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Deliverable title:	Report on existing and novel in vitro assays to detect MIEs and KEs in the qAOP network on cognitive function defects
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Leading partner:	IUF
Participating partners:	NIPH, UM, UU, 3RSMC
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Dissemination level:	Public

Contents

1. Introduction and objectives

The ONTOX project aims to deliver a generic strategy to create innovative new approach methodologies (NAMs) in order to predict systemic repeated dose toxicity effects for human safety assessment. For proof-of-concept purposes, 3 organ systems have been selected, namely the liver (WP7), the kidney (WP8), and the developing brain (WP9). WP9 aims to establish a mechanically anchored *in vitro* test battery to assess chemical compounds' developmental neurotoxic (DNT) potential. The testing battery is fit for purpose for the adverse outcome pathway (AOP) network describing the events leading to decreased cognitive function in children following prenatal exposure to chemicals. Moreover, a computational model of neural tube closure defects is being optimized and will be described in deliverable D9.3.

In order to gather the existing and novel *in vitro* assays to detect molecular initiating events (MIEs) and key events (KEs) in the qAOP network on cognitive function defects (CFD), we first compiled an AOP network based on available AOPs related to decreased cognition and impaired learning and memory. Subsequently, a literature review on CFDs (Kuchovska et al., in preparation) was carried out to have an overview of the neurodevelopmental disorders (NDDs) of interest with a focus on clinical symptoms, etiology and pathology-related information, and especially disturbed key neurodevelopmental processes (KNDP). The disturbance of any of the KNDP such as neural progenitor cell (NPC) proliferation, radial glia, neuronal, and oligodendrocyte migration, NPC differentiation into different effector cells (neurons, astrocytes, oligodendrocytes), maturation of the differentiated cell types, synaptogenesis, or neural network formation can lead to disturbed brain development and DNT. Thus these KNDPs are fit-for-purpose endpoints of an *in vitro* battery (IVB) assessing chemical effects on brain development. Moreover, the applicability domains of the IVB assays need to be properly characterized in order to increase their understanding and regulatory confidence thus enabling their acceptance and use for chemical risk assessment.

2. Results

2.1. AOP network.

To gather the existing and novel *in vitro* assays to detect MIEs and KEs in the qAOP network on CFDs, we first compiled and manually curated an AOP network based on published scientific literature and inventory of AOPs from the AOP-Wiki (Kuchovska et al., in preparation). This resulted in the AOP network (Figure 1) composed of 16 different AOPs including five AOPs that were developed at the IUF and uploaded to the AOP-Wiki (IDs 485-

489). These AOPs (IDs 485-489) describe the impairment of the KNDP of oligodendrocyte differentiation, maturation, and radial glia migration, KEs that were previously missing in the available AOPs. Figure 1 shows the AOP network including details about available assays within ONTOX to assess the MIEs and KEs as well as assays that are currently in development and assays that were developed previously and are currently part of the OECD-supported DNT IVB (OECD, 2023). The work on the AOP network curation revealed several important KNDP gaps that are currently not covered by the published AOPs. These gaps include e.g. NPC proliferation, neuronal differentiation, autophagy, neurite outgrowth, astrocyte differentiation and function, endocrine receptors regulating the KNDP, and others. To fill these gaps, we are currently investigating the KE of NPC proliferation to develop a new AOP describing the impairment of this KNDP leading to microcephaly and subsequent decreased cognition. Furthermore, the investigation of the KE relationship between microcephaly and decreased cognition would enable the addition of two more AOPs (IDs 441 and 523) to our network. Moreover, NIPH and IUF investigated and co-authored a publication that mapped the AOP-Wiki database and identified biological and disease gaps related to DNT (Jaylet et al. 2024). In this publication, we linked the AOP-Wiki DNT KEs to neurodevelopmental disorders using the 11th International Classification of Diseases system (ICD-11) provided by the World Health Organization (https://icd.who.int/browse11/l-m/en) and pointed out the gaps in missing KEs and not covered diseases.

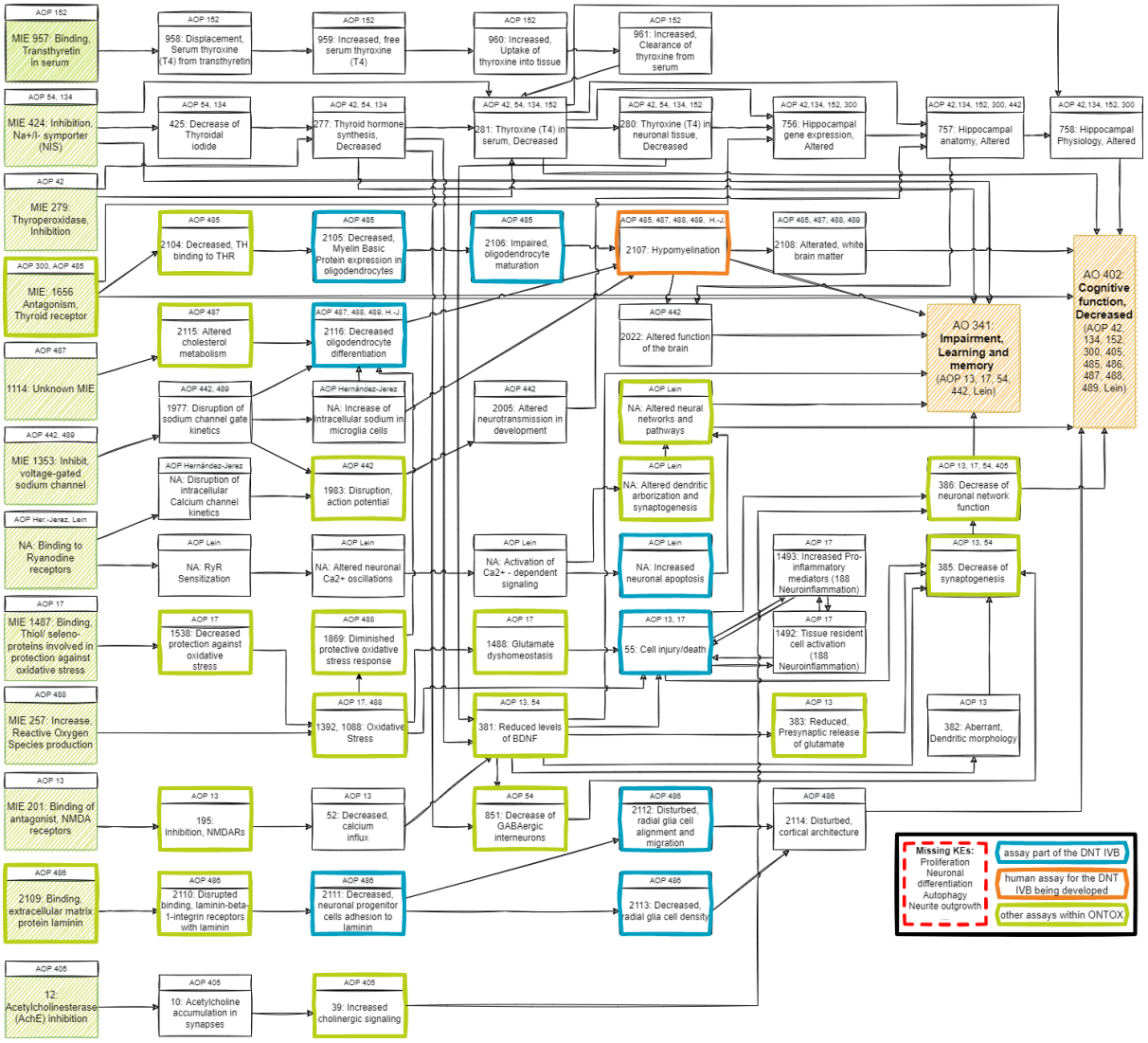

Figure 1: AOP network containing 16 AOPs focused on decreased cognition and impaired learning and memory in children following prenatal exposure to chemicals. Assays available in ONTOX to assess the KEs are shown in green and blue, and those currently developed for DNT IVB (in the framework of another project) are in orange. Moreover, assays indicated in blue are part of the OECD-supported DNT IVB. The red dashed line box indicates some of the missing KEs in the available AOPs.

2.2. *In vitro* assay development and *in vitro* battery selection

Next, we compiled the existing *in vitro* assays that were already set up and running at the IUF and that are part of the DNT IVB (OECD 2023) namely the Neurosphere Assay containing NPC1-5 assessing KNDP of NPC proliferation, radial glia, neuronal, and oligodendrocyte migration, neuronal and oligodendrocyte differentiation, and neurite outgrowth. These assays employ 3D cell models - primary neurospheres. The Toxtemps for these assays were published as supplements of the above-mentioned Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) *In Vitro* Testing Battery (OECD 2023), however, the ONTOX versions were updated with the available information required by WP11 and WP4 and sent to WP11 for addition in the ONTOX Biostudies repository. An assay for another KNDP - human synaptogenesis - that is currently not assessed in the DNT IVB was optimized at the NIPH employing human induced pluripotent stem cell (iPSC)-derived neural progenitor cells (Davidsen et al. 2021, Lauvås et al. 2022). This resulted in a Toxtemp containing a

description of several measurable endpoints namely hiNPC1-4 assays assessing human-induced NPC (hiNPC) proliferation, hiNPC differentiation into neurons and astrocytes, neurite maturation, and synaptogenesis. This Toxtemp was also forwarded to WP11 for addition in Biostudies. Subsequently, we wanted to use the mentioned differentiating hiNPC cell model to develop an assay for another KNDP - neural network formation (NNF) using microelectrode arrays (MEAs). However, the resulting electrical activity of the culture was too variable across the measured replicates. To solve this issue, we decided to employ a different cell model that proved more suitable for the human neural network formation assay (hNNF) and was developed at the IUF in the framework of another project (Bartmann et al. 2023). This cell model is commercially available (NeuCyte) and contains a precise ratio of hiPSC-derived inhibitory and excitatory neurons and primary astrocytes. The first version of the Toxtemp for this assay was written and is currently under internal IUF review and will be forwarded to WP11 for addition to Biostudies during April 2024 i.e. before the end of the second reporting period and this report's due date. All the mentioned assays are routinely complemented by a cytotoxicity (LDH) assay and/or viability (CTB) assay. The overview of the selected assays and measured KEs is shown in Table 1.

Table 1: Overview of the selected *in vitro* test methods to assess the KEs of the AOP network.

Test Method (Assay)	Test System (Cell culture)	Assessed KE	DNT Endpoint
NPC1	3D primary hNPCs	NPC proliferation	BrdU incorporation in dividing cells
NPC2a	3D primary hNPCs	radial glia migration	mean distance of radial glia (nuclei negative for neuronal and oligodendrocyte markers) from the edge of the sphere
NPC2b	3D primary hNPCs	neuronal migration	mean distance of tubulin-positive neurons from the edge of the sphere
NPC2c	3D primary hNPCs	oligodendrocyte migration	mean distance of O4-positive oligodendrocytes from the edge of the sphere
NPC3	3D primary hNPCs	neuronal differentiation	number of tubulin-positive neurons
NPC4	3D primary hNPCs	neurite growth	neurite length & area
NPC5	3D primary hNPCs	oligodendrocyte differentiation	number of O4-positive oligodendrocytes
hiNPC1	2D hiNPCs	hiNPC proliferation	number of ki67 positive neurons
hiNPC2	2D hiNPCs	hiNPC differentiation	number of nestin, tubulin and MAP2 positive neurons
hiNPC3	2D hiNPCs	hiNPC maturation	number of nestin, tubulin and synaptophysin positive cells

hiNPC4	2D hiNPCs	hiNPC synaptogenesis	Synaptophysin and PSD-95 positive cells
hNNF	NeuCyte cell model	neural network formation	network parameters (firing rate, burst duration, network burst percentage, …)
LDH	3D primary hNPCs, 2D hiNPCs, NeuCyte cell model	cell death	cytotoxicity (LDH release)
CTB	3D primary hNPCs, 2D hiNPCs	cell viability	viability (resazurin reduction)

2.3. *In vitro* assays' characterization.

Prior to regulatory approval, newly proposed non-animal methods (NAMs) must undergo a thorough characterization, including an assessment of their reliability and relevance to human biology. Thus, to increase the regulatory confidence in our NAMs, we aimed to characterize them from three different points of view. First, we carried out the scientific validation of the Neurosphere Assay focusing on 1) the relevance of the respective endpoints for brain development, 2) the confirmation of the cell type-specific morphologies observed *in vitro*, 3) the expressions of cell type-specific markers consistent with those morphologies, 4) the appropriate anticipated responses to physiological pertinent signaling stimuli and 5) the alterations in specific *in vitro* endpoints upon challenges with confirmed DNT compounds. This characterization was published by IUF in 2022 (Koch et al. 2022).
Second, we aimed to characterize the biological applicability domain of the Neurosphere Assay by investigating the involvement of 19 signaling pathways in the regulation of the KNDPs assessed in the assays. Moreover, to increase the regulatory confidence in NAMs, we demonstrated the similarity between the physiology of the test models and human biology and disease by linking the underlying molecular and cellular mechanisms and impaired functioning of signaling pathways in selected NDDs. This publication is currently under the IUF internal review (Kuchovska et al. in preparation).
Third, characterization of the hiNPCs undergoing differentiation up to 28 days by CyTOF (single cell proteomics using 42 antibodies) analysis is ongoing and the writing of a manuscript has been initiated (NIPH). The results revealed potential targets for neurodevelopmental toxicants in addition to the presence of receptors which could act as MIEs in the AOP network for cognitive defects.
Fourth, we carried out a transcriptomics characterization of the 2D and 3D hiPSC-derived cell models undergoing differentiation for up to 21 days. This thorough analysis allowed us to compare the two used cell models and reveal the dynamic developmental changes in the transcriptomes of the different differentiating cell types. Moreover, we described the presence of DNT-relevant endocrine and cholinergic receptors in these models which are currently gaps in the DNT IVB. This publication led by NIPH is currently under the co-authors' review and ready to be submitted soon (Lislien, Kuchovska, Kapr et al. in preparation).
Finally, the characterization of the total cell-associated protein content in the proliferating and differentiating hNPC and hiNPC models was carried out in order to enable the subsequent QIVIVE analysis in task 4.3. These results are planned to be published together with the cell-associated protein and lipid analysis that will be carried out by WP4 during the third reporting period.

2.4. Chemicals.

WP9 assembled the DNT chemical list containing the so-called positive and negative compounds containing pharmaceuticals, pesticides, metals, industrial chemicals, and drugs. The list is shown in Table 2. In cooperation with WP4 and WP6, relevant kinetic and human exposure data were gathered for these chemicals to inform future *in vitro* testing.

Table 2: Selected chemicals triggering DNT effects leading to CFDs and selected compounds not leading to impaired brain development.

Positive compounds	CAS number
Lead (II) acetate trihydrate	6080-56-4
Sodium arsenite	7784-46-5
Manganese (II) chloride	7773-01-5
Cadmium chloride	10108-64-2
Methylmercuric chloride	115-09-3
BDE 47	5436-43-1
BDE 99	60348-60-9
TBBPA	79-94-7
Chlorpyrifos	2921-88-2
Deltamethrin	52918-63-5
Valproic acid sodium salt	1069-66-5
Methimazole	60-56-0
Propylthiouracil	51-52-5
Sodium perchlorate	7601-89-0
Methadone hydrochloride	1095-90-5
Morphine hydrochloride	52-26-6
Dexamethasone	50-02-2
(±)-Ketamine hydrochloride	1867-66-9

Ethanol	64-17-5
Domoic acid	14277-97-5
Nicotine	54-11-5
PFOS (potassium salt)	2795-39-3
PFOA	335-67-1
PFHxS (potassium salt)	3871-99-6

Negative compounds	CAS number
D-Mannitol	69-65-8
Ibuprofen	15687-27-1
Metformin	657-24-9
Saccharin	81-07-2
Sodium benzoate	532-32-1

2.5. Physiological map of the developing brain.

This task belongs to task 1.2, however, WP9 members were leading the data search and manual literature curation work (IUF, NIPH). The resulting first version of the physiological map of the developing brain is divided into several layers. The first layer offers an overview of mapped KNDPs and cell types and contains hyperlinks leading to deeper layers of the map. The second layer is focused on important cell-cell interactions that take place during brain development and is shown in Figure 2. The third layer consists of several submaps with detailed molecular and cellular information describing the development of different cell types, namely neurons, radial glia, astrocytes, and oligodendrocytes. This multi-layered physiological map is unique in being entirely developed with human-based data thus eliminating any biases that might stem from eventual species differences in human brain development. This work is currently being covered by a manuscript in preparation in cooperation with UL.
Furthermore, neurodevelopmental disorders-related data were gathered to display them at relevant nodes of the map. For this purpose, a new plugin was developed in cooperation with the Minerva team which manages the visualization platform of the ONTOX physiological maps. Besides the neurodevelopmental disorders plugin, transcriptomics data are planned to be visualized on the maps in Minerva as well. For this aim, IUF and NIPH carried out a literature search leading to the identification of transcriptomic data of healthy fetal brain samples as well as data from NDD brain tissue. These resources were forwarded to UM and UL for further processing before their addition to the physiological map.

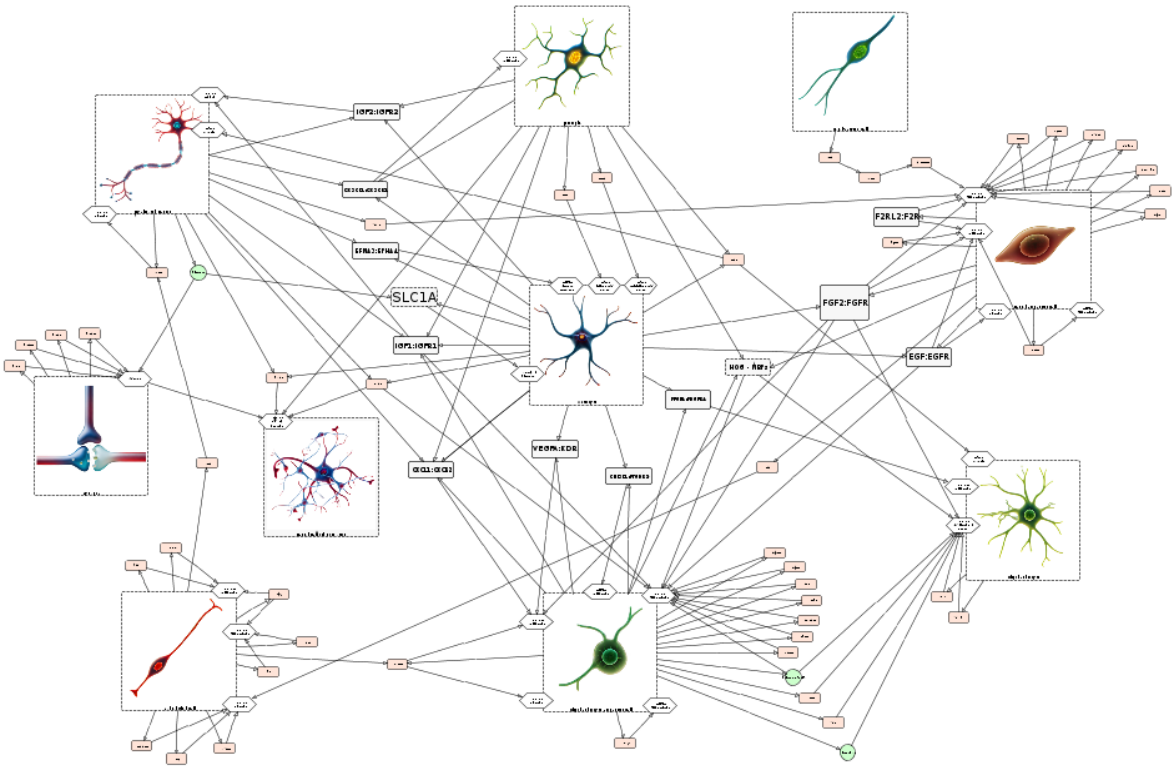

Figure 2: Cell-cell interaction physiological map of the developing brain displaying major cell types present in the developing brain.

3. Conclusions and follow-up

In conclusion, task 9.1 was successfully carried out by compiling and curating the AOP network for cognitive function defects, completing a literature review of cognitive function defects, suggesting four novel AOPs in AOPwiki (IDs 485-489), developing and selecting an *in vitro* battery to assess the KEs of the mentioned AOP network, and thoroughly characterizing the selected *in vitro* assays. Moreover, a chemical list containing CFD-inducing chemicals as well as chemicals not inducing DNT effects was prepared. Finally, a substantial time was consecrated into the development of a multiple-layered physiological map of the human developing brain.
This work resulted in two published publications, two manuscripts ready for submission, and three manuscripts in preparation. Moreover, the work was disseminated in the form of presentations and posters many times at international conferences by both IUF and NIPH (ESTIV, EUROTOX, IC-3Rs, SOT, ICT, WC12, DNT5, INA-18, and others).

4. Delays, issues and contingency

N/A

5. References

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