT testing as a pioneer discipline for protective, rather than purely predictive, toxicology. He suggested that, in many cases, it is not important to know (predict) whether a chemical promotes attention deficit or language disturbances – it would be more useful to know the highest concentration of a test chemical that does not trigger any adverse effect (Browne et al., 2024). If exposure to a chemical was limited (regulated) in a way that concentrations reached in the human body (in particular in fetal and child brains) were below[31] the lowest adverse concentration, then this approach would be "protective" for the human population. Such an approach would be a "protective strategy" and would be characterized by its focus on the necessary information for risk management rather than on fulfilling the particular data requirements (differing from regulation to regulation) of a risk assessment dossier.

Notably, this protective approach would be conservative and precautionary, assuming that not all endpoint changes measured in a testing battery would lead to human adversities. Confidence in the protectiveness of this approach depends on ensuring that all fundamental processes are covered. Once the strategy is sufficiently safe, measures may be taken to increase the specificity, to avoid false positives and prevent the "loss" of chemicals and drugs valuable for society.

In summary, Leist positioned DNT as a discipline at the forefront of NGRA – not just in terms of available technologies, but also by providing a theoretical basis for new approaches to safety science. This comprises a shift away from the sole use of apical endpoints for regulatory purposes by predicting adverse effects from more upstream events measured in NAMs (Fig. 2). His talk underscored the importance of continuing to refine these methods and integrating them into regulatory frameworks to improve human relevance of DNT testing.

## 9 AOP and mode of action frameworks

### 9.1 Background

AOPs are structured frameworks that describe the progression of a biological effect from a MIE to an adverse outcome, capturing the KEs that occur along the way. KEs are measurable biological changes that necessarily occur when a chemical triggers a certain adverse effect. Per this concept, assays that measure KEs triggered by chemicals are considered predictive for human health outcomes (Fig. 2).

It is sometimes forgotten, however, that AOPs represent general biological knowledge, not a description of the effects of a particular chemical: AOPs are compound agnostic. As they do not address specific toxicants, AOPs do not incorporate information on toxicokinetic behavior or on the concentration dependencies of chemical effects.

Mode of action (MoA) frameworks complement AOPs by focusing on the biological mechanisms or pathways that a specific chemical disrupts. MoAs can also include information on a compound's (or its metabolite's) concentration over time and space. AOPs and MoAs serve as valuable tools in regulatory decision making, particularly when integrated into IATAs – flexible, evidence-based approaches that combine data from multiple sources (e.g., *in vitro*, *in silico*, and *in vivo* studies) to provide a comprehensive assessment of chemical risks. When informed by AOPs, IATAs can better contextualize mechanistic data generated from NAMs, allowing for more accurate predictions of adverse outcomes.

### 9.2 Thyroid hormone-mediated neurodevelopmental toxicity and regulatory decision making

**Stephanie Melching-Kollmuss** (BASF SE) discussed regulatory challenges associated with assessing thyroid hormone-mediated neurodevelopmental toxicity, particularly in the context of agrochemicals. Since 2018, the EU has categorized agrochemicals as endocrine disruptors if they meet specific criteria, including demonstrating an adverse effect, showing endocrine activity (with a focus on estrogen, androgen, thyroid, and steroidogenesis pathways), and establishing a plausible link between the two. Upcoming regulations will further classify endocrine disruptors into two categories based on their relevance to human health and the environment.

The European Centre for Ecotoxicology and Toxicology of Chemicals Thyroxine (T4) Task Force investigated whether reductions in thyroid hormone levels in pregnant animals lead to neurodevelopmental outcomes in offspring, and whether thresholds exist below which such outcomes are unlikely to be relevant to humans. Thyroid hormone disruption is particularly relevant because thyroid hormone plays a key role in brain development. Many agrochemicals affect thyroid hormone levels through liver-mediated MIEs (Marty et al., 2021). However, EFSA's regulatory evaluations have primarily focused on thyroid function, rather than on later-stage neurodevelopmental effects.

The task force's review of *in vivo* studies revealed that a significant reduction in thyroid hormone levels is necessary before any neurodevelopmental effects (e.g., impairments in motor activity, learning, or memory) become evident. This suggests the existence of a threshold effect, below which reductions in thyroid hormones may not result in observable neurodevelopmental harm. Importantly, the correlation between maternal thyroid hormone levels

[31] "Below" is meant here in a sense that uncertainties in a populations' susceptibility, in modelling parameters, and in hazard assays are considered in a way that there is still a safety margin between the highest reached concentration upon population exposure and the lowest potentially adverse concentration.

and offspring neurodevelopmental outcomes was weak. Instead, directly measuring thyroid hormones in offspring provided a better predictor of neurodevelopmental effects (Marty et al., 2022).

To address the complexities of assessing thyroid hormone-mediated neurodevelopmental toxicity, the task force proposed a tiered framework incorporating both *in vivo* and *in vitro* testing. This approach emphasizes assessing patterns of effect rather than relying on single endpoints, allowing for a comprehensive understanding of how thyroid disruption may translate to neurodevelopmental outcomes. Melching-Kollmuss emphasized the need for refinement of testing methods to improve the accuracy and reliability of risk assessments, including, for example, the direct measurement of thyroid hormone levels in brain tissue, the modeling of local hormone thresholds, and investigation of species-specific differences in Phase II liver enzyme induction (Baze et al., 2024).

### 9.3 Ontology-based AI-driven innovative approach using DNT *in vitro* NAMs for NGRA

**Eliska Kuchovska** (IUF) described the ONTOX project, which is focused on creating NAMs to provide a functional, sustainable solution for advancing probabilistic human risk assessment of chemicals without animal testing. The ONTOX DNT framework is driven by a combination of AI with three key human cell-based *in vitro* assays (i.e., DNT-IVB neurosphere assay, NIPH synaptogenesis assay (Davidsen et al., 2021), and IUF neural network formation assay (Bartmann, 2023)). ONTOX's ontology framework, which serves as a knowledge base for the project, integrates data from multiple domains – including biology, chemistry, kinetics, and toxicology – into a physiological map of the developing human brain covering key brain cell types (i.e., oligodendrocytes, neurons, astrocytes, and radial glia) and their interactions during neurodevelopment (Vinken et al., 2021). This map is built exclusively from human data, ensuring its direct relevance to human biology.

ONTOX's physiological map allows researchers to visualize and explore key signaling pathways and processes relevant to neurodevelopment. For example, the map captures the effects of manipulating specific signaling pathways, such as COX-2 inhibition, on processes like oligodendrocyte differentiation. Integrating experimental data from the *in vitro* assays into this map not only characterizes the assays' biological applicability but also strengthens their human relevance and reduces uncertainty in risk assessments.

Beyond its use as a knowledge base, the ONTOX physiological map is leveraged for deriving AOPs and for predictive *in silico* models. The system integrates transcriptomic data from healthy and disease states, *in vitro* models, and physicochemical properties. The physiological map of the developing brain is connected to an AOP network that includes 16 AOPs and identifies KE links to neurodevelopmental disorders to form the multidimensional ontology. This approach provides a holistic understanding of the potential impact of chemical exposures on human brain development and represents the cornerstone of an innovative solution for probabilistic risk assessment of chemicals.

### 9.4 AOP-informed IATA as a tool to implement NAMs for DNT risk assessment

**Anna Bal-Price** (EC-JRC) discussed a collaborative effort with Iris Mangas (EFSA) to integrate *in vitro* methods into regulatory frameworks for assessing DNT hazards, particularly for pesticides. The EFSA-led initiative aims to address the limitations of *in vivo* models by leveraging an AOP-informed IATA that integrates data from multiple sources (e.g., *in vitro* assays, *in vivo* studies, and human observational data) to provide comprehensive, human-relevant risk assessment. Case studies focused on two pesticides: deltamethrin, a neurotoxic pyrethroid, and flufenacet, a non-neurotoxic herbicide serving as a negative control.

The approach began with a systematic literature review and critical data appraisal considering *in vitro*, *in vivo*, and human studies. Risk of bias was evaluated for each study, and studies were categorized into three tiers based on quality and reliability. The AOP concept was then used to contextualize and integrate this information, mapping DNT-relevant KEs and adverse outcomes. Importantly, the AOP-informed IATA utilized a Bayesian network analysis to quantify uncertainty and provide a probabilistic assessment of KE relationships, enabling a transparent, data-driven approach.

The AOP developed for deltamethrin identified multiple KEs linked to this pesticide's mode of action, including binding to voltage-gated sodium channels, disruption of neuronal network function, and impairment of behavioral outcomes like sensory-motor reflexes and memory. The IATA also highlighted missing empirical support for certain KE relationships. Inclusion of DNT-IVB data added significant value by providing missing information and reducing uncertainty. For example, assays measuring oligodendrocyte differentiation, neural network formation, and sodium channel activity were critical in demonstrating deltamethrin's DNT potential.

Bal-Price emphasized that the AOP-informed IATA approach demonstrated the utility of integrating mechanistic NAM data into regulatory assessments, improving the relevance and reliability of *in vitro* DNT assays while reducing reliance on animal testing. The final recommendation from EFSA's Panel on Plant Protection Products and their Residues is to consider including the DNT-IVB as a potential data requirement for pesticide registration, marking a significant step forward in the regulatory acceptance of NAMs for DNT risk assessment (EFSA et al., 2021).

### 9.5 Testing strategies for DNT based on the complex mechanistic knowledge of the developing brain

While *in vitro* models and AOPs have contributed to progress in understanding early DNT KEs, the connection between these events and outcomes at the brain or whole-organism behavioral levels remain poorly understood. To address this gap, **Harm Heusinkveld** (Dutch National Institute for Public Health and the Environment, RIVM)[32] introduced examples of recent projects aiming to create detailed AOPs based on human biology.

One example focused on the role of the TNFα receptor in fear behavior, illustrating how disruptions in this receptor can lead to

[32] The presentation was prepared together with Ellen Hessel (RIVM).

varying outcomes depending on the signaling pathways involved. Specifically, this work traced the MIE of TNFα receptor activation through a series of cellular and tissue-level changes, ultimately affecting long-term potentiation and fear-related behaviors in the hippocampus and amygdala. This example highlighted the complexity of neurobehavioral outcomes, showing that the same receptor disruption could lead to different behavioral endpoints based on the specific neural circuits engaged. Another example explored how deficiencies in the thyroid hormone transporter MCT-8 can result in intellectual and motor disabilities. This work integrated clinical data from patients with MCT-8 deficiency and mechanistic studies to construct a detailed AOP. The AOP linked disruptions in MCT-8 function to reduced thyroid hormone transport into the brain, leading to impaired myelination and synaptic connectivity, which in turn manifest as cognitive and motor deficits (López-Espíndola et al., 2014).

Heusinkveld also discussed the importance of integrating knowledge from both *in vitro* assays and whole-organism models. While advanced cell-based systems like MEAs and organoids offer insights into specific processes, they often fall short in representing the full complexity of organismal development. The use of whole-organism models like *C. elegans* and zebrafish can fill this gap, as these models can capture the interplay between multiple biological processes, allowing researchers to study developmental effects, behavior, and higher-order outcomes in a high-throughput manner. Zebrafish have brain regions and neurotransmitter systems that closely resemble those of humans, making them valuable tools in DNT testing strategies. In conclusion, Heusinkveld underscored the need for integrated approaches that combine human-relevant mechanistic data with whole-organism models, ultimately aiming to build more accurate and predictive testing strategies for DNT.

### 9.6 Linkages between AOP key events with *in vitro* DNT assays: Examples of GABAergic and glutamatergic modes of action

GABAergic and glutamatergic neurotransmission play critical roles in brain function and development. The GABAergic system, which primarily involves gamma-aminobutyric acid (GABA), acts as the brain's main inhibitory neurotransmitter, while the glutamatergic system, involving glutamate, serves as the principal excitatory neurotransmitter. Disruptions in these systems during neurodevelopment can lead to significant adverse outcomes, including cognitive deficits and neurodevelopmental disorders (Pitzer et al., 2023).

**Emily Pitzer** (US EPA ORD) discussed EPA's efforts to link KEs from AOPs to *in vitro* DNT assays, focusing on GABAergic and glutamatergic modes of action. The objective is to better connect MIEs with adverse outcomes observed in neurodevelopmental studies, particularly through proteomic analysis. By aligning NAMs with existing AOP frameworks, this proteomic research aims to identify where current assays can capture KEs, especially those involved in neurotransmitter systems like GABA and glutamate. Chemical probes were used to target specific neurotransmitter systems – kainic acid for the glutamatergic system and emamectin benzoate for the GABAergic system. In studies using kainic acid, which targets AMPA and kainate receptors, proteomic analysis of hippocampal tissue in adult rats revealed significant alterations in synaptogenesis pathways, indicating that this process is a critical target of glutamatergic disruptions. The team identified proteins associated with synaptic function that could serve as biomarkers for neurotoxicity and contribute to AOP development.

In parallel studies focusing on developmental exposure to emamectin benzoate, a pesticide that affects GABA-gated chloride channels, disruptions were observed in pathways related to synaptogenesis, apoptosis, and neurodegeneration in the cortex and cerebellum. Although fewer significant proteomic changes were detected at individual time points, shifts in protein expression across developmental windows revealed potential impacts on key neurodevelopmental processes. Pitzer concluded that these findings highlight the potential for linking *in vivo* proteomic data with *in vitro* assays to strengthen AOP models, though further work is needed to refine these frameworks.

## 10 Novel approaches for DNT testing

### 10.1 Background

In the pursuit of human-relevant alternatives to conventional testing methods, innovative methodologies are being developed to overcome the limitations of traditional approaches. Novel tools aim to enhance the sensitivity, accuracy, and efficiency of risk detection by incorporating machine learning, *in silico* modeling, and high-throughput screening platforms. By focusing on the integration of diverse data sources and the application of cutting-edge technologies, these approaches seek to provide more comprehensive assessments of neurotoxic effects and their underlying mechanisms. Presentations highlighted key advancements in the field, including new computational models, zebrafish-based behavioral assays, and scalable platforms for human neurodevelopmental screening.

### 10.2 Keynote 3: Computational systems models for neurovascular development

In his keynote lecture, **Tom Knudsen** (US EPA ORD) discussed interactions between the vascular and neural systems as a critical aspect of DNT and the utility of computer models for understanding the integration of biological systems. Knudson's talk covered five key areas: blood-brain barrier (BBB) development, microglial function in development, computational modeling and simulation, microglial origins, and the integration of *in vitro* and *in silico* data.

The BBB forms early in development, typically at around 6-8 weeks of human gestation, and it plays a crucial role in protecting the developing brain by regulating the transport of hormones, metabolites, and potentially harmful substances. BBB development is a highly orchestrated process involving multiple cell lineages interacting through intricate signaling pathways. By identifying genes evolutionarily important for BBB development and assembling them into ontological maps, a biologically driven framework was generated for assembling a computer model of the neurovascular system.

Microglia, the brain's resident immune cells, are among the first cells to colonize the developing brain, arriving even before key developmental processes such as angiogenesis, neurogenesis, and synaptogenesis commence (Reemst et al., 2016). Microglia, which originate from the yolk sac and later the fetal liver, play multifaceted roles crucial to brain development, including supporting the formation and maintenance of the BBB (Ginhoux et al., 2013; Squarzoni et al., 2014; Saili et al., 2017). These cells are essential for the proper patterning and integrity of the brain's vasculature, a role that underscores their significance in both neurodevelopmental health and disease. Microglia also help to maintain the BBB's selective permeability. Depleting microglia in mouse models resulted in disrupted vascular development and a compromised BBB, leading to increased permeability to substances that would otherwise be restricted from entering the brain (Naphade et al., 2023). Microglia undergo dynamic state changes throughout development and in response to chemical exposures and diseases, and they interact closely with neurons and endothelial cells, influencing processes like synaptogenesis and vascular remodeling (Masuda et al., 2020; Rawlinson et al., 2020).

Knudsen's work aims to leverage these insights to build a computational model simulating interactions between microglia and other cell types within the developing neurovascular unit. By understanding how microglia contribute to the formation and maintenance of the BBB and the brain's vascular network, researchers can better predict how disruptions – whether due to genetic mutations or chemical exposures – might lead to neurodevelopmental disorders. Knudsen introduced a cell agent-based model known as the computational neurovascular unit (cNVU), which simulates the interactions among various cell types, including endothelial cells, neuroprogenitor cells, and microglia, within the developing brain. This two-dimensional model leverages a detailed understanding of cellular signaling pathways to replicate the processes of angiogenesis and neurovascular development *in silico* (Naphade et al., 2023).

The model demonstrates how angiogenic sprouts grow towards the neurogenic niche, branching and arborizing as they interact with microglia. When these microglia adhere to endothelial cells, they shift to an activated state, altering their signaling properties and influencing the vasculature growth and patterning. The cNVU model allows researchers to manipulate various components, such as knocking out specific signaling molecules, to observe effects on vascular development. For instance, removing VEGF-A, secreted by neural progenitor cells, prevents invasion of vasculature into the neurogenic niche, leading to a failure in proper vascular development. Similarly, knocking out CSF1, a critical signal for microglial function, reduces arborization of the vasculature, mirroring the *in vivo* observations in mouse models in which microglia were depleted.

The power of this model lies in its ability to simulate and predict the outcomes of complex biological interactions. For example, simulations of the effects of the pesticide mancozeb on neurovascular development accurately predicted disruptions in microglial infusion and vascularization, findings that were later confirmed in human cell-based models. This validation underscores the utility of the cNVU model in not only understanding neurodevelopmental processes but also in predicting how chemical exposures might perturb these processes, leading to DNT (Zurlinden et al., 2020). The model's versatility was further demonstrated by its application to the early developmental stages of microglia, tracing their origins from yolk sac blood islands and mapping their migration to the brain (Ginhoux et al., 2010, 2013). By incorporating dynamic gene networks and homeobox gene regulation into the model, researchers can explore how various genetic and environmental factors influence the specification and behavior of mesodermal populations, including microglial precursors. This capability allows for detailed fate mapping and exploration of "what-if" scenarios, providing insights into how early-stage disruptions might impact later neurodevelopment. Knudsen emphasized that models like the cNVU that reflect the complexity of cellular interactions can help researchers predict, in a virtual environment, how chemicals and other factors might disrupt neurodevelopment.

### 10.3 Biological interpretation model for DNT

Acknowledging the challenges of integrating and interpreting large, diverse datasets to determine if a compound has the potential to be developmentally neurotoxic, **Kelly Carstens** (US EPA, ORD) discussed the complex data landscape emerging from the DNT-IVB. Assays measuring similar neurodevelopmental processes (e.g., neurite outgrowth) in different test systems may produce varying results due to differences in cell types, species, and experimental conditions. For example, only 4 concordant hit calls were observed between 20 chemicals tested in 5 neurite outgrowth assays. Additionally, due to differing data processing approaches (Blum et al., 2025), PoD values often vary across assays, complicating the process of identifying the most sensitive endpoints and understanding the true DNT potential of a substance. Data variability also exists at the endpoint level. Carstens' analysis indicated, e.g., that the IUF NPC1b proliferation endpoint (measurement of bromodesoxyuridine incorporation after 72 h) indicates particularly high test compound potencies, compared to other assays. The MEA network formation assay (NFA) emerged as the most sensitive assay overall.

The MEA NFA was further shown to lack strong differential patterns of bioactivity across its 17 measured endpoints, suggesting that feature reduction – to fewer, more informative endpoints – might enhance the efficiency and clarity of bioactivity evaluations (Martin et al., 2024; Cöllen et al., 2025). A similar analysis of the acute MEA assay showed more distinct bioactivity patterns associated with primary mode(s) of action, indicating that the two assays provide different types of information, which is important to consider in terms of context of use.

ML- and AI-driven approaches are promising for developing predictive models from complex DNT-IVB data. Unsupervised ML, which involves grouping data without predefined labels, was initially applied to identify patterns within assay data. Techniques like K-means clustering revealed some biological patterns but also showed considerable overlap between *in vivo* positive and negative predictions, suggesting that further refinement is needed. In contrast, supervised ML, in which data are labeled as positive or negative based on known DNT outcomes, has yielded more accurate models. Specifically, the naïve Bayes model demonstrated

good performance, with a sensitivity of around 72% and a specificity of 93%. Encouraged by these results, ongoing efforts are focused on developing a Bayesian network model, aggregating data from multiple assays to provide probabilistic predictions for DNT outcomes. While these models are still in the early stages, they hold promise for integrating complex datasets into meaningful predictions. Future work will concentrate on refining models, improving data processing, and better integrating diverse data types to enhance predictive accuracy.

### 10.4 Multi-behavioral phenotyping and DNT fingerprints for the identification of chemical MoA in early-life-stage zebrafish

Zebrafish are a potentially valuable model for DNT testing due to their ability to encompass all key developmental processes, their rapid development outside the mother, and their metabolic competence, which allows for realistic chemical exposure studies. Zebrafish also show strong genetic conservation with humans, making them highly relevant for human DNT testing (Howe et al., 2013). **Tamara Tal** (Helmholtz Centre for Environmental Research – UFZ) discussed development of the visual and acoustic motor response (VAMR) NAM in zebrafish, a behavioral battery based on the widely used light-dark transition test but expanded to include additional behavioral endpoints like startle responses to visual and acoustic stimuli, non-associative habituation learning, and memory retention. The model tests zebrafish larvae for changes in locomotor activity in response to light-dark transition, providing a robust phenotypic readout of neurodevelopmental processes.

The VAMR NAM system generates "behavioral fingerprints" for chemicals, capturing a unique profile of effects across various endpoints. This fingerprinting approach allows researchers to differentiate chemicals with similar modes of action by comparing their behavioral effects across the entire battery. For example, two NMDA receptor antagonists, MK801 and APV, both impair habituation learning in zebrafish but exhibit distinct effects on locomotor activity during light and dark phases. This distinction provides a nuanced understanding of the chemicals' neuroactive properties and highlights the ability of the VAMR NAM to capture subtle differences in chemicals' ability to alter behavior.

Novel modes of action can also be identified using the VAMR system, as demonstrated by a case study involving chlorophene, an environmental chemical that initially impaired habituation learning in the VAMR NAM. The team hypothesized that chlorophene might impair learning by acting as an NMDA receptor antagonist, but follow-up studies in mice found no evidence of NMDA receptor involvement. Returning to the behavioral battery, chlorophene was shown to cause dark-phase sedation in zebrafish, a behavior typically linked to GABA receptor agonists. This finding was later confirmed in both mouse and human *in vitro* models, underscoring the utility of the VAMR to uncover unexpected mechanisms of action. In conclusion, Tal emphasized that the VAMR NAM offers several advantages, including rapid testing (within five days), low cost, and the ability to capture complex behavioral responses that better align with those seen in rodent guideline studies. The system could complement existing *in vitro* DNT testing frameworks by identifying false negatives and providing mechanistic insights into chemical modes of action. Future directions include integrating this assay into larger screening programs, comparing data across chemical test sets, and refining the assay for application in different behavioral paradigms.

### 10.5 Application of *in silico* models for DNT bioactivity and prenatal toxicokinetics: fluoxetine case study

*In silico* approaches, which involve using computer-based models to predict biological activity and toxicokinetics, are increasingly valuable in DNT IATA. **Sue Marty** (Dow) provided two examples of *in silico* models to assess the bioactivity and prenatal toxicokinetics of chemicals: PBK modeling and chemoinformatic modeling for developing quantitative structure-activity relationships (QSAR).

Marty presented a case study using IATA to predict the bioactivity of fluoxetine, a common antidepressant approved for use during pregnancy. Cheminformatic QSAR modeling identified multiple potential targets of fluoxetine, including mitochondrial function and various neurotransmitter receptors. A review of *in vitro* ToxCast data found that the lowest $AC_{50}$ value was related to mitochondrial membrane potential, aligning with computational predictions. A PBK model was then used to predict fetal blood concentrations, initially predicting a narrow margin of safety compared to the *in vitro* bioactivity threshold. However, accounting for fluoxetine's active metabolite led to predicted fetal concentrations that effectively eliminated the safety margin, suggesting that *in vivo* exposure could reach bioactive levels.

Given the relevance of bioactive metabolites, Marty assessed the ability of *in silico* models to predict metabolites and their activities. Using six parent-metabolite pairs previously tested in the DNT-IVB, cheminformatics was used to predict the activity of the parent compound, then the GastroPlus®[33] model was used to predict metabolites, whose activity was also subsequently predicted using cheminformatic models. Results were then compared to those from the DNT-IVB. For example, she reviewed methimazole and its metabolite ethylene thiourea (ETU). While GastroPlus predicted ETU formation, neither cheminformatics nor *in vitro* assays identified thyroid hormone-related effects, which are central to ETU's known mechanism. This highlighted gaps in existing models for predicting endocrine-related neurodevelopmental toxicity. In another example, retinol's conversion to trans-retinoic acid was well-predicted by both GastroPlus and cheminformatics, and *in vitro* data effectively captured expected DNT endpoints such as neural outgrowth. However, in the case of organophosphates like chlorpyrifos and its oxon metabolite, only the parent compound was consistently detected in the DNT-IVB, leaving some key metabolites undetected. In conclusion, Marty emphasized that while *in silico* predictions have demonstrated utility, they need to be part of a broader weight-of-evidence approach within IATA frameworks.

[33] https://www.simulations-plus.com/software/gastroplus/

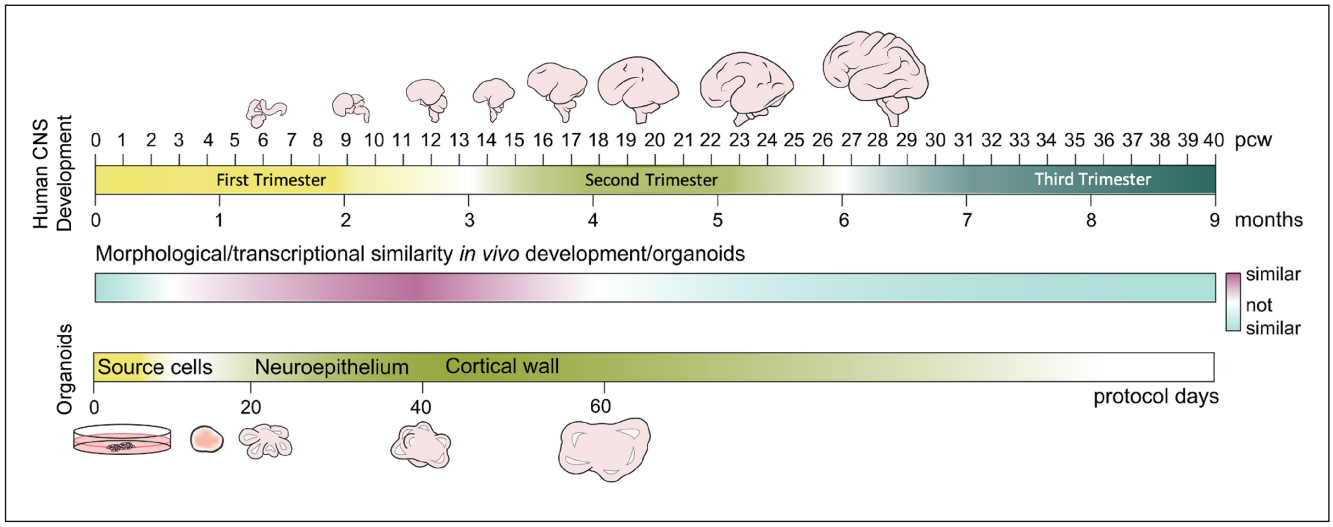


**Fig. 5: Comparison between human *in vivo* brain development and the corresponding stages of brain organoid development**
The timeline compares human *in vivo* brain development with the corresponding stages in brain organoid development. A cyan-to-purple gradient is used to indicate relative similarities between the two timelines, informed by cell biology and transcriptomics data from multiple studies. This gradient is a qualitative representation, not a precise metric. Note: The human brain and brain organoids are not shown to scale. Pcw, post-conception weeks. This figure (protected by a CC-BY license) was reproduced from Kelava and Lancaster (2016).

### 10.6 RosetteArray® for qHTS of human neurodevelopmental risk

**Randolph Ashton** (University of Wisconsin Madison) described the RosetteArray® platform, a scalable, standardized assay designed for high-throughput screening of human neurodevelopmental risk factors. This platform leverages the process of rosette morphogenesis – early stages of neural tube formation – from human pluripotent stem cells (hPSCs) (Grubb, 2006). By integrating micro-patterned substrates, the platform controls the size and formation of neuroepithelial rosettes, allowing for reproducible modeling of early neural development from the forebrain to the spinal cord. The RosetteArray platform offers multiple readouts, including cell viability, neurodifferentiation, rosette formation, and structural integrity (Lundin et al., 2024). These data points can provide insights into potential mechanisms of toxicity by examining the dose-response curves generated from the assay.

RosetteArray focuses on a developmental window covering the first six weeks of human embryogenesis, during which disruptions can lead to neural tube defects and other neurodevelopmental disorders (Fig. 5; Kelava and Lancaster, 2016). To demonstrate its biological relevance, compounds with known correlations to neural tube defect risks were tested, such as methotrexate, valproic acid, and benomyl. The assay effectively identified these risks, with some metrics showing increased sensitivity in detecting morphogenetic disruptions. Another case study involved dolutegravir, an antiretroviral drug linked to neural tube defects in clinical trials. RosetteArray detected the drug's effects within its therapeutic range, and supplementation with folate was shown to mitigate the risk, highlighting the assay's capacity to model real-world clinical scenarios.

To examine genetic factors in combination with chemical exposures, stem cell lines were generated with mutations in the MTHFR (methylenetetrahydrofolate reductase) gene, associated with neural tube defects. Homozygous mutant lines exhibited increased sensitivity to methotrexate, consistent with clinical findings. Additionally, knockout of the SCRIB gene, linked to another critical pathway in neural tube defect risk, generated region-specific disruptions in rosette formation, indicating significant impacts on spinal cord development but not on forebrain structures.

Ashton highlighted the applicability of the RosetteArray platform in studying neurodevelopmental disorders, particularly ASD. The platform was used to investigate the role of a mutation in the SYNGAP1 (Synaptic Ras GTPase Activating Protein 1) gene, which is associated with ASD (Satterstrom et al., 2020). RosetteArray detected decreased rosette formation in patient-derived cell lines with the mutation, a finding consistent with cortical organoid models but observable within just eight days (Birtele et al., 2023; Jourdon et al., 2023).

In conclusion, Ashton emphasized that the RosetteArray platform offers a standardized approach to assess the intersection of genetic and chemical factors in neurodevelopmental disorders. Exemplary studies have indicated that this model is well-suited for both research and screening applications, particularly in the context of early neurodevelopmental events.

## 11 Conclusions

The 5th International Conference on Developmental Neurotoxicity Testing (DNT5) served as a pivotal gathering for the exchange of ideas, research findings, and practical strategies aimed at advancing the field of DNT testing in general and promoting the implementation of the DNT-IVB in particular. By bringing together experts from regulatory agencies, validation institutions, academia, industry, and advocacy groups, the conference fostered collab-

oration and highlighted the critical role of NAMs in modernizing DNT assessments.

Participants generally expressed a very optimistic attitude and were highly confident that past success in the field would continue[34]. This optimism was based on experience with various case studies. The fast progress of the DNT-IVB was seen as encouraging. Many tasks that appeared difficult, or even impossible 20 years ago, have now been accomplished. The history of NAM-based DNT testing showed that many of the difficulties could be overcome by the collaborative efforts of diverse stakeholders, working together to find solutions. The DNT5 conference talks and discussions outlined some of the key opportunities and research directions for the upcoming years, and participants enthusiastically anticipate the next updates, e.g., at DNT6 or within the OECD working group on DNT. Issues left open were whether and how additional NAM should be integrated, how information from the DNT-IVB can be integrated with other types of information (e.g., IVIVE or other bioactivities/adversities), and which approach will be taken to apply the complex, multidimensional data in *de novo* risk assessment (Cöllen et al., 2025).

Participant excitement was grounded on a realistic approach, acknowledging that ongoing effort is needed to refine existing models, expand the use of NAMs in regulatory frameworks, and to address key challenges such as validation, transferability, integration into classification and labelling (e.g., the globally-harmonized system (GHS) or European CLP), and application for risk assessments. As the field evolves, continued collective action, robust scientific inquiry, and interdisciplinary cooperation will be required to shape the future of DNT testing, ultimately striving towards safer, more ethical approaches that better protect human health and the environment.

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## Disclaimer

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## Conflict of interest

Drs Randolph Ashton and Gavin Knight are co-founders and executives at Neurosetta® LLC, which commercializes the RosetteArray® platform for DNT and disease modeling/drug discovery applications. Ellen Fritsche is a shareholder and scientific managing director of the company DNTOX GmbH, a contract research organization, which provides DNT IVB assay services. Several of the co-authors work for companies that have an interest in product safety. They disclose this situation (as seen from the affiliations). None of the authors from academia or industry declare a conflict of interest.

## Data availability

Data have not been generated in the course of this project. Background information supporting the conclusions of this report are available from the corresponding author upon reasonable request.

## Acknowledgements

We gratefully acknowledge the funding of the meeting and its workshops by the Doerenkamp-Zbinden foundation, the German Research Foundation, and the 3R-Network of the *Land Baden-Württemberg*. The work was also facilitated by grants from the BMBF (161L0243B, 016LW0146K) and the European Union's Horizon 2020 research and innovation program under grant agreements No. 964537 (RISK-HUNT*3R*), No. 964518 (ToxFree), No. 101057014 (PARC), No. 825759 (ENDpoiNTs), and No. 963845 (ONTOX). ONTOX and RISK-HUNT*3R* projects are part of the ASPIS project cluster (https://aspiscluster.eu/). The Division of Translational Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services ZIA ES103387-02 are under Contract No. HHSN273201500010C.

The work presented by Drs Randolph Ashton and Gavin Knight was supported by NIEHS R42ES033912 grant awarded to Neurosetta LLC and US Environmental Protection Agency grant 83573701, NSF CAREER Award #1651645, and an Innovation in Regulatory Science Award #1014150 from the Burroughs Wellcome Fund awarded to Randolph Ashton (University of Wisconsin-Madison). The work present by Dr. Kanda was supported by research grants from the Ministry of Health, Labour and Welfare (MHLW) (19KD1003 and 22KD1002).