[FHI, KM] [1]

Institution: Norwegian Institute of Public Health (FHI/NIPH)
Administrative unit: Division of Climate and Environmental Health
Title of case study: New Approach Methodologies for use in the hazard identification and characterisation of chemicals – a case study on Developmental Neurotoxicity
Period when the underpinning research was undertaken: 2019-present
Period when staff involved in the underpinning research were employed by the submitting institution:
Period when the impact occurred: 2023

1. Summary of the impact

An increasing number of chemicals are entering the market and society demands that these chemicals are safe for human health. The public opinion (voiced by the European Parliament) demands to reduce the use of experimental animals in the safety testing of chemicals without compromising safety. New Approach Methodologies for hazard assessment have been proposed as a way forward. We have specifically worked on a model using human neural stem cell-based methods to study effects of environmental chemicals on neurodevelopmental processes vital for healthy brain development. This assay will be included in a revised DNT in vitro battery (https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf).

2. Underpinning research

The developing nervous system is considered to be more susceptible to chemical perturbations than the adult brain, due to the complex processes that occur during brain development (Myhre and Hessel, 2022). These processes are typically time-sensitive and include differentiation of the neural progenitor cells into neurons and glial cells, synaptogenesis, neuronal network formation, etc. (Tal et al., 2023; Lauvås et al 2022). Disturbance of any of these processes may lead to adverse neurodevelopment. Information on the developmental neurotoxic potential of many chemicals is lacking and systematic testing for developmental neurotoxicity (DNT) is not mandatory in the EU for pesticides, biocides, pharmaceuticals, or industrial chemicals. Using animals as model organisms for human development is of limited value due to species differences in brain development as well as the associated difficulties with data interpretation and extrapolation. There is no formally accepted alternative to in vivo animal studies for the identification of the neurotoxic potential of chemicals for regulatory purposes. To protect children's healthy brain development, regulatory agencies need fast, affordable, versatile, ethical constraints-free New Approach Methodologies (NAMs) that can accurately evaluate substance toxicity to close the data gap on the DNT potential of untested compounds. The NAMs should allow both hazard identification as well as hazard characterisation and contribute to the next generation risk assessment. Over the past decades, there has been an intense focus on the development of alternatives to animal methods within the DNT community, resulting in the DNT in vitro battery (IVB) https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf. The assays allow to studyseveral of the key neurodevelopmental processes vital for brain development. This major endeavour resulted in recently published OECD-supported guidance on the evaluation of data on DNT IVB, however, no guideline exists at this moment for the regulatory use of data produced with the DNT IVB. Therefore, NAMs for regulatory testing must be fully developed, recognized, and endorsed. The data gaps identified in the current DNT IVB are described in https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf. NIPH has recently used human induced pluripotent stem cell (hiPSC)-derived NPC in a 2D culture system producing neurons and astrocyte mixed cultures. With this test system, a test method for studying synaptogenesis in addition to other neurodevelopmental processes that are gaps in the current DNT IVB was set up (Lauvås et al. 2022; Davidsen et al. 2021; Pistollato et al. 2017). Data gaps present in the current

DNT in vitro battery are addressed in ongoing projects like ONTOX and PARC with the aim to develop a 2nd generation DNT IVB.
It is quite unique that we have now established an IVB for DNT that was shown to deliver robust results for use in regulatory assessments. The developing brain is a complicated organ and the development of this IVB, and future improvements and refinements, is the result of the efforts of a dedicated group of researchers supported by funding agencies that are united in their willingness to make this happen. NIPH is proud to be part of this initiative and is now taking an even more prominent role in further work.

3. References to the research

To close existing gasps and develop in vitro DNT NAMS for a 2nd generation DNT IVB, the research unit has succeeded in acquiring external funding in the EU funded projects ONTOX and PARC, and recently an EFSA funded 4-years project to fill gaps in the current DNT IVB related to glia cell function in brain development and toxicity. The purposes in the EU projects are to develop developmental neurotoxicity in vitro NAMs to develop key events in the pathways to cognitive defects, and to develop a 2nd generation DNT IVB battery for improved risk assessment of chemicals. Employees from our department is highlighted bold letters in the selected publications below.

Tamara Tal, **Oddvar Myhre**, Ellen Fritsche, Joëlle Rüegg, Kai Craenen, Kiara Aiello-Holden, Caroline Agrillo, Patrick J. Babin, Beate I. Escher, **Hubert Dirven**, Kati Hellsten, Kristine Dolva, Harm J. Heusinkveld, Yavor Hadzhiev, Selma Hurem, Karolina Jagiello, Beata Judzinska, Nils Klüver, Anja Knoll-Gellida, Britta A. Kühne, Marcel Leist, **Malene Lislien**, Jan L. Lyche, Ferenc Müller, Winfried Neuhaus, Giorgia Pallocca, Bettina Seeger, Ilka Scharkin, Stefan Scholz, Ola Spjuth, Monica Torres-Ruiz, Kristina Bartmann (2023). New approach methods to assess developmental and adult neurotoxicity for regulatory use: A PARC Work Package 5 project (Frontiers in Toxicology (submitted).

Lauvås AJ, **Lislien M, Holme JA, Dirven H,** Paulsen RE, Alm IM, Andersen JM, Skarpen E, Sørensen V, Macko P, Pistollato F, Duale N, **Myhre O** (2022). Developmental neurotoxicity of acrylamide and its metabolite glycidamide in a human mixed culture of neurons and astrocytes undergoing differentiation in concentrations relevant for human exposure. *Neurotoxicology* 92, 33-48.

Myhre O, Hessel EVS (2022). Editorial: Toxicants and neurodevelopmental disorders. *ReproductiveToxicology* 110, 68-69.

Davidsen N, Lauvås A, Myhre O, Ropstad E, Carpi D, Mendoza de Gyves E, Berntsen HF, **Dirven H**, Paulsen RE, Bal-Price A, Pistollato F (2021). Exposure to human relevant mixtures of halogenated persistent organic pollutants (POPs) alters neurodevelopmental processes in human neural stem cells undergoing differentiation. *Reproductive Toxicology* 100, 17-34.

Myhre O, Låg M, Villanger G, Oftedal B, Øvrevik J, Aase H, Paulsen RE, Bal-Price A, **Dirven H** (2018). Early life exposure to air pollution particulate matter (PM) as risk factor for attention deficit/hyperactivity disorder (ADHD): Need for novel strategies for mechanisms and causalities. *Toxicology and Applied Pharmacology* **354**, 196-214.

Pistollato F, Canovas-Jorda D, Zagoura D, Price A (2017). Protocol for the Differentiation of Human Induced Pluripotent Stem Cells into Mixed Cultures of Neurons and Glia for Neurotoxicity Testing. *J Vis Exp* DOI: 10.3791/55702.

4. Details of the impact

Epidemiological data indicate that toxicant exposures in Europe contribute substantially to neurobehavioral deficits and diseases, with an estimated cost of >€150 billion /year, emphasising the advantages of developing new NAMs for testing and identifying hazards, get a mechanistic

understanding to explore causal relationships and introduction of knowledge-based regulations to control exposure.
New knowledge of risk factors and their underlying mechanisms is likely to be vital to minimise new cases of neurodevelopmental disorders and cognitive deficits. Our projects are therefore highly relevant and timely and has the potential to become a demonstrator how toxicological hazards could be addressed by NAMS, also for other health outcomes.
Animal experiments using mainly rats are currently the gold standard in DNT testing. DNT testing for a large number of environmental toxicants is not fit-for-purpose with the current guideline studies because i) they are time- and cost-intensive (1 year/compound may cost up to 1.000.000 EUR), ii) it is ethically questionable (testing one substance may require up to 140 dams and 1000 juveniles), iii) there are uncertainties in its methodologies, evaluation, and regulation; iv) their predictivity for protection of the human brain is questionable due to the differences in brain function/complexity, exposure, neurodevelopmental timing, toxicokinetics and toxicodynamics between rodents and humans.
To protect children's brains, regulatory agencies need fast, affordable, versatile, ethical constraint-free NAMs that can accurately evaluate substance toxicity in line with the Chemical Strategy Sustainability goals of the European Commission and the 3Rs-Principle (EUSAAT, https://eusaat.eu/), accesses 29th January 2024) to close the data gap on the DNT potential of untested compounds. The NAMs should allow both hazard identification as well as hazard characterization and contribute to the next generation risk assessment. Regulators should have high confidence that the proposed strategies substituting animal testing gives the same level of protection as animal studies.
Brain development is a highly complex procedure that covers time and a large variety of neurodevelopmental processes. Over the past decades, there has been an intense focus on the development of alternatives to animal methods, resulting in the DNT *in vitro* battery (IVB) https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf. These assays model many, although not all, of the key neurodevelopmental processes vital for healthy brain development and employ cell models of mostly human origin. This major endeavor resulted in recently published OECD-supported recommendations on the evaluation of data on developmental neurotoxicity *in-vitro* battery, however, no guideline exists at this moment for the regulatory use of data produced with the DNT IVB. Therefore, to identify DNT alerts and prioritize substances for testing at a lower tier level in a more efficient and predictive manner to adhere to the domain of applicability, NAMs for regulatory testing must be fully developed, recognized, and endorsed.
Despite its complexity, key neurodevelopmental processes that are vital for brain development were identified (https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf).
For testing if a substance exerts adverse effects on the developing brain without using whole animals, key neurodevelopmental processes known to be vital for healthy brain development are mimicked by assays in relevant test systems *in vitro*.
There are currently **biological gaps** in coverage of key neurodevelopmental processes in the DNT IVB that have been acknowledged, including

- assays for Neural Progenitor Cell (including radial glia) proliferation of different developmental stages
- Astrocyte development and function;
- Synaptogenesis (astroglia and microglia contribution, species)
- Myelination (astroglia and microglia contribution)
- Neural Network Formation (astroglia and microglia contribution, species);
- Microglia presence (microglia addition to existing test systems for synaptogenesis, neural network formation, myelination)

that produce uncertainty with regards to the biological applicability domain of the battery (Crofton and Mundy 2021 https://doi.org/10.2903/sp.efsa.2021.EN-6924). The hNPC model at our department aims to close the gaps in particular related to replacement of rat synaptogenesis in

the current DNT IVB with human based assay for synaptogenesis, and the role of astroglia and microglia cells for synaptogenesis in the EU funded projects PARC and ONTOX. Many countries world-wide, including several EU member states and EFSA as an EU agency as well as the US-EPA, have endorsed this activity and recognised its high priority.

5. Sources to corroborate the impact

Blum, J., S. Masjosthusmann, K. Bartmann, F. Bendt, X. Dolde, A. Dönmez, N. Förster, A. K. Holzer, U. Hübenthal, H. E. Keßel, S. Kilic, J. Klose, M. Pahl, L. C. Stürzl, I. Mangas, A. Terron, K. M. Crofton, M. Scholze, A. Mosig, M. Leist, and E. Fritsche (2022). Establishment of a human cell-based in vitro battery to assess developmental neurotoxicity hazard of chemicals, Chemosphere, 311: 137035.

Commission, European (2020). Chemicals Strategy for Sustainability Towards a Toxic-Free Environment, 1-24 https://ec.europa.eu/environment/strategy/chemicals-strategy_en.

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Davidsen, N., A. J. Lauvås, O. Myhre, E. Ropstad, D. Carpi, E. M. Gyves, H. F. Berntsen, H. Dirven, R. E. Paulsen, A. Bal-Price, and F. Pistollato (2021). Exposure to human relevant mixtures of halogenated persistent organic pollutants (POPs) alters neurodevelopmental processes in human neural stem cells undergoing differentiation. Reprod Toxicol, 100, 17-34.

Escher SE, Partosch F, Konzok S, JenningsP, Luijten M, Kienhuis A, de Leeuw V, Reuss R, Lindemann K-M, Hougaard Bennekou S (2022). External Scientific Report. Development of a Roadmap for Action on New Approach Methodologies in Risk Assessment, 1-153.

Lauvås, A. J., M. Lislien, J. A. Holme, H. Dirven, R. E. Paulsen, I. M. Alm, J. M. Andersen, E. Skarpen, V. Sørensen, P. Macko, F. Pistollato, N. Duale, and O. Myhre (2022). Developmental neurotoxicity of acrylamide and its metabolite glycidamide in a human mixed culture of neurons and astrocytes undergoing differentiation in concentrations relevant for human exposure, Neurotoxicology, 92, 33-48.

Masjosthusmann, S. et al (2020). Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity. EFSA Supporting Publications 17, 1938E, 1-152.

Myhre O, Låg M, Villanger G, Oftedal B, Øvrevik J, Aase H, Paulsen RE, Bal-Price A, Dirven H (2018). Early life exposure to air pollution particulate matter (PM) as risk factor for attention deficit/hyperactivity disorder (ADHD): Need for novel strategies for mechanisms and causalities. Toxicology and Applied Pharmacology 354, 196-214.

Pistollato, F., D. Canovas-Jorda, D. Zagoura, and A. Price (2017). Protocol for the Differentiation of Human Induced Pluripotent Stem Cells into Mixed Cultures of Neurons and Glia for Neurotoxicity Testing, J Vis Exp DOI: 10.3791/55702.