Test method development for the identification of endocrine disruption-mediated developmental neurotoxicity

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Entwicklung von Testmethoden zur Identifizierung von endokriner Disruption-vermittelter Entwicklungsneurotoxizität

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Tag der mündlichen Prüfung:

„Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less”

Marie Curie

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1 Introduction

1.1 Brain development

The brain is one of the most complex biological systems. Its development is characterized by the spatiotemporal organization of a multitude of intricate processes (Fig. 1). The brain consists of billions of neurons that form networks and neuronal connections, the basis for action, feelings, thought, and sensations (Stiles and Jernigan 2010; Stiles 2017). Brain development itself begins during the third gestational week (GW) and continues until late adolescence (Silbereis et al. 2016; Stiles 2017).

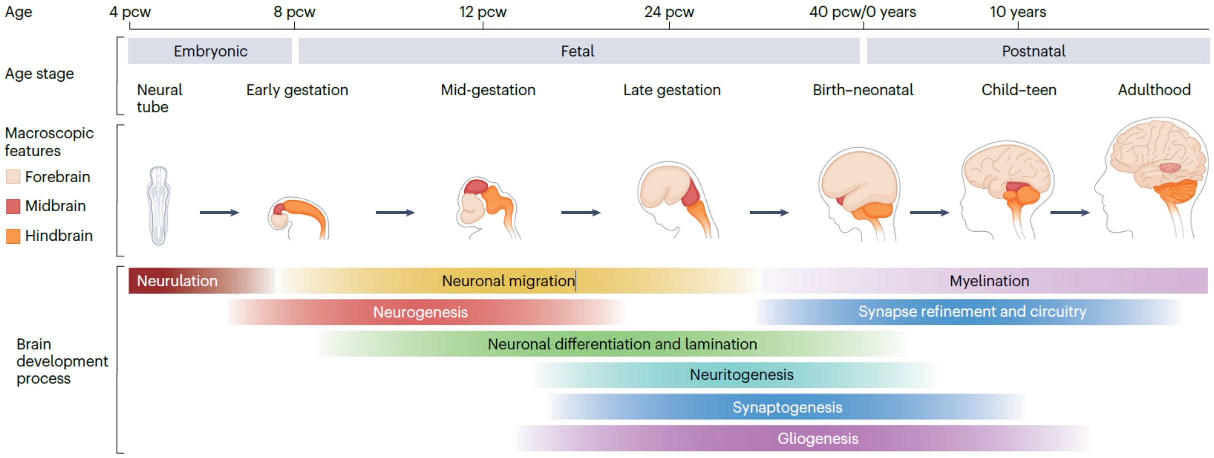

Figure 1: Stages of human brain development. Different processes of human brain development begin during different developmental timepoints pre- and postnatally. Abbreviations: pcw postconceptional weeks. (Adapted from (Zhou et al. 2024).

Prenatal development can be divided into two distinct phases, the embryonic and the fetal developmental phase. Embryonic development marks the onset of organ formation, while fetal development is the major phase of organ maturation. The different developmental phases, both pre- and postnatally, are characterized by distinct key neurodevelopmental processes (KNDPs) which overlap and are to a large extent dependent on each other (Fig. 1) (Andersen 2003; Silbereis et al. 2016). During the third GW, the ectoderm gives rise to neural progenitor cells (NPCs) capable of differentiating into the different cell types of the nervous system. Subsequently, central nervous system (CNS) development is initiated by neurulation and neural tube formation which precedes the formation of the brain and spinal cord (Copp et al. 2003; Stiles and Jernigan 2010). Neurulation is characterized by an enlargement of the NPC population and a transition into radial glia cells. These progenitor cells subsequently differentiate into glial cells (gliogenesis), such as oligodendrocytes and astrocytes, or into neurons (neurogenesis) (Stiles and Jernigan 2010; Silbereis et al. 2016). Radial glia cells migrate radially from the ventricular zone into the developing cortex, forming a scaffold, facilitating the migration of neurons, astrocytes, and oligodendrocytes along the scaffold (Borrell and Götz 2014; Fernández et al. 2016). The radial glia cells can be divided into apical and basal radial glia cells with the abundance of basal radial glia cells causing a divergence of the fibers during growth and subsequently a fan-like scaffold for cells to migrate along, resulting in the development of gyri and sulci. This so-called gyrencephaly is prevalent in for example primates and humans and is characterized by an increased cortical surface area and thus higher brain mass and number of neuronal cells. In contrast, a lower amount of basal radial glia cells can be found in lissencephalic species such as rodents where fibers follow a parallel trajectory (Sun and Hevner 2014; Borrell and Götz 2014). In addition, the timing of development and maturation of the brain varies across mammalian species. While at birth cortical neurogenesis and synaptogenesis have passed their peak in humans, it is still ongoing in mice (Workman et al. 2013). Moreover, the neural maturity of the human brain two months before birth correlates with the maturation state of newborn macaque monkeys and rodents two weeks after birth which further complicates extrapolation from other species to humans (Clancy et al. 2001).

With the onset of neurogenesis, the NPC proliferation pattern changes from a symmetrical pattern yielding two identical NPCs to an asymmetrical pattern generating both one NPC and one neuron. While the NPC remains in the ventricular zone, the neurons migrate into the outer layers of the neocortex. Neuronal migration therefore follows an inside-out pattern ultimately resulting in a six-layered neocortical mantle (Stiles and Jernigan 2010; Borsani et al. 2019). At their target location, neurons terminally mature into different subtypes of inhibitory and excitatory neurons and form connections to other neurons via synapses. These neural networks form the basis for signal transmission and communication within the brain via electrochemical information. Although synaptogenesis lasts from fetal development until adolescence, more than 50 % of neurons undergo apoptosis before birth (Stiles and Jernigan 2010). In addition, to increase the efficiency of signal transduction, about 50 % of synapses are disassembled during adulthood in a process called synaptic pruning (Stiles and Jernigan 2010; Borsani et al. 2019).

Neurogenesis occurs mainly prenatally between embryonic day 70 and 92 while radial glia cell fate changes from a neurogenic to a gliogenic cell fate in a process called the gliogenic switch. Moreover, glial progenitor cells continue proliferating, migrating, and differentiating after birth (Stiles and Jernigan 2010; Rash et al. 2019). Glial cells comprise a major cell type within the brain with astrocytes playing a critical role in forming the blood-brain barrier, neurotransmission, and metabolic regulation within the brain (Jessen 2004; Molofsky and Deneen 2015). On the other hand, oligodendrocytes are specialized cells producing myelin to insulate axons subsequently facilitating faster signal transduction due to saltatory transmission. In addition, oligodendrocytes maintain axonal integrity and neuronal survival (Jessen 2004; Emery 2010; Stiles and Jernigan 2010).

Since brain development is a multi-faceted process, it is orchestrated by a variety of different signaling pathways that regulate the different KNDPs and their interplay. One of the most important signaling pathways during brain development is the sonic hedgehog (SHH) signaling pathway. The presence of SHH is necessary for the dorsoventral patterning of the forebrain and regulates proliferation during corticogenesis. Moreover, SHH plays an important role in gliogenesis with SHH regulating the differentiation of progenitor cells into astrocytes and oligodendrocytes (Yabut and Pleasure 2018). On the other hand, notch signaling regulates neural stem cell maintenance and cell fate determination. Activation of notch signaling promotes glial and represses neurogenic cell fate, while notch inhibition elicits opposing effects by promoting neurogenesis and inhibiting gliogenesis (Engler et al. 2018; Koch et al. 2022). Furthermore, fibroblast growth factor (FGF) in combination with retinoic acid (RA) signaling regulates the induction and subsequent patterning of the hindbrain. FGF8 represses differentiation and neural patterning, while RA inhibits FGF8 signaling thus inducing neuronal differentiation and neural patterning (del Corral et al. 2003; Frank and Sela-Donenfeld 2019). In addition, the Wnt/β-catenin signaling pathway is another important pathway for mid-, hindbrain, and spinal cord development and plays a central role in neurogenesis and neuronal cell fate specification. Wnt signaling is implicated in the proliferation of NPCs, and its downregulation results in a switch from a proliferative to a differentiative state (Noelanders and Vleminckx 2017).

1.2 Development neurotoxicity (DNT)

Since physiological brain development relies on a complex interplay of different signaling pathways and KNDPs, disruption of any of the underlying processes of brain development by physical or chemical insult can adversely affect the developing brain subsequently causing functional or structural impairment, so called developmental neurotoxicity (DNT) (Giordano and Costa 2012). Moreover, due to the multitude of unique KNDPs occurring pre- and early postnatally, the developing brain is more susceptible to insults than the adult brain (Grandjean and Landrigan 2006). DNT is associated with neurodevelopmental disorders including autism spectrum disorder (ASD) or attention-deficit hyperactivity disorder (ADHD). These neurodevelopmental disorders can not only negatively influence the quality of life but also adversely affect society and economy if their prevalence in society increases (Grandjean and Landrigan 2006, Grandjean and Landrigan 2014; Giordano and Costa 2012).

In addition, the immune system and the mechanisms for metabolism, and excretion of xenobiotics are still not fully developed in the fetus and chemicals can be transported from the mother to the embryo/fetus thus further increasing the risk for the developing brain (Vizcaino et al. 2014; Mallozzi et al. 2016). Moreover, modifications of single genes can cause neurodevelopmental disorders, so called monogenic disorders. These disorders affect more than 1 % of children and range from diseases adversely affecting brain development such as monogenic ASD or fragile X syndrome to degenerative diseases such as certain forms of Parkinson's disease or amyotrophic lateral sclerosis (Chen et al. 2020). While the occurrence of neurodevelopmental disorders can partially be traced back to these genetic factors, environmental chemical exposure is discussed to have substantial contribution (Grandjean and Landrigan 2006, Grandjean and Landrigan 2014). Currently, 13 chemicals have been identified to specifically cause DNT in humans with for example ethanol exposure causing fetal alcohol spectrum disorders (FASD) or arsenic exposure being associated with adverse neurobehavioral performance in newborns (Grandjean and Landrigan 2014; Wang et al. 2018; Popova et al. 2023). Other cases of DNT compounds include polychlorinated biphenyls (PCB) that correlate with reduced cognitive function and ADHD-related behavior (Schantz et al. 2003; Jacobson and Jacobson 2003; Vreugdenhil et al. 2004).

Current chemical risk assessment for DNT is performed in *in vivo* test guidelines (TG), namely the developmental neurotoxicity study (OECD TG 426) and the cohort 2 of the extended one generation toxicity study (EOGRTS, TG 443) both addressing DNT specifically (OECD 2007, 2018a). However, neither the TG 426 nor the TG 443 cohort 2 are mandatory studies during chemical risk and hazard assessment and only need to be performed if a concern was identified in previous toxicity studies such as the 90 days repeated dose toxicity study (OECD TG 408) (OECD 2018b). These triggers include neurotoxicity, endocrine disruption (ED), or weight of evidence (WoE) suggesting any of the beforementioned triggers (Bal-Price et al. 2018b). Moreover, the DNT guideline studies have been raising concerns not only due to their apparent ethical issues but also due to their high costs and long duration for testing with the cost exceeding 1 million Euro over a duration of more than one year (Meigs et al. 2018; Paparella et al. 2020). In addition, they do not provide mechanistic information, and species differences in brain development including differing developmental windows, or brain composition and structure, highlighted by i.e., gyrencephaly and humans and lissencephaly in rodents, further question the predictivity of *in vivo* DNT TGs for humans (Paparella et al. 2020).

One of the potential mechanisms for DNT is disruption of the endocrine system. The endocrine system plays a pivotal role during physiological brain development with disruption of different signaling pathways such as the RA and glucocorticoid pathway being associated with neurodevelopmental deficits ranging from microcephaly to memory deficits, anxiety disorders, and ADHD (Moisiadis and Matthews 2014; Pirozzi et al. 2018).

1.3 Endocrine signaling during brain development

Some signaling molecules such as sex steroids, glucocorticoids or thyroid hormones (TH) are secreted by glands (e.g., adrenal gland or thyroid) transported to their target tissue via the bloodstream where they elicit cell type specific reactions, generally binding to nuclear receptor (NR) and subsequently regulating target gene expression. These molecules are thus part of the endocrine system. The endocrine system plays a pivotal role in maintaining the physiological development of the developing and adult brain (Gkikas et al. 2017; Miranda and Sousa 2018; Ponzi et al. 2020; Oliveira et al. 2021). The endocrine system develops as one of the first systems during human development and originates from all three germ layers. The gonads and adrenal cortex which produce sex steroids stem from mesoderm, the pituitary develops from ectoderm and other endocrine glands that produce amine and peptide hormones originate from endoderm (Betts et al. 2022). During early embryonic development, a major site for hormone synthesis and secretion, the so called feto-placental unit, develops. This unit functions as a link between the fetus and mother and coordinates respiratory and excretory functions, the exchange of metabolic products and nutrients, and is dependent on neuronal and hormonal factors from both mother and fetus. The endocrine system develops and matures during embryonic and fetal development with i.e., the thyroid gland and pituitary emerging by GW 12 and GW 7 respectively. Although the placenta prevents most maternal hormones from reaching the fetus due to its unique functions, the maternal hormones themselves are necessary for the development of the fetal endocrine system by regulating the synthesis of hormones in the placenta and fetus (Tal and Taylor 2000).

Endocrine active compounds, or hormones, are organic substances which circulate the body at low concentrations inducing specific responses at target cells and tissues (Gronemeyer et al. 2004; Gkikas et al. 2017). Endocrine active compounds can largely be divided by structure and source into steroidal and non-steroidal hormones. Steroid hormones consist of the sex hormones, androgen and estrogen, oxysterols, mineralcorticoids, and glucocorticoids which are lipophilic and can cross the cell membrane freely (Cole et al. 2019). Non-steroid hormones such as retinoids or amino acid derivates such as triiodothyronine (T3) and thyroxine (T4) are hydrophilic substances that cannot cross the cell membrane (Feher 2017). These hormones act via membrane-bound receptors or are transported into the cell by specific transport proteins (Fig. 2) (Kedishvili 2016; Cole et al. 2019). Lastly, fatty acid binding proteins (FABP) facilitate the transport and uptake of peroxisome proliferator-activated receptor (PPAR) ligands which consist primarily of fatty acids and their metabolites (Tan et al. 2002; Hostetler et al. 2009). Since the blood-brain barrier (BBB) functions as a barrier to separate the brain from peripheral tissues, it also prevents most substances from reaching the brain uncontrolled. Therefore, hormones must first cross the BBB to enact their functions in brain development and homeostasis. Due to their lipophilic nature, unbound steroid hormones such as sex steroids or glucocorticoids can pass the BBB freely by transmembrane diffusion. On the other hand, non-lipophilic hormones such as TH cross the BBB via active transport facilitated by peptide transporters (Banks 2012; Hampl et al. 2015). Furthermore, the developing brain is much more prone to chemical insult since the BBB is not fully matured and is thus more permeable for chemicals to reach the brain (Schettler 2001). Within cells, hormones commonly act by binding to NRs that subsequently bind DNA response elements and induce target gene expression (Robinson-Rechavi et al. 2003). These NRs are part of a superfamily of transcriptional regulators and are involved in organ development and different physiological functions, such as cell or organ homeostasis, i.e., salt and water resorption in kidneys by regulating the expression of effector genes (Gkikas et al. 2017; Frigo et al. 2021). NRs can act as monomers, form homodimers, such as androgen receptor (AR) and estrogen receptor (ER), or heterodimers. The main partner for heterodimerization is the retinoid X receptor (RXR), which forms heterodimers with the retinoic acid receptor (RAR), liver X receptor (LXR), PPAR, vitamin D receptor (VDR) or the thyroid hormone receptor (THR) (Gronemeyer et al. 2004; Evans and Mangelsdorf 2014). While the RXR forms permissive heterodimers with the LXR or PPAR which can be activated by ligand binding to any of the two receptors, non-permissive heterodimers, formed with RAR, THR or VDR can only be activated by ligand binding to the partner receptor, not the RXR itself (Evans and Mangelsdorf 2014).

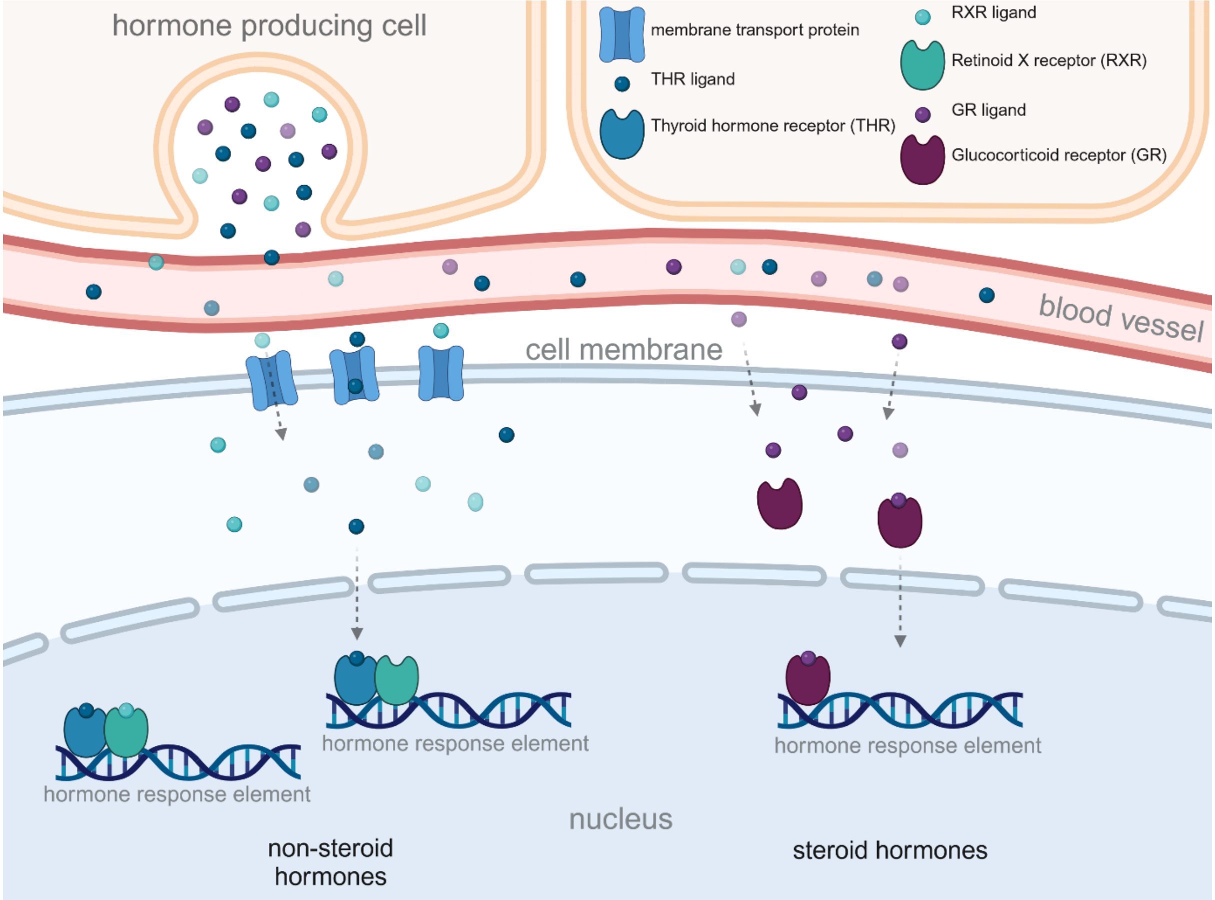

Figure 2: Modes for endocrine signaling. After hormone-producing cells release the signaling molecules into the blood stream they are transported to target cells. While non-steroid hormones such as thyroid hormones and retinoid X receptor ligands pass the cell membrane via membrane-bound transport proteins, steroid hormones such as glucocorticoids pass the cell membrane freely due to their lipophilic character. Within the cells, the ligands bind to receptors which are present as monomers or dimers. The receptors are either present in the nucleus and bound to hormone response elements (LXR, PPAR, THR, RAR, RXR) or they are present in the cytosol (AR, ER, GR). If the receptor is present in the cytosol, the ligand-receptor complex translocates into the nucleus where it binds to hormone response elements of the DNA and subsequently induces target gene expression. Created with Biorender.com

Different hormones elicit different functions and actions during brain development. For example, TH, which are produced in the thyroid gland and transported via the bloodstream to target tissues such as the brain, mediate the differentiation and maturation of oligodendrocytes (Rodríguez-Peña 1999; Baxi et al. 2014). On the other hand, RA acts as a paracrine and autocrine signaling molecule which is taken up as the non-active retinol and transported to target cells. In the target cells, retinol is metabolized to the active compound all-*trans* retinoic acid (*at*RA). *at*RA binds to RAR which are present as RAR-RXR heterodimers bound to RAR response elements in the DNA and subsequently regulates target gene expression (Maden 2002; Duester 2008; Rhinn and Dollé 2012). These target genes elicit responses ranging from mediating neural tube formation to regulating hindbrain patterning in combination with FGF8 (del Corral et al. 2003; Wilson et al. 2003; Glover et al. 2006; Janesick et al. 2015). Moreover, *at*RA is implicated in developing CNS vasculature via WNT signaling (Bonney et al. 2016;

Pawlikowski et al. 2019). Furthermore, RA is necessary for neuronal differentiation by initiating a cell cycle stop and the subsequent shift from proliferation to differentiation in NPCs (Janesick et al. 2015).

Glucocorticoids are steroid hormones that are synthesized from cholesterol and regulate different physiological processes and functions including development, inflammation, and stress response (Vitellius et al. 2018; Timmermans et al. 2019). Due to their lipophilic nature, glucocorticoids cross cell membranes of target cells directly. Within the cells, the glucocorticoids bind to glucocorticoid receptors (GR) in the cytosol, that subsequently translocate into the nucleus, bind to GR response elements as either monomers or homodimers and regulate target gene expression (Timmermans et al. 2019). Glucocorticoids are downstream effectors of the hypothalamus-pituitary-adrenal axis and play a pivotal role in the maturation of different organs such as lungs, liver, or the central nervous system during fetal development (Miranda and Sousa 2018). Moreover, glucocorticoids coordinate a switch from proliferation to differentiation, regulate neuronal migration by altering myosin function, and induce neurite outgrowth by regulating gene expression associated with microtubules and the actin cytoskeleton (Datson et al. 2008; Fukumoto et al. 2009; Miranda and Sousa 2018).

Hormonal involvement vastly differs between developmental windows and species. For example, the thyroid system regulates a variety of processes in different species such as cardiovascular function in humans, the neurological development of mammals or postembryonic metamorphosis in amphibians (Zoeller and Rovet 2004; Jabbar et al. 2017; Thambirajah et al. 2019). Moreover, the developmental window stipulates functions of the endocrine system. This can be seen by a developmental stage-dependent TH metabolism after glucocorticoid exposure in rats. While exposure to glucocorticoids in rat fetuses does not affect circulating T3 or T4 levels or deiodinase activity in the brain, postnatal exposure to glucocorticoids increases deiodinase activity in the brain and reduces circulating T3 and T4 levels, thus revealing different susceptibility of TH physiology and homeostasis to glucocorticoid treatments depending on the timepoint of exposure (Van der Geyten and Darras 2005).

Although the endocrine system is present in most species, differences have evolved. The role of RA in the development of cortical neurons and the prefrontal cortex is conserved in humans, chimpanzees, and mice, yet the expansion of the frontal cortex characterized by a layer 4 is specific for humans and primates (Shibata et al. 2021). Furthermore, sexual differentiation in rodent brains occurs shortly before and after birth and is guided by estrogens regulating apoptosis and cell survival as well as neurite growth via MAP kinase activation (McCarthy 2008). On the other hand, sexual differentiation in primates occurs from mid to late gestation and is guided by androgens determining development of masculine sexual behavior and even causing masculinization of female genitalia (Wallen 2005).

Disruption of the endocrine system during development can cause severe adverse effects leading to neurodevelopmental disorders. For example, TH insufficiency causes congenital hypothyroidism (Chen and Hetzel 2010; Noyes et al. 2019; LaFranchi 2021). Furthermore, up- and downregulation of TH signaling is associated with adverse brain development defined by reduced IQ, grey matter and cortex volume (Korevaar et al. 2016). On the other hand, both excess and insufficiency of RA are associated with altered hindbrain patterning and neural tube malformations such as *spina bifida* (DeLoia and Solter 1990; Glover et al. 2006).

1.4 Endocrine disruption (ED)

Currently, the effects of only a few endocrine signaling pathways on the developing brain, namely androgen, estrogen, RA, and TH signaling, have been thoroughly investigated, while the impact of the multitude of other endocrine pathways is only sparsely understood (Kajta and Beyer 2003; Bernal 2005, Bernal 2007; Glover et al. 2006; Frank and Sela-Donenfeld 2019; Brann et al. 2022; Alemany 2022). Substances interfering with the endocrine system are termed endocrine disrupting chemicals (EDCs). EDCs can adversely affect the endocrine system at low concentrations and in certain cases i.e., PCBs, were shown to bioaccumulate in the environment (Masuo and Ishido 2011; Montano et al. 2022). Despite their ban in the 1970s PCBs are still ubiquitously found in the environment due to their high persistence and low biodegradability highlighted by half-lives of up to over 40 years (Venier and Hites 2010; Seegal et al. 2011). Although EDCs pose a significant health concern for humans and the environment, linking EDC exposure to developmental disorders remains difficult. However, increasing evidence suggests the influence of EDCs in reproductive, metabolic, neurological, and cardiovascular disorders (Ahn and Jeung 2023). For example, the synthetic estrogen diethylstilbestrol (DES) was given to pregnant women from the 1940s to 1970s to prevent miscarriage and complications during pregnancy (Patisaul et al. 2018). Long-term studies revealed the development of severe reproductive abnormalities ranging from cancers in the reproductive tract and increased risk for the development of breast cancer in daughters and testicular cancers and malformations of the urogenital tract in sons of DES-treated women (Palmer et al. 2006; Harris and Waring 2012). As a further example, PCBs were shown to reduce T4 levels in rats and elicit agonistic and antagonistic effects on the THR (Naveau et al. 2014; Ghassabian and Trasande 2018). Moreover, PCB exposure, measured by cord-blood concentrations, has been linked to neurodevelopmental deficits characterized by reduced cognitive function and intelligence in children (Jacobson et al. 1990; Darvill et al. 2000; Stewart et al. 2003). In addition, epidemiological studies indicate that exposure to pyrethroid pesticides adversely affects neurobehavioral development (Viel et al. 2015, Viel et al. 2017). These effects are hypothesized to be partially caused by ED with studies observing altered TH levels after exposure to pyrethroids (Kaul et al. 1996; Giray et al. 2010; Leemans et al. 2019).

With emerging evidence of EDC exposure correlating with adverse effects such as neurodevelopmental disorders, increasing efforts have been put into identifying EDCs in the human exposome (Sánchez et al. 2024). Currently, the framework for testing and assessment of endocrine disruptors focuses primarily on estrogenic, androgenic, thyroidogenic, and steroidogenic (EATS) modalities and their effects on reproduction. Therefore, current risk assessment for EDCs mostly disregards other endocrine signaling pathways such as the retinoid or glucocorticoid system, other target organs and processes such as brain development (Browne et al. 2020; Grignard et al. 2020; Martyniuk et al. 2022). While first *in vitro* test methods target receptor binding and transactivation of ER, AR, and THR, other assays target certain enzymes important for hormone synthesis such as the US EPA TG OPPTS 890.1200 (Aromatase Assay) (OECD 2018c). More mechanistic *in vivo* ED assays such as the TG 440 (Uterotrophic Assay) and the TG 441 (Hershberger Assay) assess only reproductive organs and give limited mechanistic information on the endocrine mode of action MoA (OECD 2009, OECD 2018c, OECD 2018d; Coady et al. 2017). In addition, only the TG 422 (Combined repeated dose toxicity study with the reproduction/ developmental toxicity screening test) opens the opportunity to assess TH-dependent perturbations of the developing brain by measurement of TH concentrations in the blood without however deriving a MoA (OECD 2015). Lastly, the more complex *in vivo* TGs, namely the TG 426 and TG 443, while assessing effects on brain development or reproductive toxicity, give limited information on an underlying endocrine MoA, thus making it difficult to identify EDCs using these TGs (Coady et al. 2017). Furthermore, these studies do not allow for a reliable identification of ED-DNT which, in combination with the lack of comprehensive testing for DNT leaves a potentially hazardous data gap (Bal-Price et al. 2018b). Furthermore, interspecies differences can affect the susceptibility to EDCs with for example a higher rate of TH synthesis and lower TH half-life in rats compared to humans (Jahnke et al. 2004; Patisaul et al. 2018).

To advance testing and assessment of endocrine disruptors, the European Horizon 2020's Eurion Cluster was formed. The Eurion Cluster consists of 8 projects which focus on different aspects of new testing and screening methods to identify EDCs and subsequently contribute to the development of new, international, strategies for testing and assessment of endocrine disrupters. These projects focus on TH disruption (Kortenkamp et al. 2020; Moroni et al. 2020), female reproductive toxicity of EDCs (van Duursen et al. 2020), metabolic effects, and metabolic disorders caused by EDCs (Audouze et al. 2020; Küblbeck et al. 2020; Legler et al.

2020), optimization of guidelines for human health and environmental testing of EDCs (Holbech et al. 2020) and ED-DNT (Lupu et al. 2020). In addition to these projects, new platforms identified the necessity to advance regulatory testing of endocrine disruptors and facilitate the process of validation and integration of these test methods into regulatory frameworks (Grignard et al. 2022).

1.5 Alternatives in toxicity testing

Due to the above-mentioned challenges of the current toxicological hazard and risk assessment, a paradigm shift away from *in vivo* studies, which are not only resource- and time-intensive but also cause severe ethical issues, has been recommended. This Next Generation Risk Assessment (NGRA) is exposure-driven, hypothesis-led and based on high-throughput *in vitro, in chemico* and *in silico* methods (Luo et al. 2023). Moreover, a focus on cell-based and biochemical screening assays enables the screening of a higher amount of chemicals compared to classical *in vivo* rodent studies (Paparella et al. 2020).

However, since alternative methods are not widely accepted for their regulatory use, integrated approaches for testing and assessment (IATA) are being developed which use data from multiple sources for hazard identification and characterization as well as safety assessment of chemicals (Bal-Price et al. 2018b). This data is derived from different *in silico, in chemico, in vitro,* and *in vivo* methods in a WoE-based approach to facilitate decision-making on the potential hazard or risk of a test substance (OECD 2017a). For example, after the ban on animal testing for cosmetic ingredients in 2013 great efforts have been made to develop and validate alternative methods based on the key events leading to skin sensitization. The obtained data is then used in an IATA to identify the capacity of chemicals to act as skin sensitizers (de Ávila et al. 2019; Gądarowska et al. 2022). A similar shift in toxicology has been adopted in DNT testing culminating in the assembly of cell-based *in vitro assays* into a DNT *in vitro* testing battery (DNT-IVB) consisting of 17 *in vitro* assays from the US Environmental Protection Agency (EPA), and two working groups from the Leibniz – Research Institute for Environmental Medicine (IUF) and the University of Konstanz (UKN) (Fritsche et al. 2018; Sachana et al. 2019; Crofton and Mundy 2021). The DNT-IVB assays assess eight KNDPs based on mainly human cells. These KNDPs are proliferation (NPC1, hNP1), migration (UKN2, NPC2a-c), differentiation into neurons (NPC3) and oligodendrocytes (NPC5), neurite outgrowth (NPC4, UKN4, UKN5, hN ini, Cortical ini), apoptosis (hNP1), synaptogenesis (rCortical, rSynaptogenesis), and neural network formation (rCortical MEA) (Fig. 3). While some assays seem redundant investigating the same endpoint, differences in cell model and cell type, for example, migration of radial glia cells (NPC2a), neurons (NPC2b), and oligodendrocytes (NPC2c) derived from primary human NPCs, or migration of human induced pluripotent stem cell (hiPSC)-derived neural crest cells (UKN2), exhibit different chemical sensitivities since they address different chemical MoAs. Moreover, these redundancies were identified to increase the predictivity of the DNT-IVB (Masjosthusmann et al. 2020).

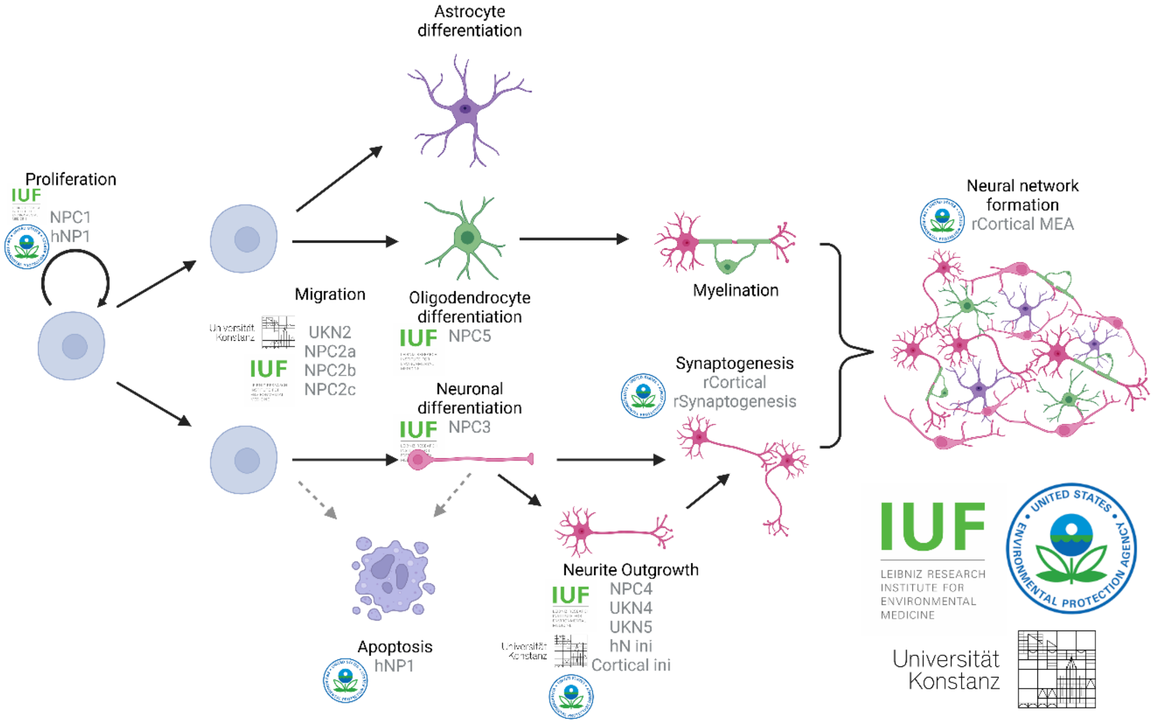

Figure 3: Key neurodevelopment processes (KNDPs) of brain development. Neural stem cells proliferate and differentiate into glial and neuronal progenitor cells. Those progenitor cells then migrate to target locations and terminally differentiate into the respective effector cells of the brain. Differentiated oligodendrocyte mature and begin myelinating axons of neurons. Differentiated neurons interact via synapses ultimately forming neural networks which consist of neurons, oligodendrocytes and astrocytes. The DNT-IVB covers different KNDPs of brain development. Created with Biorender.com

To confirm the transferability, reproducibility and biological relevance of toxicological test methods, a strict validation process is being required by the OECD before allowing the application of these methods for current regulatory risk and hazard characterization (OECD 2005; Griesinger et al. 2016). The classic validation process relies on the comparison to animal data and is a time-consuming process, especially regarding the transferability of the test method to other laboratories in form of ring trials. On the other hand, novel approaches for NAMs suggest focusing on a more scientific, mechanistic process (Hartung et al. 2013; van der Zalm et al. 2022). This approach targets the underlying mechanistic rationale focusing on MoA, adverse outcome pathways (AOP), and pathways of toxicity (Leist et al. 2012; Hartung et al. 2013; Bal-Price et al. 2018a). Since intra-laboratory reproducibility and biological relevance are requirements to ascertain the performance and readiness of NAMs, the evaluation of interlaboratory transferability is essential also for the validation of NAMs (OECD 2005). Therefore, adapting the validation process for novel test methods using a pre-validation approach is an important step in advancing the development and use of *in vitro* test methods without expending large amounts of resources or animals in the process (OECD 2005; Gibb 2008; Krewski et al. 2020). One example for an envisioned application of NAMs in toxicological hazard assessment is the DNT-IVB. The DNT-IVB is planned for use for the initial screening and prioritization of substances with missing information on their DNT potential and for follow-up screens after identification of DNT potential in *in silico* quantitative structure-activity relationship (QSAR) and Read-Across approaches. Moreover, the DNT-IVB is planned to support single compound hazard assessment for either a target-focused approach for DNT *in vivo* guideline studies or to assess the ambiguity of existing *in vivo* data and ultimately replace *in vivo* DNT studies in defined approaches (Crofton and Mundy 2021).

1.6 The Neurosphere assay

One test method that is part of the DNT-IVB is the human Neurosphere assay, a high-content medium-throughput assay which allows for the investigation of chemical effects on several KNDPs (Fig. 4) (Moors et al. 2009; Masjosthusmann et al. 2020; Koch et al. 2022). Furthermore, the assay is established in male and female NPCs derived from different species, i.e., humans, rodents, and rabbits, thus enabling the assessment of sex and species differences (Baumann et al. 2014, 2016; Masjosthusmann et al. 2018; Barenys et al. 2021; Koch et al. 2022; Pla et al. 2022).

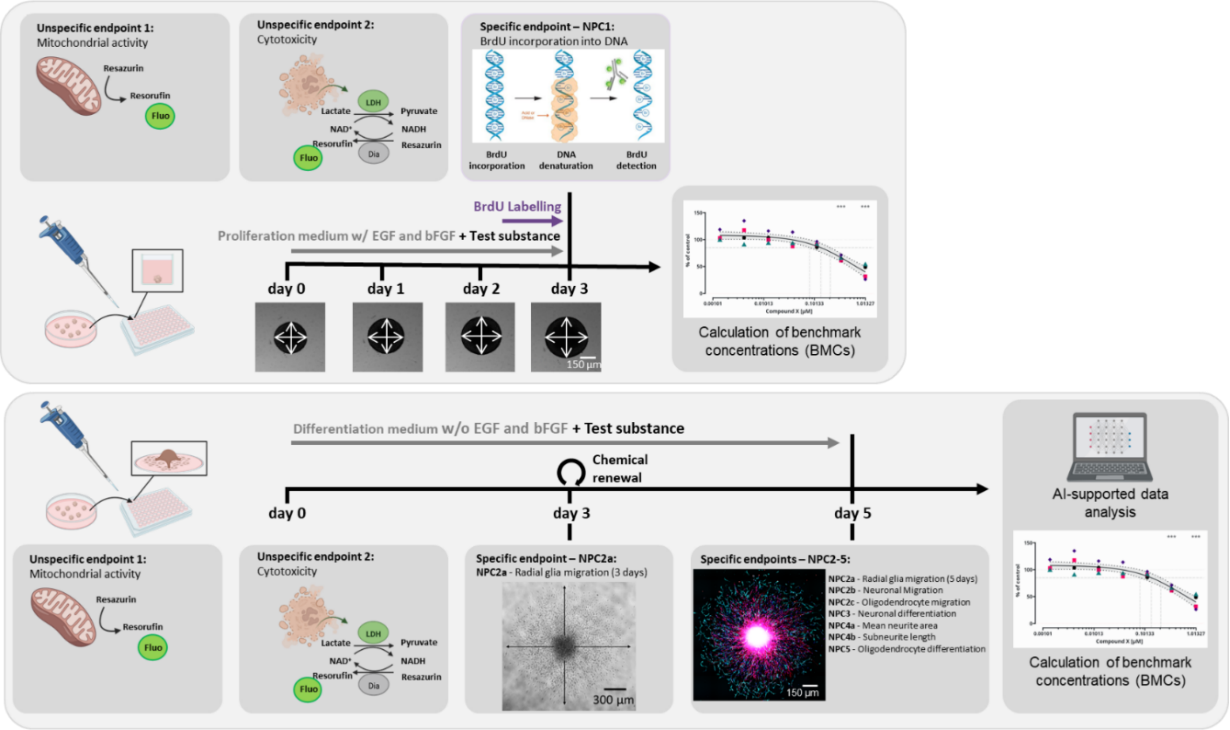

Figure 4: Experimental setup of the Neurosphere Assay. hNPCs generated from gestational week (GW) 16-19 fetal brain cortices (Lonza, Belguim) are cultivated in the presence of growth factors epidermal growth factor (EGF) and fibroblast growth factor (FGF) as three-dimensional cell aggregates, so called neurospheres. The neurospheres are mechanically passaged two to three days prior to an experiment in order to yield equally sized spheres. Proliferation is assessed in presence of growth factors by BrdU incorporation into newly synthesized DNA (NPC1). Differentiation is assessed by plating neurospheres on an extracellular matrix consisting of poly-D-lysine and laminin in absence of growth factors. Cells then migrate radially out of the sphere core and differentiate into effector cells, namely astrocytes, neurons, and oligodendrocytes. Immunocytochemical staining and two convolutional neural networks (CNN) enable the assessment of radial glia (NPC2a), neuronal (NPC2b), and oligodendrocyte migration (NPC2c), differentiation into neurons (NPC3) and oligodendrocytes (NPC5), as well as neurite length (NPC4a) and neurite area (NPC4b) after five days of cultivation. In addition, cytotoxicity and mitochondrial activity assays are performed in order to exclude any unspecific effects due to cytotoxicity. Using AI-supported data analysis, benchmark-concentrations for different compounds and endpoints can be calculated. Abbreviations: hNPC, human neural progenitor cell; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDL, poly-D-lysine; AI, artificial intelligence; BMC, benchmark concentration. Created with Biorender.com

In previous studies, species differences in terms of sensitivity to DNT chemicals were confirmed since rat-derived NPCs (rNPCs) showed a higher sensitivity toward methylmercury-induced reduction of neuronal differentiation than human-derived NPCs (hNPCs) (Masjosthusmann et al. 2019). In addition, sodium arsenite was revealed to elicit species specific effects. Whereas hNPC proliferation was the most sensitive endpoints (MSE) in hNPCs, in rNPCs the KNDP neuronal differentiation was most sensitive to arsenite exposure (Baumann et al. 2016; Masjosthusmann et al. 2019). Moreover, previous studies included the investigation of different flame retardants, Chinese herbal medicine, and the characterization of ED-DNT focusing on species differences in aryl hydrocarbon receptor (AhR) and TH signaling (Schreiber et al. 2010; Gassmann et al. 2010; Dach et al. 2017; Klose et al. 2020, Klose et al. 2022, Klose et al. 2023). Here, species differences in AhR signaling during neurodevelopment *in vitro* became apparent, since AhR activation reduced migration and proliferation in mouse-derived NPCs (mNPCs) while hNPCs and AhR knock-out mNPCs remained unaffected by AhR activation (Gassmann et al. 2010). Moreover, hNPCs were shown to be more susceptible to disruption of T3-mediated oligodendrocyte maturation than rat NPCs, and the flame retardant tetrabromobisphenol A (TBBPA) was identified as an EDC causing DNT via THR antagonism (Klose et al. 2020). In conclusion, previous studies suggest that the human Neurosphere Assay is capable to detect EDCs eliciting ED-DNT especially via THR disruption, however, a comprehensive analysis of the hormonal dependencies of the modelled KNDPs was lacking and thus is one focus of this thesis.

1.7 Aims

The current framework for EDC risk assessment has severe limitations regarding the coverage of the plethora of hormone receptors expressed in the developing brain, thus questioning its predictivity in identifying EDCs that cause ED-dependent DNT. Moreover, species differences between rodents and humans regarding brain development, both in a non- endocrine and endocrine context, further limit the predictivity of the current EDC risk assessment process that mainly relies on rodent models. This thesis was performed as part of the Horizon 2020 research and innovation program ENDpoiNTs, funded by the European Commission, focusing on the closure of the scientific data gap regarding ED-DNT and the development of novel human-relevant test methods that shall identify EDCs harming the developing brain with higher predictivity for humans than the current EDC risk assessment process. In order to contribute to this common effort, this thesis applied the DNT-IVB Neurosphere Assay to investigate its applicability for the identification of EDCs in detail. The following aims were pursued to (i) increase the confidence in the biological relevance of the Neurosphere Assay and its predictivity to identify DNT chemicals, (ii) identify its applicability domain regarding the detection of ED-DNT focusing on underlying sex- and species-specificities and (iii) assess its values within a novel testing battery comprising different *in vitro* and *in silico* ED-DNT NAMs:

1. A scientific and mechanistic validation of the Neurosphere Assay to confirm biological relevance of the modelled KNDPs as well as an adequate response to biological stimuli and DNT reference chemicals.
2. The investigation of hormonal dependencies of the KNDPs modelled within the Neurosphere Assay to close the scientific data gap regarding the influence of hormones during brain development.
3. The study of sex-and species-specificities in terms of the impact of hormone receptor signaling during brain development.
4. The development of new ED-DNT NAMs based on the hormone-sensitive KNDPs modelled within the Neurosphere Assay.

2 Manuscripts

The present thesis consists of the following three manuscripts:

The first manuscript 2.1, ‘Scientific validation of human Neurosphere Assays for developmental neurotoxicity evaluation’ (Koch et al. 2022) describes the five-step scientific validation of the ‘Neurosphere Assay’ (NPC1-6) and two additional, human induced pluripotent stem cell (hiPSC)-based, assays (iNPC1-2a).

The second manuscript 2.2, ‘Nuclear hormone receptors control fundamental processes of human fetal neurodevelopment: Basis for endocrine disruption assessment’ (Koch et al. in preparation) explores the impact and importance of hormone receptor signaling and endocrine disruption on different KNDPs in NPCs in a species overarching context.

The third manuscript 2.3, ‘The NPC1_RAR_GR assay identifies putative endocrine disrupting chemicals (EDCs) perturbing glucocorticoid receptor (GR) or retinoic acid receptor (RAR) signaling in developing human brain cells’ (Schlüppmann et al. in preparation) presents the development of a novel human-based test method to identify EDCs disturbing GR and RAR signaling.

2.1 Scientific validation of human Neurosphere Assays for developmental neurotoxicity evaluation

Katharina Koch, Kristina Bartmann, Julia Hartmann, Julia Kapr, Jördis Klose, Eliška Kuchovská, Melanie Pahl, Kevin Schlüppmann, Etta Zühr, Ellen Fritsche

There is a call for a paradigm shift in developmental neurotoxicity (DNT) evaluation, which demands the implementation of faster, more cost-efficient, and human-relevant test systems than current in vivo guideline studies. Under the umbrella of the Organisation for Economic Co-operation and Development (OECD), a guidance document is currently being prepared that instructs on the regulatory use of a DNT in vitro battery (DNT IVB) for fitfor- purpose applications. One crucial issue for OECD application of methods is validation, which for new approach methods (NAMs) requires novel approaches. Here, mechanistic information previously identified in vivo, as well as reported neurodevelopmental adversities in response to disturbances on the cellular and tissue level, are of central importance. In this study, we scientifically validate the Neurosphere Assay, which is based on human primary NPCs (hNPCs) and an integral part of the DNT IVB. It assesses neurodevelopmental key events (KEs) like NPC proliferation (NPC1ab), radial glia cell migration (NPC2a), neuronal differentiation (NPC3), neurite outgrowth (NPC4), oligodendrocyte differentiation (NPC5), and TH-dependent oligodendrocyte maturation (NPC6). In addition, we extend our work from the hNPCs to human induced pluripotent stem cell-derived NPCs (hiNPCs) for the NPC proliferation (iNPC1ab) and radial glia assays (iNPC2a). The validation process we report for the endpoints studied with the Neurosphere Assays is based on 1) describing the relevance of the respective endpoints for brain development, 2) the confirmation of the cell type-specific morphologies observed in vitro, 3) expressions of cell type-specific markers consistent with those morphologies, 4) appropriate anticipated responses to physiological pertinent signaling stimuli and 5) alterations in specific in vitro endpoints upon challenges with confirmed DNT compounds. With these strong mechanistic underpinnings, we posit that the Neurosphere Assay as an integral part of the DNT in vitro screening battery is well poised for DNT evaluation for regulatory purposes.

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Scientific Validation of Human Neurosphere Assays for Developmental Neurotoxicity Evaluation

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There is a call for a paradigm shift in developmental neurotoxicity (DNT) evaluation, which demands the implementation of faster, more cost-efficient, and human-relevant test systems than current *in vivo* guideline studies. Under the umbrella of the Organisation for Economic Co-operation and Development (OECD), a guidance document is currently being prepared that instructs on the regulatory use of a DNT *in vitro* battery (DNT IVB) for fit-for-purpose applications. One crucial issue for OECD application of methods is validation, which for new approach methods (NAMs) requires novel approaches. Here, mechanistic information previously identified *in vivo*, as well as reported neurodevelopmental adversities in response to disturbances on the cellular and tissue level, are of central importance. In this study, we scientifically validate the Neurosphere Assay, which is based on human primary neural progenitor cells (hNPCs) and an integral part of the DNT IVB. It assesses neurodevelopmental key events (KEs) like NPC proliferation (NPC1ab), radial glia cell migration (NPC2a), neuronal differentiation (NPC3), neurite outgrowth (NPC4), oligodendrocyte differentiation (NPC5), and thyroid hormone-dependent oligodendrocyte maturation (NPC6). In addition, we extend our work from the hNPCs to human induced pluripotent stem cell-derived NPCs (hiNPCs) for the NPC proliferation (iNPC1ab) and radial glia assays (iNPC2a). The validation process we report for the endpoints studied with the Neurosphere Assays is based on 1) describing the relevance of the respective endpoints for brain development, 2) the confirmation of the cell type-specific morphologies observed *in vitro*, 3) expressions of cell type-specific markers consistent with those morphologies, 4) appropriate anticipated responses to physiological pertinent signaling stimuli and 5) alterations in specific *in vitro* endpoints upon challenges with confirmed DNT compounds. With these strong mechanistic underpinnings, we posit that the Neurosphere Assay as an integral part of the DNT *in vitro* screening battery is well poised for DNT evaluation for regulatory purposes.

Keywords: developmental neurotoxicity, neural progenitor cells, neurons, oligodendrocytes, new approach methodologies, 3D *in vitro* models, human induced pluripotent stem cells, radial glia cells

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1 INTRODUCTION

During the last years, enormous scientific and regulatory efforts have been focusing on the establishment of a novel procedure for developmental neurotoxicity (DNT) evaluation (Sachana et al., 2019). The two main drivers for these efforts were the extremely high costs that current DNT guideline studies demand and the resulting overall lack of data, including mechanistic information, that exists for chemicals concerning their influence on brain development. In addition, unique features of the human brain and its development (Rice and Barone Jr., 2000; Dehay and Kennedy, 2007, 2009; Somel et al., 2011; Florio and Huttner, 2014; Pollen et al., 2015; Borrell, 2019) strongly support the general endeavor to use human-derived models for risk decisions in 21st-century toxicity evaluation (National Research Council, 2007). There is a vast amount of data on different compound classes including metals, pesticides, and drugs linking compound exposure to adverse neurodevelopmental outcomes in children, like a drop in IQ or memory and attention deficits (Vorhees et al., 2018). Nevertheless, so far only 110–140 chemicals have been evaluated using *in vivo* DNT guideline studies (Makris et al., 2009; Paparella et al., 2020), while for the majority of the human exposome this data is lacking (Sachana et al., 2021a). Moreover, the contribution of chemical exposure to human neurodevelopmental diseases like autism spectrum or attention deficit hyperactivity disorder has so far only been heavily discussed on an associative basis but not finally mechanistically substantiated (Grandjean and Landrigan, 2006; Abbasi, 2016; Bennett et al., 2016; Gould et al., 2018; Moosa et al., 2018; Cheroni et al., 2020; Masini et al., 2020). Considering the social, societal and economic consequences that DNT entails (Bellinger, 2012; Grandjean and Landrigan, 2014), it is obvious that strategies are needed that allow a faster, more cost-efficient and human-relevant assessment of DNT for closing this obvious data gap.

Efforts for the implementation of DNT *in vitro* alternative methods for hazard identification and risk assessment have been evolving over more than 15 years (Coecke et al., 2007; Lein et al., 2007; Crofton et al., 2011; Bal-Price et al., 2012; Bal-Price et al., 2015a; Fritsche, 2017; Fritsche et al., 2017). According to the proposed paradigm shift in DNT testing (Sachana et al., 2019), a DNT *in vitro* battery (IVB) was assembled based on neurodevelopmental key events (KE; Fritsche et al., 2018b) and assay readiness (Bal-Price et al., 2018). DNT test methods have been assembled (Harrill et al., 2018; Masjosthusmann et al., 2020) and are the basis for a currently prepared guidance document of the Organisation for Economic Co-operation and Development (OECD) with the purpose to shape a framework facilitating the regulatory use of DNT *in vitro* data with fit-for-purpose applications (Crofton and Mundy, 2021). The guidance document rests on two pillars, i.e. 1) the data generated through the experimental work (Masjosthusmann et al., 2020) and 2) the development of a variety of case studies including integrated approaches to testing and assessment (IATA) for screening and prioritization. The OECD guidance document is planned to be published in the first quarter of 2022.

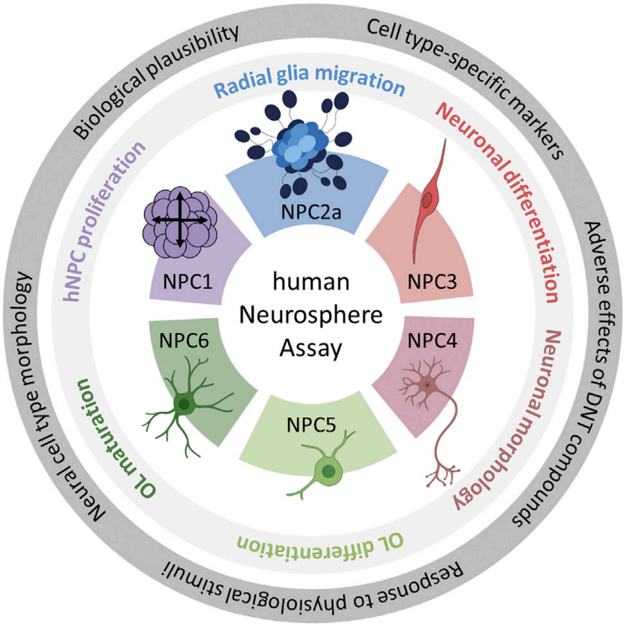

FIGURE 1 | Schematic description of the human Neurosphere Assay test methods and the identification of their scientific bases. The outer ring highlights the five questions which were the basis of the scientific validation of all six assays. The inner ring contains the neurodevelopmental key events modelled in the individual NPC assays. Each color represents one NPC assay. The color scheme is kept throughout the manuscript. Abbreviation: OL, oligodendrocytes. Created with biorender.com.

One crucial issue for OECD application of methods is validation (Coecke et al., 2007; Gourmelon and Delrue, 2016; Griesinger et al., 2016). While the traditional practice for assay validation is lengthy and relies on animal data, new approach methods (NAMs) need novel validation approaches. Here, mechanistic information previously identified *in vivo*, as well as reported neurodevelopmental adversities in response to disturbances on the cellular and tissue level, are of central importance (Hartung et al., 2013; Leist and Hartung, 2013). Here the scientific basis of a test method provides the mechanistic rationale for the predictive capacity of the assay. In addition, reliability, defined by the quality of the test method, is a crucial parameter. The scientifically sound, reliable test method also has to be fit-for-purpose implying that the regulatory question is known (Leist et al., 2010, 2014). Lab-to-lab transferability of assays has always been one crucial aspect of assay reliability. However, when e.g. certain robotics equipment is available only in one place, ring trials cannot be performed (Judson et al., 2013).

In this study, we validate the Neurosphere Assay, a high content assay for DNT evaluation, which is part of the DNT IVB (Masjosthusmann et al., 2020), using a mechanistic rationale approach. The Neurosphere Assay is based on human fetal neural progenitor cells (hNPCs) which are cultivated as proliferative neurospheres that have the potential to differentiate into brain effector cells including neurons, astrocytes and oligodendrocytes. Six early human fetal neurodevelopment key events (KEs) which are associated with DNT, are assessed in assays termed NPC1-6. Human NPC proliferation (NPC1ab) is a prerequisite for brain formation, with disturbances causing alterations in brain morphology and microcephaly (de Groot et al., 2005). Radial glia cell migration (NPC2a) generates a scaffold for migrating neurons during the course of corticogenesis and ensures normal brain structure and function. Alterations in this KE cause developmental brain disorders such as heterotopia and lissencephaly (Barkovich et al., 2005). Neuronal differentiation (NPC3) and neurite outgrowth (NPC4) are key cellular features associated with the functional maturation of the CNS. Disturbances in both directions (promotion or inhibition) are considered as adverse and are associated with depressive mood disorders and intellectual disabilities (Song and Wang, 2011; Guidi et al., 2018). Oligodendrocyte differentiation (NPC5) and thyroid hormone-dependent oligodendrocyte maturation (NPC6) are indispensable for the insulation of neuronal axons with disturbances causing demyelination diseases like leukomalacia that severely affect neuronal functioning (Baumann and Pham-Dinh, 2001; Volpe et al., 2011). Besides these DNT-relevant neurodevelopmental KEs, the Neurosphere Assay assesses the mitochondrial function and detects cytotoxicity upon chemical exposure to discriminate specific from unspecific effects (**Figure 1**).

All individual test method evaluations are automated, and concerning the experimental procedures, i.e. most pipetting steps are performed by a liquid handling system. The NPC2-5 assays are multiplexed. Quantification of differentiated neurons and oligodendrocytes is achieved by automated image analyses of immunostained cells that migrate out of the spheres in 96-well plates using convolutional neuronal networks (CNN; Förster et al., 2021). All endpoints are then analyzed using the Omnisphero software (Schmuck et al., 2017). This automated endpoint evaluation system allows an investigator bias-free, objective and low hands-on-effort identification of specific brain cells that used to be only possible by manual counting. In addition to cell identification, sphere-related endpoints like radial migration can be assessed. We further add data on the novel human induced pluripotent stem cell (hiPSC)-derived NPC (hiNPC) assays (hiNPC1/2) investigating similar endpoints. This hiPSC-based test system provides an unlimited cell source that is thoroughly characterized in a quality-controlled banking process (Tigges et al., 2021) that contributes to increasing the reproducibility of the test results. Furthermore, since iPSCs are reprogrammed from somatic cells (e.g. fibroblasts), the cell source raises fewer ethical concerns regarding its derivation process than primary cell material. However, hiNPCs represent a more immature developmental stage than fetal NPCs, hence they also have distinct applicability domains concerning neurodevelopmental timing.

Here we present the scientific basis for the individual NPC test methods. That the endpoints tested with the Neurosphere Assay are biologically indispensable for normal brain development (biological plausibility) was described in detail earlier (Fritsche et al., 2018b). Therefore, we now focus on the morphology of the different neural cell types, the expression of cell type-specific markers, the responses of the neurodevelopmental processes to physiological stimuli by using signaling pathway modulators and the predictive power to identify adverse effects of known DNT compounds. Together with the biological relevance of the endpoint, these five aspects build the scientific basis of the Neurosphere Assay.

2 MATERIALS AND METHODS

2.1 Reagents

Test compounds applied for the validation process are summarized in **Table 1**. Details for each specific assay are described below.

2.2 Basic hNPC and hiNPC Cell Culture

Primary human NPCs (hNPCs) were isolated from cortices of gestational week 16–19 fetuses and purchased from Lonza Verviers SPRL, Belgium (#PT-2599). The hiPSCs were purchased from Alstem (iPS11) and WiCell (IMR-90, Clone-4). The neural induction of hiPSCs into human-induced neural progenitor cells (hiNPCs) was performed in our laboratory as described in detail in Nimtz et al. (2020). The hNPCs and hiNPCs were cultured as free-floating neurospheres in proliferation medium consisting of DMEM (#31966-021, Thermo Fisher, United States) and Hams F12 (#31765-027, Thermo Fisher, United States) in a 2:1 ratio (v:v) supplemented with 2% B27 (#17504044, Thermo Fisher, United States), 20 ng/ml EGF (#PHG0313, Thermo Fisher, United States), 20 ng/ml FGF basic (#233-FB, R&D Systems, United States), and 100 U/ml penicillin and 100 µg/ml streptomycin (#P06-07100, Pan-Biotech, Germany). Neurospheres were cultured under standard cell culture conditions at 37°C and 5% CO_2 in 10 cm diameter cell culture dishes coated with poly-2-hydroxyethyl methacrylate (poly-Hema; #P3932, Merck, United States). For cell passaging, once per week, neurospheres were mechanically dissociated into cubicles of 0.2 mm edge length using a McIlwain tissue chopper (#TC752, Campden Instruments, United Kingdom). Neurospheres were supplied with fresh culture medium three times per week, by replacing half of the culture medium.

2.3 The Neurosphere Assay (NPC1-6)

2.3.1 Proliferation (NPC1ab)

hNPC and hiNPC proliferation (NPC1ab assay) was scientifically validated by assessing the incorporation of bromodeoxyuridine (BrdU, NPC1b, #11669915001, Roche, Switzerland) into the DNA and by measuring the increase in sphere size (NPC1a; 0, 24, 48, and 72 h) using the Cellomics ArrayScan and the provided HCS Studio Cellomics software (version 6.6.0; Thermo Fisher Scientific). In brief, proliferating NPCs of 300 µm diameter were cultivated for 3 days in 100 µl proliferation medium containing EGF and FGF basic (detailed composition described in the basic cell culture section). One NPC neurosphere was cultivated in 100 µl medium in a well of a poly-Hema-coated 96-well plate and 4–5 technical replicates were prepared for each experimental condition. Proliferating NPCs were further exposed to the EGF receptor inhibitor PD153035 (0.01, 0.16 and 0.64 µM), known

TABLE 1 | List of chemicals used in the experimental part, including their sources, catalog numbers, stock concentrations, and solvents.

Reagents	Source	Catalog Number	Solvent	Stock
Ascorbic acid	Merck	A92902	H2O	100 mM
Bisindolylmaleimide 1 (Bis-I)	Merck	203290	DMSO	20 mM
Cadmium chloride	Toxcast library	DTXSID6020226	DMSO	20 mM
N-[N-(3,5-Difluorophenacetyl)-Lalanyl]- S-phenylglycine t-butyl ester (DAPT)	Merck	D5942	DMSO	40 mM
Deltamethrin	Merck	45423	DMSO	20 mM
Epidermal growth factor (EGF)	Thermo Fisher	PHG0313	DPBS +/+	10 µg/ml
Fibroblast growth factor (FGF) basic	R&D Systems	233-FB	0.1% BSA, 1 mM DTT in DPBS +/+	10 µg/ml
Methylmercury(II) chloride	Merck	33368	H2O	20 mM
Narciclasine	Cayman Chemicals	20361	DMSO	20 mM
NH-3	Nguyen et al. (2002) Singh et al. (2016)	-	DMSO	1 mM
PD153035	Merck	SML0564	DMSO	5 mM
PP2	Merck	P0042	DMSO	10 mM
Rotenone	Santa Cruz Biotechnology	203242	DMSO	100 mM
L-3,3′,5 triiodothyronine (T3)	Merck	T2877	1:1 (v/v) 96% EtOH : 1 M HCl	0.3 mM
Tetrabromobisphenol A (TBBPA)	Merck	330396	DMSO	50 mM

DNT-positive chemicals with known modes of action, i.e. cadmium chloride (0.03–20 µM) and rotenone (0.01–2.22 µM), or the respective solvent (solvent control) over the whole 3 days. As an endpoint-specific positive control, confirming that the assay detects reductions in NPC proliferation, NPCs were cultivated in medium without growth factors (w/o growth factors). For the assessment of the sphere size, images of neurospheres were taken daily using an inverted microscope CKX41 (Olympus) with a ×100 magnification. Detailed descriptions of the NPC1ab and iNPC1ab assays can be found in the DNT *in vitro* testing battery report (Masjosthusmann et al., 2020) and in (Hofrichter et al., 2017), respectively. Besides proliferation, cell viability (CellTiter-Blue Assay (CTB), #G8081, Promega, Madison, United States) and cytotoxicity (CytoTox-ONE Homogeneous Membrane Integrity Assay; #G7891, Promega, Madison, United States) were assessed simultaneously.

Flow cytometry analyses were performed to confirm the cell type-specific marker expression in proliferating hNPC and hiNPC neurospheres. Neurospheres (hNPCs in passage 4, hiNPCs in passage 5) were singularized with accutase (Stemcell Technologies, Canada) for 20 min at 37°C on an orbital shaker (800 rpm) and stained with viability stain 510 (#564406, BD Bioscience, Germany), anti-Nestin-Alexa647 antibody (#560341, BD Bioscience, Germany) and anti-Sox2-PerCP-Cy5.5 antibody (#561506, BD Bioscience, Germany). The antibodies were all diluted 1:20 in BD Pharmingen stain buffer (BD Bioscience, Germany) except for the viability stain (1:1000 in DPBS −/−, #14190144, Thermo Fisher, United States). Samples were analyzed using a BD FACSCanto II (BD Bioscience, Germany) and FlowJo software (10.8.0). Debris, doublets, and dead cells were discarded during the gating process. 20000 cells were analyzed per sample.

2.3.2 hNPC Differentiation and Immunocytochemical Staining (NPC2-5)

For the initiation of cell differentiation into neurons, oligodendrocytes, and astrocytes (Moors et al., 2009; Breier et al., 2010), 0.3 mm hNPC neurospheres were transferred into 96-well plates coated with 0.1 mg/ml poly-D-lysine (#P0899-50MG, Merck, United States) and 12.5 µg/ml laminin (#L2020-1MG, Merck, United States). The following differentiation medium was prepared and used for neurosphere cultivation: DMEM (#31966-021, Thermo Fisher, United States) and Ham’s F12 (#31765-027, Thermo Fisher, United States) in a 2:1 ratio (v:v) supplemented with 1% N2 (#17502-048, Thermo Fisher, United States) and 100U/mL penicillin and 100 µg/ml streptomycin (#P06-07100, Pan-Biotech, Germany). After 5 days of differentiation, migrated cells were fixed with 4% paraformaldehyde for 30 min at 37°C and stained with antibodies against β(III)tubulin (neurons) and O4 (oligodendrocytes) as previously described in detail by (Klose et al., 2021b; 2021a). In brief, unspecific binding sides on the fixated cells were blocked with 10% goat serum (GS, #G9023-10ml, Merck, United States) in PBS for 30 min at 37°C. Primary antibodies against β(III)tubulin (1:400, rabbit anti-β(III)tubulin monoclonal antibody [EP1569Y]-Alexa Fluor® 647, #ab190575, Abcam, United Kingdom) and O4 (1:400, mouse anti-O4 IgM, #MAB1326, R&D systems, United States) were incubated overnight in PBS containing 0.01% Triton-X and 2% GS at 4°C. After three washing steps with PBS, the cells were incubated with the secondary antibody for the O4-staining (1:400, goat anti-mouse IgM-Alexa Fluor® 488, #A-21042, Thermo Fisher, United States) and Hoechst33258 (1:100, #94403-1ML, Merck, United States) in PBS containing 2% GS for 60 min at 37°C. For the staining of radial glia, fixated cells were blocked with 10% GS in PBS for 30 min at 37°C and stained with antibodies against nestin (1:200, Alexa Fluor® 647 mouse anti-nestin,

#560393, BD Biosciences, United States), Ki67 (1:500, Ki67 (8D5) mouse mAb, #9449, Cell Signaling Technologies, United States) or GFAP (1:200, anti-GFAP mouse (GA5) antibody, #G9269, Merck, United States) overnight in PBS containing 0.01% Triton-X and 2% GS at 4°C. After three washing steps with PBS, the cells were incubated with the secondary antibodies for Ki67 (1:400, goat anti-mouse IgG 488, #A-11001, Thermo Fisher, United States) and GFAP (1:400, goat anti-rabbit IgG 488, #A-11008, Thermo Fisher, United States) and Hoechst33258 (1:100, #94403-1ML, Merck, United States) in PBS containing 2% GS for 60 min at 37°C. All pictures of immunocytochemical stainings were acquired with the High Content Analysis (HCA) platform Cellomics ArrayScan using a 200-fold magnification, a resolution of 552×552 pixel and the provided HCS Studio Cellomics software (version 6.6.0; Thermo Fisher Scientific).

2.3.3 hiNPC Differentiation and Migration (hiNPC2a+3)

Differentiation of hiNPCs was performed as described above for primary hNPCs, with the exception that hiNPCs were cultivated in CINDA medium containing DMEM (#31966-021, Thermo Fisher, United States) and Ham's F12 (#31765-027, Thermo Fisher, United States) in a 2:1 ratio (v:v) supplemented with 1% N2 (#17502-048, Thermo Fisher, United States), 2% B27 (#17504044, Thermo Fisher, United States), 100 U/ml penicillin and 100 µg/ml streptomycin (#P06-07100, Pan-Biotech, Germany), 5 mM creatine monohydrate (#C3630, Merck, United States), 100 U/mL Interferon-γ (#300-02, PeproTech, Germany), 20 ng/ml neurotrophin-3 (#450-03, PeproTech, Germany), 300 µM dibutyryl-cAMP (#D0260, Merck, United States) and 20 µM ascorbic acid (#A5960, Merck, United States). The neural induction of human induced pluripotent stem cells (hiPSCs) into human induced neural progenitor cells (hiNPCs) is described in detail in Bartmann et al. (2021) and Nimtz et al. (2020). After 3 days of differentiation, cells were fixed with 4% paraformaldehyde for 30 min at 37°C, washed four times with PBS, and stained with S100β antibody (1:500, rabbit anti-S100 beta antibody [EP1576Y], #ab52642, Abcam, United Kingdom) in 0.05% PBS-T with 3% GS overnight at 4°C, followed by five PBS washing steps, before incubation with the secondary antibody (1:500, goat anti-rabbit IgG 488, #A-11008, Thermo Fisher, United States) in PBS with 2% GS and 1% Hoechst 33258 (1:100, #94403-1ML, Merck, United States) for 60 min at room temperature. After 5 washing steps with PBS, cells were stained with the conjugated β(III)tubulin antibody (1:400, rabbit anti-β(III)tubulin monoclonal antibody [EP1569Y]-Alexa Fluor® 647, #ab190575, Abcam, United Kingdom) in PBS with 2% GS. After 5 washing steps with PBS, images of immunocytochemical stainings were acquired as described for primary hNPCs.

For the scientific validation of the hiNPC migration assay, hiNPCs were exposed to either EGF (0.5–1 ng/ml) alone or in combination with the EGFR-inhibitor PD153035 (1–2 µM), the SRC- kinase inhibitor PP2 (10 µM), narciclasine (0.0001–0.1 µM) or the respective solvent (solvent control). The migration distance was assessed after 24, 48 or 72 h as described for hNPC migration below.

2.3.4 hNPC Migration (NPC2)

Upon plating of hNPC neurospheres on PDL-laminin matrices, NPCs radially migrate out of the sphere core, thereby adapting a radial glia-like morphology and forming a circular migration area. The migration distance of radial glia cells (RG, NPC2a) is assessed manually after 72 h using bright-field microscopy and automated after 120 h by analyzing the ICC stainings with the software Omnisphero as previously described by Schmuck et al. (2017). In brief, RG migration is assessed manually on bright-field pictures, taken with the Cellomics ArrayScan using a 50-fold magnification, by measuring the radial distance of the furthest migrated cells to the sphere core as number of pixels following conversion into µm using Fiji Image J software (Schneider et al., 2012). After 120 h, RG migration is evaluated automatically by defining the area of Hoechst33258-stained nuclei as the migration area of this particular sphere using the Omnisphero software. Additionally, the migratory capacity of neurons (NPC2b) and oligodendrocytes (NPC2c), defined as the mean distance of all neurons/oligodendrocytes within the migration area divided by the RG migration distance, is automatically assessed after 120 h. The validation of the NPC2b and NPC2c assay is not included in this manuscript.

For the scientific validation of the NPC2a assay, hNPCs were exposed to human-relevant pathway modulators as well as known DNT-positives during the 5 days of differentiation. Neurospheres were differentiated in presence of 1) epidermal growth factor (EGF, 0.5–1 ng/ml) alone or in combination with the EGF receptor-inhibitor PD153035 (1–2 µM), 2) the Src-kinase inhibitor PP2 (10 µM), 3) increasing concentrations of methylmercury (MeHg, 0.003–2.22 µM) or 4) the respective solvent (solvent control). Besides RG migration analysis, cytotoxicity was assessed.

2.3.5 Neuronal and Oligodendrocyte Differentiation and Neuronal Morphology (NPC3-5)

Multiplexed with the assessment of RG migration after 120 h (NPC2a), further endpoints can be assessed in an automated way using different software tools. The endpoints NPC3-5 model neuronal differentiation (NPC3) and morphology (NPC4), as well as oligodendrocyte differentiation (NPC5) after 120 h of differentiation.

After staining of the differentiated cells with the above-mentioned antibodies and subsequent image acquisition with the Cellomics ArrayScan (see section "*hNPC Differentiation and Immunocytochemical staining*"), a series of separate images were edited together to create one image per well, including all three channels (nuclei (Hoechst33258), neurons (Alexa647®), oligodendrocytes (Alexa488®)). For this step, the high-content analysis (HCA) tool Omnisphero was used (Schmuck et al., 2017). Based on the cells with Hoechst-positive nuclei migrating out of the sphere core and the formed circular migration area, RG migration was calculated for each sphere after 120 h. Neuronal (NPC3) and oligodendrocyte (NPC5) differentiation is defined by the number of cells stained for β(III)tubulin and O4, respectively, as a percentage of the total nuclei count within the migration area. The stained neurons and oligodendrocytes are identified using two convolutional neural networks (CNN) based on the Keras architecture implemented in Python 3, which were trained by historical handpicked data (Förster et al., 2021). The number of nuclei was determined using the SpotDetector (V4.1) bio-application of the HCS Studio Cellomics software (version 6.6.0, Thermo Fisher Scientific). All neurons identified by the CNN were additionally analyzed regarding their morphology by assessing their neurite length and area (NPC4).

For the scientific validation of the NPC3 assay, neurospheres were differentiated in presence of DAPT (0.01 µM–10 µM), narciclasine (0.00014 µM–0.1 µM), or the respective solvent (solvent control). For the scientific validation of the NPC4 assay, neurospheres were differentiated in presence of narciclasine (0.00014 µM–0.1 µM), bisindolylmaleimide 1 (0.027–20 µM) or the respective solvent (solvent control). For the scientific validation of the NPC5 assay, neurospheres were differentiated in presence of DAPT (0.01 µM–10 µM), 100 µM ascorbic acid, deltamethrin (0.027–20 µM), tetrabromobisphenol A (0.027–20 µM) or the respective solvent (solvent control).

2.3.6 Oligodendrocyte Maturation Assay (NPC6)

The methodology is described in detail in (Dach et al., 2017; Klose et al., 2021b). In brief, hNPCs were plated on 8-chamber slides (five spheres per chamber) and 24-well plates (10 spheres per well) in differentiation medium containing either solvent or 3 nM triiodothyronine (T3) and incubated for 5 days on PDL-laminin matrices. To test for thyroid hormone disruption, hNPCs were additionally differentiated in presence of T3 with or without increasing concentrations of the thyroid hormone receptor antagonist NH-3 (4–400 nM) or the flame retardant TBBPA (0.01–1 µM). After 5 days, immunocytochemical stainings for oligodendrocytes (O4) and cell nuclei (Hoechst33258) were performed in the 8-chamber slides as described above. Imaging of stained 8-chamber slides was performed using the Cellomics ArrayScan VTI instrument (Thermo Fisher Scientific) and the software Omnisphero (Schmuck et al., 2017). For two defined areas (1098 mm x 823 mm size) within the migration area, the oligodendrocyte number was calculated and expressed as a percentage of the total number of nuclei. Oligodendrocyte percentages were averaged per sphere and the mean and standard deviation were calculated for the five spheres per chamber.

From the spheres plated within the 24-well plate, total RNA was extracted and 150 ng were transcribed into cDNA using the RNeasy Mini Kit (#74106, Qiagen, Germany) and the Quantitect Reverse Transcription Kit (#205313, Qiagen, Germany) according to the manufacturer's instructions. Quantitative real-time polymerase chain reactions (qRT-PCR) were performed with the QuantiFast SYBR Green PCR Kit (#204054, Qiagen, Germany) and the Rotor-Gene Q Cycler (Qiagen, Germany) using primers for ACTB (fw: CAGGAAGTCCCTTGCCATCC, rev: ACCAAAAGCCTTCATACATCTCA), MBP (fw: CAGAGC GTCCGACTATAAATCG, rev: GGTGGGTTTTCAGCGTCT A). Gene expression was quantified with the copy number method and MBP expression was normalized to 10.000 ACTB copy numbers (Dach et al., 2017; Klose et al., 2021b).

The maturation quotient (Q_M) is then calculated as MPB copy numbers divided by the percentage of oligodendrocytes within the hNPC differentiated culture. Therefore, an increase in the Q_M represents an increase in oligodendrocyte maturation.

2.3.7 Mitochondrial Activity and Cytotoxicity Assays

Mitochondrial activity and cytotoxicity were assessed in parallel to the specific endpoints of the Neurosphere Assay to discriminate specific compound effects from unspecific effects originating from necrosis or reduced cell viability. After the respective days of chemical exposure, mitochondrial activity was assessed using the Alamar blue assay (CellTiter-Blue Assay (CTB), #G8081, Promega, United States). In parallel, cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH) from cells with damaged membranes (CytoTox-ONE Homogeneous Membrane Integrity Assay; #G7891, Promega, United States). As lysis control for the LDH assay, neurospheres were incubated for 45 min with 0.2% Triton X-100. Fluorescence was measured with a Tecan infinite M200 Pro reader (ex: 540 nm; em: 590 nm). The relative fluorescence unit (RFU) values of the replicates were averaged and medium without cells was used to correct for background fluorescence. Of note, impaired radial glia migration and reduced nuclei count correlate with a reduced CTB value as a consequence of the diminished cell number within the migration area (Fritsche et al., 2018a; Klose et al., 2021a). Therefore, in the case of a compound inhibiting radial glia migration or reducing the nuclei count, the CTB assay is an inadequate measure of viability and thus the LDH assay alone should be used as the reference to identify DNT-specific effects (Klose et al., 2021a). In the figures, except for **Figures 5F+G** and **Figure 7F**, only cytotoxicity is displayed.

2.3.8 Statistics

For all hNPC experiments, at least two different individuals (hNPC donors) were used and for all hNPC and hiNPC experiments, at least three independent biological replicates with at least three technical replicates each were performed. Experiments were defined as independent if they were generated with hNPCs from different individuals or a different passage number. Results are presented as mean ± SEM unless otherwise indicated. For calculating dose-response curves, a sigmoidal curve fit was applied using GraphPadPrism software. Statistical significance was calculated using one-way ANOVA with Bonferroni's post hoc tests or two-tailed Student's t-tests ($p \leq 0.05$ was termed significant).

3 RESULTS AND DISCUSSION

In the next paragraphs, we will guide through the endpoints of the Neurosphere Assays, starting with the human NPC assays that are based on primary human fetal NPCs. Succeeding, we present endpoints of a novel iNeurosphere Assay, which is based on human induced pluripotent stem cells. During method development, we proceeded according to the Guidance Document on Good *In Vitro* Method Practices (GIVIMP) principles, to ensure predictivity and reproducibility of the test methods (OECD, 2018; Pamies et al., 2022). The description of each

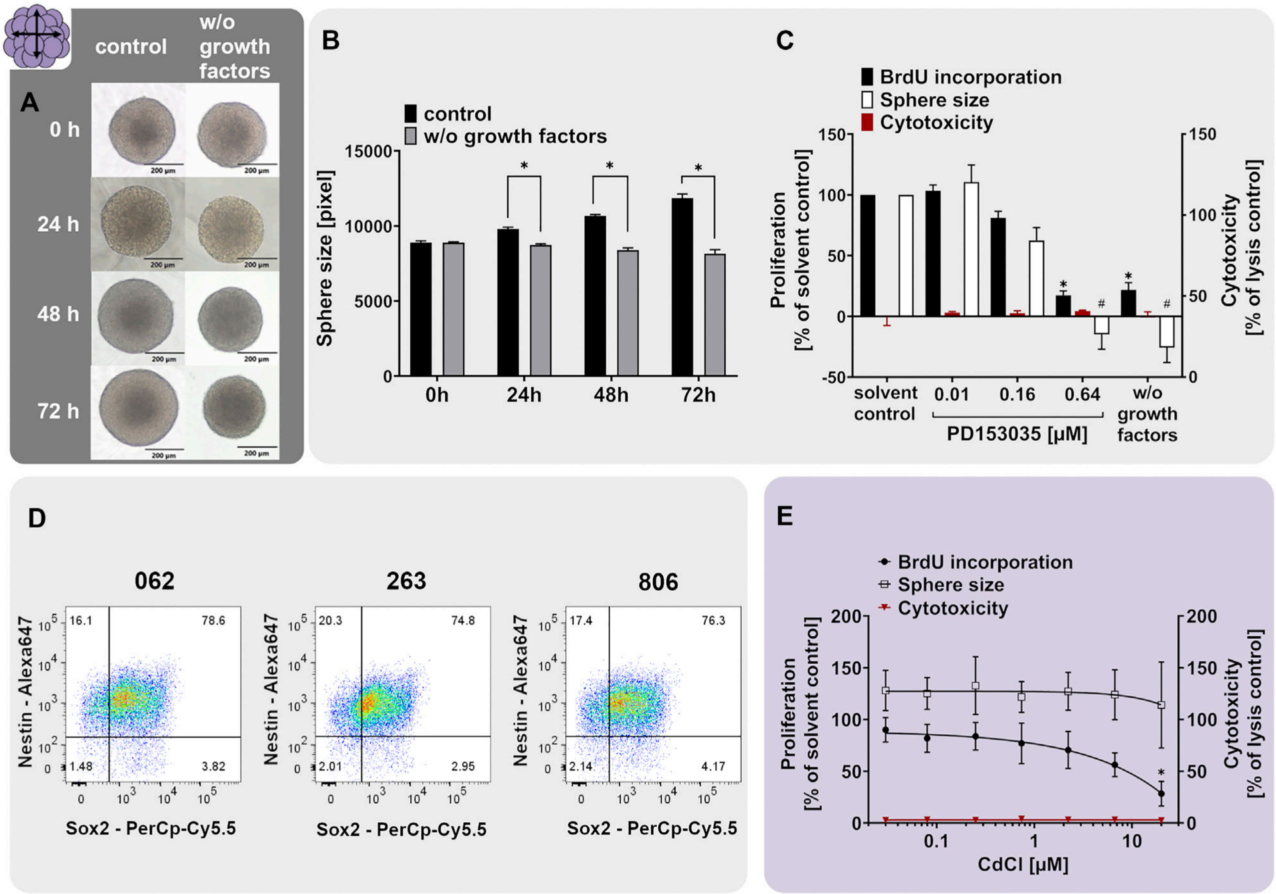

FIGURE 2 | The NPC1ab assay identifies chemicals disturbing NPC proliferation. **(A+B)** Primary hNPC neurospheres (Lonza, Verviers, Belgium) were cultivated for 3 days in proliferation medium containing 20 ng/ml of the growth factors EGF and FGF (control) or in medium without growth factors (w/o growth factors). Representative pictures **(A)** and quantifications of the sphere size **(B)**, as assessed within the NPC1a assay, showed that growth factors are necessary for hNPC proliferation. **(D)** Proliferating hNPC neurospheres issued from three different individuals (062, 263, and 806) were analyzed using flow cytometry analysis, confirming high expression of the neural stem/progenitor markers nestin and SOX2. The percentage of double-positive cells is indicated in the upper right quartile. **(C, E)**. Exposure of proliferating hNPCs for 3 days to increasing concentrations of **(C)** the EGFR inhibitor PD153035 (0.01–0.64 µM) or **(E)** cadmium chloride (0.027–20 µM) concentration-dependently decreased hNPC proliferation compared to the respective solvent controls (adapted from Masjosthusmann et al., 2020). Proliferation was assessed by sphere size increase (NPC1a) and BrdU incorporation into the DNA (NPC1b). The values of the chemical-treated conditions are expressed as % of the respective solvent controls. Cytotoxicity (LDH release) was assessed in parallel and is depicted as % of a lysis control (spheres treated with 0.2% Triton-X100). Data are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA **(C, E)** and two-tailed Student's t-tests **(B)**. A *p*-value below 0.05 was termed significant. * and # significantly changed compared to the solvent control of the respective endpoint if not marked otherwise.

Neurosphere Assay endpoint follows the same rationale. First, the relevance of the respective endpoint for brain development is described. Second, *in vitro* morphologies and expressions of respective markers corresponding to the individual cell types and test methods are shown. Third, endpoint responses to a selection of physiologically pertinent signaling stimuli during neurodevelopmental processes are demonstrated. These data underscore the biological relevance of the individual endpoints. Fourth, examples of adverse effects of DNT compounds on neurosphere endpoints are displayed. These data are important building blocks for scientific validation of DNT test methods since they contribute to the scientific basis and applicability domains of the studied neurodevelopmental processes and hence increase confidence in their usage.

3.1 NPC Proliferation (NPC1)

Proliferation is one of the essential neurodevelopmental processes during brain development and comprises the increase in cell number through cell growth and division (Homem et al., 2015). Disturbances in both directions (decrease and increase of proliferation) may result in neurodevelopmental disorders such as microcephaly or megalencephaly, respectively. Microcephaly is manifested by a severe reduction in brain size and was linked to prenatal exposure of human fetuses to the Zika virus (Devakumar et al., 2018). Megalencephaly, on the other hand, is defined as increased growth of cerebral structures during development and is associated with metabolic disorders such as L-2-hydroxyglutaric aciduria (Pavone et al., 2017). Both microcephaly and megalencephaly may result in severe neurological disabilities such as global developmental delay, seizures, deficits in language development and social interactions (Guerrini and Dobyns, 2014).

Neurospheres are valuable 3D test systems to study NPC proliferation since they are highly proliferative in suspension culture in the presence of growth factors (Reynolds et al., 1992). For our specific neurosphere test system (Lonza, Verviers, Belgium), expression of the cell type-specific CNS neural stem and progenitor cell markers nestin and SRY-box 2 (SOX2) in proliferating hNPCs was confirmed (**Figure 2D**). Nestin is an intermediate filament protein type IV (Lendahl et al., 1990) used as a molecular marker for neuroepithelial stem cells and CNS progenitors. When human multipotent CNS progenitors differentiate into neurons and glial cells, nestin expression is rapidly downregulated *in vivo* (Dahlstrand et al., 1995) confirming its usefulness as a neural progenitor cell marker. SOX proteins comprise a group of transcription factors conserved throughout evolution. SOX2 is a marker for proliferating CNS progenitors and its overexpression inhibits neuronal differentiation (Pevny and Placzek, 2005). The primary hNPCs used in this study were double-positive for nestin and SOX2 as shown in **Figure 2D**. In total, 76.6, 74.8, and 76.3% of cells issued from the three different individuals, respectively, were double-positive for the two markers, and only 1.48, 2.01, and 2.14% of cells expressed none of them, hence confirming their resemblance to neural progenitors *in vivo*. The expression of nestin and SOX2 was assessed in primary hNPCs previously (Hofrichter et al., 2017). Although the percentage of cells expressing neither of the two markers was comparable in these two studies, the average percentage of double-positive cells was lower in the present study (75.9% in the present study versus 96.3% (Hofrichter et al., 2017)). This might be explained by a higher passage number (4) of hNPCs used in this study (i.e. the highest passage usually used within the Neurosphere Assay) in comparison with passage 0 used in the study of (Hofrichter et al., 2017). In addition to their expected marker expression, hNPCs exert the expected morphology (**Figure 2A**). Neurospheres of a few hundred µm in diameter consist of individual cells (e.g. one neurosphere with 300 µm in diameter contains 2.6 x 10^3 cells; (Moors et al., 2009)) and display a perfectly round shape with no disintegrated borders.

The proliferative capacity of hNPCs was assessed by cultivating them in either medium supplemented with (control) or deprived of (w/o growth factors) the human growth factors EGF and FGF basic (20 ng/ml each). Human NPCs cultivated for 3 days in control medium increased their size on average by 33%, whereas hNPCs cultivated in growth factor-deprived medium (w/o growth factors) did not proliferate and even slightly shrunk in size by 8.2% (**Figures 2A,B**). Direct measurements of proliferation by BrdU incorporation indicated a 78.1% decrease in BrdU incorporation in spheres growing in the absence (w/o growth factors) compared to the presence (solvent control) of growth factors (**Figure 2C**). The proliferative capacity of Lonza hNPCs was reported previously (Moors et al., 2009; Baumann et al., 2015; Klose et al., 2021a).

The neurodevelopmental process of proliferation is guided by various signaling pathways including the epidermal growth factor receptor (EGFR) signaling (Ayuso-Sacido et al., 2010). To elucidate if EGFR mediates this proliferative cell response in hNPC, we assessed hNPC proliferation in presence of the EGFR inhibitor quinazoline PD153035. PD153035 antagonized the EGF-induced hNPC proliferation, as assessed via BrdU incorporation and sphere size increase, without inducing cytotoxicity. This confirms that EGFR signaling regulates hNPC proliferation *in vitro* (**Figure 2C**). EGFR signaling generally regulates cell proliferation, growth, differentiation and cell survival (Oda et al., 2005). In the developing brain, the EGFR is increasingly expressed over time (Romano and Bucci, 2020) and is mainly found in proliferating and migratory brain regions (Kornblum et al., 1997; Caric et al., 2001). The EGFR is therefore indispensable for proper rodent brain development (Romano and Bucci, 2020). Specifically, the proliferation of murine neural stem cells and nestin$^+$ progenitor cells was previously increased by EGFR signaling *in vitro* (Sun et al., 2005; Ayuso-Sacido et al., 2010). *In vivo*, EGF induced proliferation of stem cells and progenitors in the murine fourth ventricle and central canal of the spinal cord (Martens et al., 2002) and ependymal precursor cells of the adult rat spinal cord (Kojima and Tator, 2000). Moreover, PD153035 reportedly suppressed proliferation of murine neural stem cells *in vitro* (Tropepe et al., 1999). These data—especially from the *in vivo* studies—support the importance of the EGFR pathway for NPC proliferation.

As a chemical exerting adverse effects on hNPC proliferation, we selected cadmium chloride. Prenatal exposure to cadmium chloride is associated with a lower child intelligence score (Kippler et al., 2012b), memory deficits, and learning disabilities in children (Tian et al., 2009). In rodents, cadmium causes behavioral and neurotoxicological changes (Dési et al., 1998). Hence, it is listed amongst the compounds triggering DNT (Mundy et al., 2015; Aschner et al., 2017). Cadmium is acting via the induction of oxidative stress, thus causing cell death and affecting mTOR, Erk1/2, and JNK signaling pathway activity (Kippler et al., 2012a; Leal et al., 2012). In mouse neural stem/progenitor cells, cadmium remarkably influenced the expression of genes related to cell growth, proliferation, cell cycle, and survival (Deng et al., 2020). In the present study, concentration-dependent inhibition of hNPC proliferation was observed following exposure to cadmium chloride compared to the solvent control without any observed cytotoxicity (**Figure 2E** adapted from Masjosthusmann et al. (2020)). Of note, the effects on NPC1b (BrdU incorporation) were much more pronounced compared to NPC1a (sphere size), highlighting that NPC1b is the more sensitive endpoint since differences in DNA replication by far precede the microscopic changes. The hNPC proliferation assay previously identified numerous compounds eliciting adverse effects on the proliferation process e.g. MeHgCl, arsenic, methylazoxy methanol acetate, $NaAsO_2$ (Baumann et al., 2016), the flame retardants EHDPHP and TCP (Klose et al., 2021a) and a variety of compounds in a large screening study (Masjosthusmann et al., 2020). These studies support the usefulness of the 3D hNPC test system for assessing the effects of compounds on NPC proliferation.

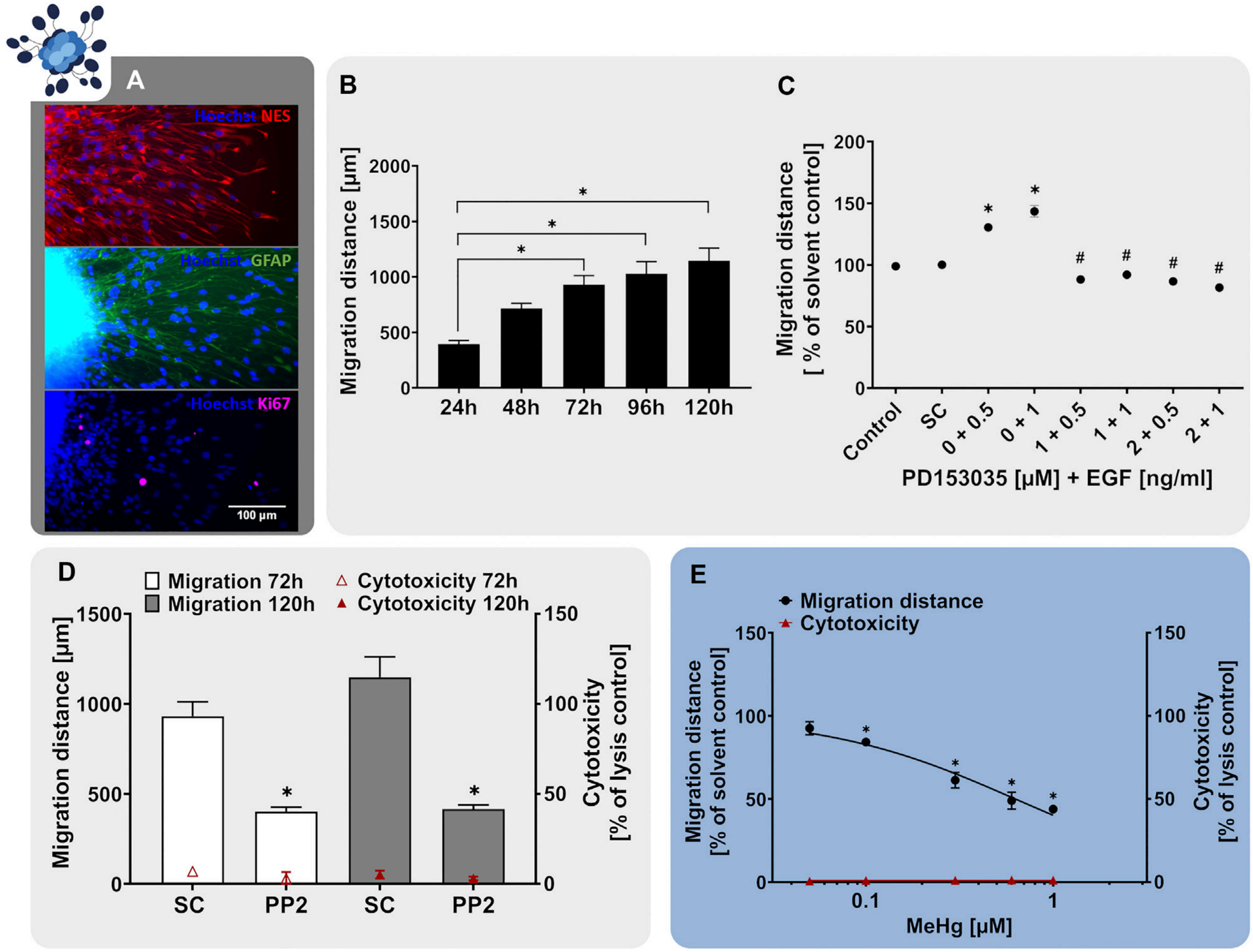

FIGURE 3 | The NPC2a assay identifies chemicals disturbing radial glia migration. Primary hNPCs were differentiated on PDL-laminin-coated 96-well plates in the absence of growth factors. **(A)** After 24 h, immunocytochemical stainings for nestin, GFAP and Ki-67 were performed confirming radial glia-typic marker expression and morphology. Nuclei were counterstained with Hoechst33258. **(B)** Over the 5 days of differentiation, cells radially migrate out of the sphere core and form a circular migration area. The migration distance of hNPCs increased gradually over time, as assessed by determining the distance from the sphere core to the furthest migrated cells at four opposite positions in brightfield images every 24 h **(C)** hNPC migration over 5 days was assessed in presence of EGF (0.5–1 ng/ml) alone, in combination with the EGFR inhibitor PD153035 (1–2 µM), or the respective solvent. While EGF increased hNPC migration compared to the solvent control, PD153035 inhibited the EGF-induced effect. **(D)** A negative effect of the SRC-family kinase inhibitor PP2 on hNPC migration was confirmed by differentiating hNPCs for 3 and 5 days in presence of 10 µM PP2 or the respective solvent (SC). **(E)** hNPCs differentiation in presence of increasing concentrations of methyl-mercury (MeHg, 0.005–1 µM) for 3 days concentration-dependently reduced the migration distance (adapted from Fritsche et al., 2018a). For **(D,E)**, cytotoxicity (LDH release) was assessed in parallel and is depicted as % of a lysis control (differentiated hNPCs treated with 0.2% Triton-X100). Data are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA **(B, E)** and two-tailed Student's t-tests **(C, D)**. A *p*-value below 0.05 was termed significant. *significantly changed compared to the respective solvent control. $^{\#}$significantly changed compared to the respective EGF concentration.

3.2 Radial Glia Migration (NPC2a)

Fetal cortex development is characterized by different migratory processes mainly involving radial glia cells (RG) and neurons (Borrell and Götz, 2014; Falk and Götz, 2017). Human RG exhibit two distinct functions, which are prerequisites for cortex development, especially the higher organization of the human brain: 1) due to their self-renewing capacity, RG increase the cortical cell pool before terminally differentiating into neurons and glial cells. This leads to cortical expansion, increases neurogenesis and causes the characteristic folded cerebral cortex architecture in gyrencephalic species like humans. 2) due to their migratory capacity, RG form scaffolds for migrating neurons and hence represent the pillars of cortex formation (Borrell and Götz, 2014; Falk and Götz, 2017). As a consequence of disturbance of RG function during human brain development, neurodevelopmental disorders such as heterotopia and lissencephaly can develop (Barkovich et al., 2005; Matsumoto et al., 2017; Ferent et al., 2020).

Neurospheres are well-suited cell systems for studying neural cell migration since without any additional cues the cells start wandering out of the spheres once they are plated on a suitable matrix (Zhou and Chiang, 1998; Kukekov et al., 1999). In our human neurosphere test system, we established a RG migration test method (NPC2a assay) that specifically measures the migration distance travelled by RG cells (Moors et al., 2007, 2009; Baumann et al., 2015). In our studies, plating of hNPC neurospheres on poly-D-lysine/laminin-coated matrices initiates cell migration in radial trajectories, forming a circular migration area around the sphere core. After 24 h, the migrated cells exhibit the characteristic elongated RG-like morphology and express the RG-markers nestin and GFAP as well as the proliferation marker Ki-67 (**Figure 3A**). *In vivo*, RG are highly polarized and have a particular elongated morphology since they form processes extending from the apical to the basal side of the cortex (Ferent et al., 2020). In accordance with that, the nestin- and GFAP-positive cells migrating out of the hNPC sphere core display an active growth cone protrusion which diverges from the cell body to explore the vicinal environment (**Figure 3A**; Baumann et al. (2015)). The migratory potential of the hNPC-derived RG is preserved *in vitro* over the time-course of at least 120 h (**Figure 3B**). However, a decrease in migration speed can be observed after the first 24 h. Since the specific RG architecture provides a scaffold supporting neuronal migration during cortex development, the correct formation and maintenance of the RG scaffold is crucial for the organization of neuronal networks and disturbances correlate with cortical malformations such as human lissencephaly, polymicrogyria and heterotopia (Ferent et al., 2020). Therefore, RG migration (NPC2) is a fundamental neurodevelopmental key event, which is indispensable in a predictive testing battery identifying chemical-induced DNT.

During human brain development, migratory processes are regulated by various signaling pathways, whose activity should be preserved within a predictive *in vitro* model. Similar to NPC proliferation, migration of neural stem cells is regulated by EGF, exerting its actions through the EGFR (Ayuso-Sacido et al., 2010). The EGFR is expressed not only in proliferating but also in differentiating hNPC and was identified as a human-relevant key regulator in a gene-gene interaction network involved in hNPC migration together with SRC-kinase (Masjosthusmann et al., 2018). Studies on EGFR knockout mice reported a decrease in brain size, supporting the involvement of the EGFR in migratory processes during cortical development (Kornblum et al., 1998). Moreover, intraventricular administration of EGF caused migration of subependymal NPCs from the lateral ventricle into the adjacent neural tissue in the adult mouse brain (Craig et al., 1996). Similarly, exposure to EGF (0.5–1 ng/ml) after neurosphere plating enhanced hNPC migration compared to the solvent control (**Figure 3C**). In addition, co-administration of the EGFR-inhibitor PD153035 (1–2 μM) antagonized the EGF-induced migratory stimulating effect demonstrating EGF action on NPC migration via the EGFR. As a second human-relevant key regulator of migration, which is expressed in hNPCs (Masjosthusmann et al., 2018), we assessed the effects of SRC-family kinase inhibition on RG migration. SRC-family kinases are fundamental for brain development and disruption of their activity correlates with defects in radial migration and reeler-like malformations of cortical development (Jossin et al., 2003; Kuo, 2005; Wang et al., 2015). Exposure of migrating hNPCs to the SRC-family kinase inhibitor PP2 reduced hNPC migration to 40% of the solvent control without causing any signs of cytotoxicity (**Figure 3D**; Moors et al., 2007). Our results indicate that human-relevant signaling pathways involved in neurodevelopmental migratory processes *in vivo* (EGFR and SRC) are preserved in the hNPC-derived cells *in vitro* supporting the applicability of the NPC2a assay to study cell migration during development.

In addition to studying signaling pathways, the NPC2a assay is also able to identify chemicals evidently disturbing migratory processes upon chemical exposure. Prenatal exposure to MeHg causes severe neurological symptoms including intellectual disabilities and cerebral palsy in children (Harada, 1978). Investigations of brain autopsy samples confirmed that exposure to MeHg perturbed cell migration and disorganized neocortical layering (Choi et al., 1978), which was verified in animal models (Kakita et al., 2002). Dysplasia and abnormal cortical cytoarchitecture have been attributed to a MeHg-mediated genetic reprogramming of signaling pathways regulating neural development. Hence disrupting the cerebral cortical organization, disturbing migratory processes and causing heterotopia (Choi et al., 1978; Rand et al., 2009; Faustman et al., 2012). One signaling pathway affected by MeHg exposure is the Notch receptor pathway, which controls cell fate decisions, proliferation, migration and neurite outgrowth during neural development (Bland and Rand, 2006). Moreover, MeHg disturbs the cytoskeletal organization involved in cell migration by disrupting the assembly and polymerization of microtubules (Choi, 1991). Exposure of differentiating hNPCs to MeHg resulted in a concentration-dependent reduction of RG migration at *in vivo* relevant concentrations (**Figure 3E** adapted from Fritsche et al. (2018a), Moors et al. (2009), Baumann et al. (2016)). No cytotoxicity was observed in the tested concentration range, indicating a specific effect of MeHg on hNPC migration. As demonstrated with MeHg, the NPC2a assay is able to detect specific alterations in cell migration and therefore allows for the detection of chemically-induced disruption of migration in the context of brain development. Besides RG migration (NPC2a) the Neurosphere Assay covers the endpoints neuronal (NPC2b) and oligodendrocyte migration (NPC2c). However, for these endpoints, the identification of signaling pathways and model substances is still ongoing, which is the reason why they are not included in this study.

3.3 Neuronal Differentiation and Morphology (NPC3+4)

During cortex development, NPCs including RG cells eventually lose their proliferative capacity and terminally differentiate into neural effector cells, i.e. neurons and glia cells (oligodendrocytes and astrocytes). Neurons then migrate alongside the scaffold of RG to their final destinations, to generate the different cortical layers (Rakic, 1972; Gilmore and Herrup, 1997). The generation of sufficient numbers of neurons is a prerequisite for the functionality of neuronal networks and associated learning and memory functions (Berdugo-Vega et al., 2020). Therefore, disturbed neurogenesis manifests in several behavioral

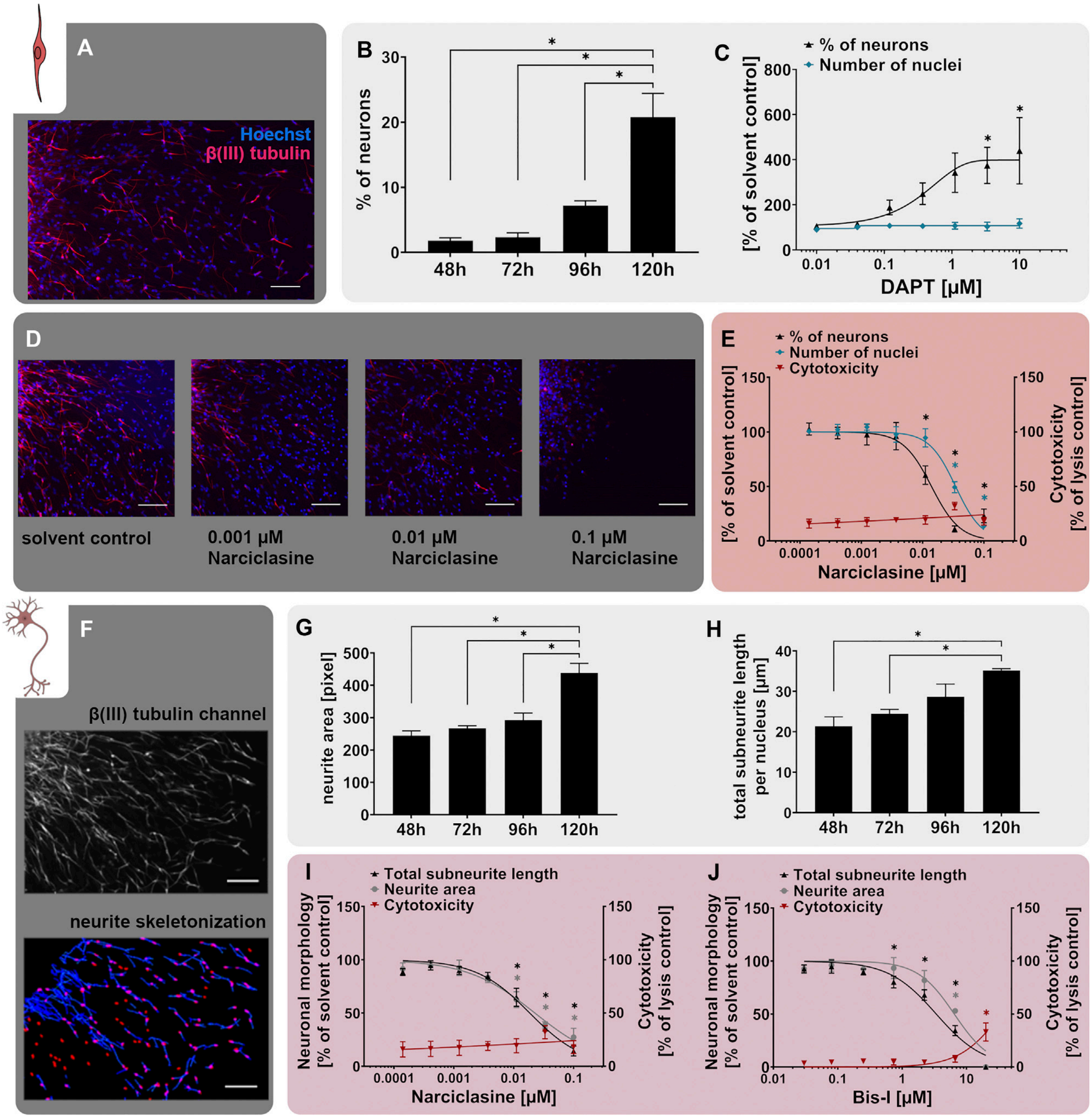

FIGURE 4 | The NPC3 and NPC4 assays identify disruption of neuronal differentiation and morphology. Primary hNPC neurospheres were differentiated on PDL-laminin-coated matrices for 5 days without growth factors. **(A)** Immunocytochemical stainings for β(III)tubulin (neurons, red) and Hoechst33258 (nuclei, blue) confirmed neuronal marker expression and morphology. Scale bar: 100 µm. **(B)** Neuronal differentiation, assessed as the percentage of β(III)tubulin-positive neurons compared to the total nuclei count within the migration area, increased gradually over the 5 days of differentiation. **(C)** hNPCs differentiation for 5 days in presence of increasing concentrations of the Notch inhibitor DAPT (0.01 µM–10 µM) concentration-dependently decreased neuronal differentiation compared to the solvent control. **(D,E)** Treatment with the RhoA activator narciclasine (0.00014 µM–0.1 µM) for 5 days concentration-dependently decreased neuronal differentiation compared to the solvent control (SC). Representative pictures of β(III)tubulin- and Hoechst33258-stained cells **(D)** and concentration-response curves **(E)** are shown (adapted from Masjosthusmann et al. (2020). Scale bar: 100 µm. Cytotoxicity (LDH release) was assessed in parallel and is depicted as % of a lysis control (differentiated hNPCs treated with 0.2% Triton-X100). **(F)** The NPC4 assay detects the neuronal morphology of hNPC-derived β(III)tubulin-positive neurons. After the neurons were annotated by the convolutional neural network of the AI, neurite length and area were calculated by the Omnisphero software (Schmuck et al., 2017). **(G+H)** Neurite area and total subneurite length gradually increased over the 5 days of differentiation. **(I+J)** Both the RhoA activator narciclasine and the PKC inhibitor bisindolylmaleimide 1 (Bis-I) concentration-dependently decreased the neurite area and subneurite length compared to the respective solvent control in hNPCs differentiating over 5 days (adapted from Masjosthusmann et al., 2020). Cytotoxicity (LDH release) was assessed in parallel and is depicted as % of a lysis control (differentiated hNPCs treated with 0.2% Triton-X100). Data are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA. A p-value below 0.05 was termed significant. * significantly changed compared to the respective solvent control.

disorders such as depression (Song and Wang, 2011) or the intellectual disabilities of patients with Down Syndrome (Guidi et al., 2018; Stagni et al., 2018). In addition, an elevation in neurogenesis is a major driver of epileptogenesis (Jessberger and Parent, 2015). Hence, the correct balance of neurogenesis is crucial for normal brain development.

As a very simplified model, the Neurosphere Assay mimics cortex development *in vitro* since during the time-course of hNPC differentiation, neurons arise and migrate along the scaffold of RG cells (Fritsche et al., 2018b). Such young neurons are typically bipolar in shape and display neurites that show very limited branching (**Figures 4A,E,F** and Budday et al., 2015). Using high content imaging (HCI) and a subsequent artificial intelligence (AI; developed in collaboration with Prof. Dr. Axel Mosig (Ruhr University Bochum), Förster et al., 2021) we define neuronal identity in the mixed-culture neurosphere migration area due to immunocytochemical stainings with β(III)tubulin (**Figure 4A**). Over time, neurons progressively appear in the migration zone representing approximately 20% of the mixed culture after 5 days (**Figure 4B**), which is the time point of endpoint analysis in the NPC3 test method.

Neurogenesis during brain development is primarily regulated by the Notch signaling pathway, which is evolutionarily highly conserved and operates at many stages of human brain development (Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006; Pierfelice et al., 2011). Stimulation of the Notch pathway could be correlated with impaired neuronal differentiation *in vivo* (Zhou W. et al., 2016; Zhang et al., 2018). In contrast, inhibited Notch signaling is known to accelerate neuronal differentiation *in vitro* and *in vivo* (Borghese et al., 2010). This can be pharmacologically excited by the Notch receptor inhibitor N-[N-(3,5-Difluorophenacetyl)-Lalanyl]-S-phenylglycine t-butyl ester (DAPT) through blockage of the presenilin-y-secretase complex (Dovey et al., 2001). Moreover, the Notch pathway is involved in the formation of long-term memory and is thus a putative actuator of developmental disorders (Costa et al., 2005). According to our comprehensive transcriptomic analysis, differentiating hNPCs express Notch receptors 1-3 (Masjosthusmann et al., 2018). Here we show that similar to our previously published work after 72 h of differentiation (Masjosthusmann et al., 2018), DAPT increases neuronal numbers to 187% and 439% of the respective solvent control at 0.12 and 10 μM DAPT, respectively, after 5 days of differentiation. Of note, the total cell number was not affected, indicating that the increase in neuronal numbers is at the expense of another cell type within the mixed culture (**Figure 4C**). The higher sensitivity of hNPC towards DAPT in this study is possibly due to the longer experimental time. Moreover, here we use different individuals compared to the previous study. These results indicate that the human-relevant Notch signaling pathway, which is one of the main drivers of neuronal differentiation *in vivo*, is also active in the hNPCs *in vitro*.

The positive effect of Notch inhibition on neuronal differentiation is thought to be - at least in part - attributed to suppression of the Rho GTPase RhoA (Peng et al., 2019). Consistent with this notion, narciclasine, an activator of RhoA, reduced neuronal differentiation of primary hNPCs cultured for 5 days in a concentration-dependent manner, together with a less sensitive reduction of the nuclei number (**Figures 4D,E** adapted from Masjosthusmann et al. (2020)). Increased RhoA activity correlated with reduced neuronal differentiation of murine neural stem cells and human iPSCs (Yang et al., 2016; Bogetofte et al., 2019). In contrast, inactivated RhoA signaling was sufficient to stimulate axon regeneration and recovery of hindlimb function after spinal cord injury in mice (Dergham et al., 2002) supporting the concept of RhoA activity as an inhibitory driver of neurogenesis.

Besides the generation of adequate neuronal numbers during neurogenesis, neuronal maturation, especially neurite outgrowth, and branching are equally important for the functional maturation of the CNS. Perturbations of which are assumed to be linked to neurodevelopmental disorders like autism spectrum disorder in humans (Zikopoulos and Barbas, 2010). The NPC4 assay measuring neurite outgrowth builds upon the neuronal differentiation assay (NPC3) and evaluates the neurite morphology within the multicellular differentiated neurosphere culture. After AI-based identification of β(III)tubulin$^+$ neurons, their morphological features, e.g. neurite length and neurite area, are evaluated (**Figure 4F**) using the Omnisphero software (Schmuck et al., 2017). During the 5 days of hNPC differentiation, neurite maturation is characterized by an elongation of neurites and an increase in neurite area (**Figures 4G+H**).

Consistent with the above-mentioned effect of RhoA activation on neuronal differentiation (NPC3), narciclasine also reduced both neurite area and neurite length (**Figure 4I** adapted from Masjosthusmann et al. (2020)) within the NPC4 assay. This is in line with previous studies, reporting that narciclasine reduced neurite outgrowth via the Rho-associated protein kinase (ROCK) pathway in neurons differentiated from LUHMES human neuronal precursor cells (Krug et al., 2013). Moreover, a contactin-1 knock-down-dependent increase in RhoA activity caused morphological alterations in rat cortical neurons *in vivo* (Chen et al., 2018). Neurite outgrowth is further regulated by protein kinase C (PKC), a serine/threonine kinase, which controls various cellular responses by phosphorylation of substrate molecules and alteration of gene transcription (Nishizuka, 1986; Newton, 1995). While PKC activation induced neurite outgrowth in rat pheochromocytoma (PC-12) cells and primary rat spinal cord neurons from embryonic day 14 (Hundle et al., 1995; Yang et al., 2010), inhibition of PKC with the PKC inhibitor bisindolylmaleimide 1 (Bis-I) reduced neurite growth in PC-12 cells (Das et al., 2004), rat cortical neurons and human iPSC-derived neurons (Druwe et al., 2016). Similar effects were identified in the NPC4 assay upon exposure of hNPCs to Bis-I, which reduced neurite length and area compared to the solvent control (**Figure 4J** adapted from Masjosthusmann et al. (2020)).

Neuronal differentiation and maturation are tightly regulated processes, which are controlled by a variety of different signaling pathways, whose perturbation can cause severe adverse

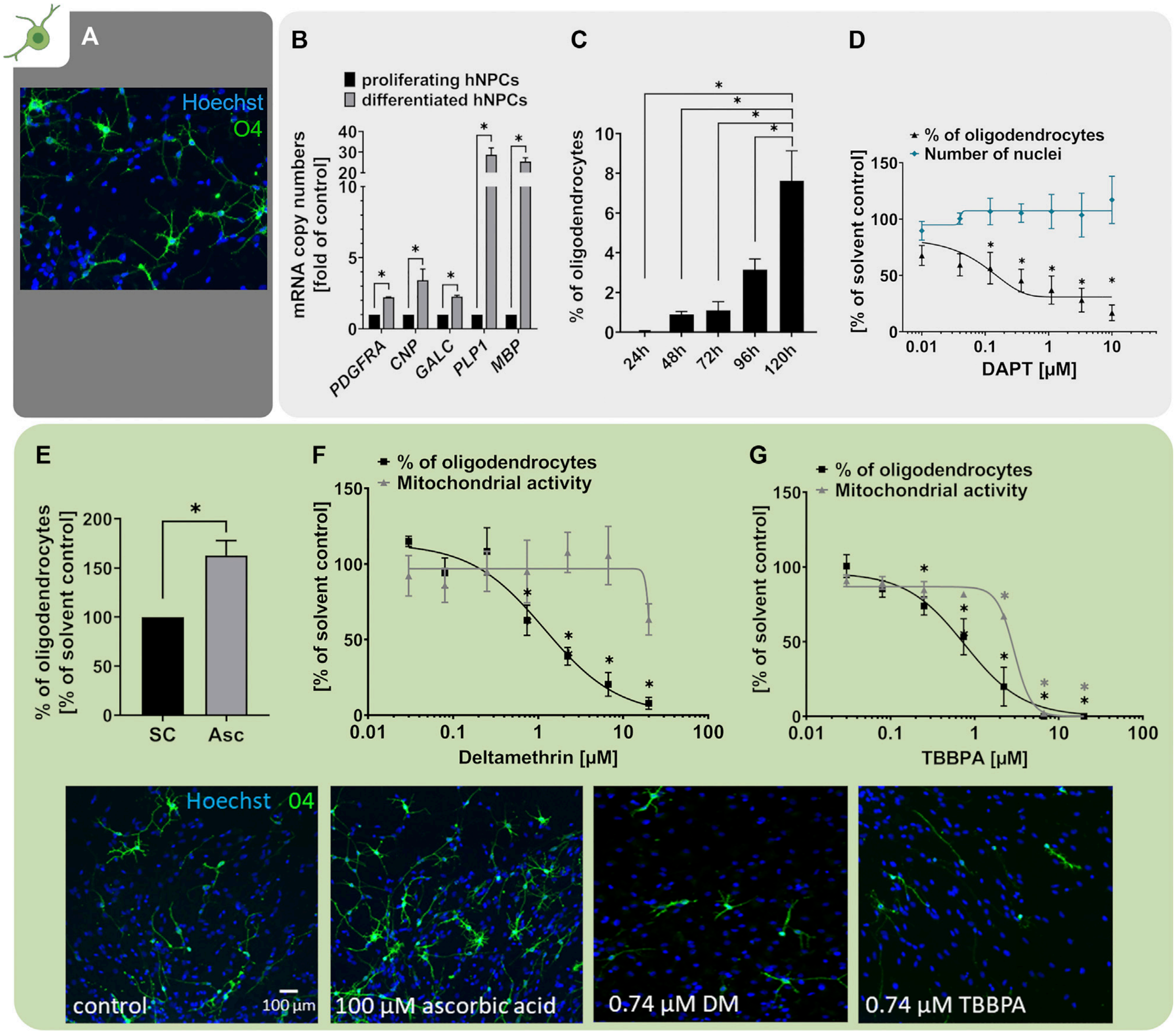

FIGURE 5 | Oligodendrocyte differentiation is assessed with the NPC5 assay. **(A)** Primary hNPCs were differentiated for 5 days in differentiation medium without growth factors on PDL-laminin-coated plates. Immunocytochemical stainings were performed to identify cells of the OL lineage (O4) and cell nuclei (Hoechst33258). **(B)** mRNA expression of OL lineage markers *PDGFRA*, *CNP*, *GALC*, *PLP1* and *MBP* was assessed in proliferating hNPCs and hNPC differentiated for 60 h using quantitative real-time PCR. Expression was calculated as copy numbers (CN) per CN of the reference gene *ACTB* multiplied by 10.000. The expression in differentiated hNPCs is displayed as fold of expression in proliferating hNPCs. Expression of all markers increased during hNPC differentiation (adapted from Klose et al. (2021a)). **(C)** Oligodendrocyte differentiation, assessed as the percentage of O4-positive OLs compared to the total nuclei count within the migration area, increased gradually over the 5 days of differentiation. **(D)** hNPCs differentiation for 5 days in presence of increasing concentrations of the Notch inhibitor DAPT (0.01 µM–10 µM) concentration-dependently decreased OL differentiation compared to the solvent control. **(E)** hNPCs differentiation in presence of 100 µM ascorbic acid (Asc) increased the percentage of OLs in the differentiated culture. **(F+G)** Both exposure to the insecticide deltamethrin (DM, 0.027–20 µM, adapted from Masjosthusmann et al., 2020) and the flame retardant tetrabromobisphenol A (TBBPA, 0.027–20 µM, adapted from Klose et al., 2021a) during the 5 days of differentiation concentration-dependently decreased OL differentiation compared to the respective solvent controls. For deltamethrin and TBBPA the mitochondrial activity was assessed in parallel and is depicted as % of solvent control. For **(E–G)**, representative pictures of O4$^+$ OLs exposed to solvent control or the respective treatments are shown. Data are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA **(C,D,F,G)** and two-tailed Student's t-tests **(B,E)**. A *p*-value below 0.05 was termed significant. *significant compared to the respective solvent control.

neurodevelopmental effects. The NPC3 (neuronal differentiation) and NPC4 (neuronal morphology) assays respond to known pathway modulators regulating neurogenesis and neurite outgrowth *in vivo* and are therefore predictive assays to identify chemicals disturbing neuronal development.

3.4 Oligodendrocyte Differentiation (NPC5)

Myelinating oligodendrocytes (OLs) are responsible for the formation of insulating myelin sheaths, thus accelerating the conduction of electrical impulses along axons and preserving axonal integrity during neurodevelopment and beyond. OLs derive from NPCs and RG cells differentiating into oligodendrocyte precursor cells (OPCs) and terminally into pre-myelinating OLs (pre-OLs) and myelin-producing mature OLs (Emery, 2010; van Tilborg et al., 2018). The OL-derived myelin ensheathing neuronal axons is indispensable for the development and function of the human brain (reviewed in Barateiro et al. (2016)). However, both pre-OLs and myelinating OLs are susceptible to various stressors including oxidative stress, astrogliosis, excitotoxicity and inflammation (reviewed in van Tilborg et al. (2016)) rendering them susceptible to a variety of exogenous stressors. Disturbances in oligodendrogenesis during neurodevelopment are associated with hypomyelination and white-matter deficits manifesting in clinical pathologies including the Allan-Herndon-Dudley Syndrome (Sarret et al., 2010) and periventricular leukomalacia (PVL; Back et al., 2001). Since the pool of OLs in humans remains stable after childhood, especially interference in OL development during the neurodevelopmental period is crucial (Yeung et al., 2014). The generation of pre-myelinating OLs can be modelled in hNPCs *in vitro* (NPC5). Differentiation of hNPCs over 5 days generated cells expressing the OL-marker O4, which exhibit the typical OL morphology with multiple branched processes necessary to ensheath neuronal axons (**Figure 5A**). Compared to undifferentiated hNPC neurospheres, differentiating hNPCs significantly increased mRNA expression of the OL markers *PDGFRA* (platelet-derived growth factor alpha, PDGFRα), *CNP* (CNPase), *GALC* (Galactosylceramidase), *PLP1* (proteolipid protein 1), and *MBP* (myelin basic protein) already after 60 h (**Figure 5B** adapted from Klose et al. (2021a)). While *PDGFRA* is predominantly a marker of immature OPCs, especially *MBP* is a myelin-associated gene increasingly expressed during oligodendrocyte maturation (Barateiro and Fernandes, 2014; Marinelli et al., 2016). Based on the marker expression and the highly branched morphology (**Figures 5A,B**), we conclude that our pre-OLs exhibit a certain degree of maturity. Similar to the neuronal differentiation described above, also the percentage of OLs within the multicellular hNPC-derived migration area increased over the differentiation time resulting in approximately 8% OLs after 5 days (**Figure 5C**; Moors et al., 2009).

It is well studied that several signaling pathways, including the Notch pathway, regulate NPC differentiation into OPCs (reviewed by He and Lu, 2013). A study on zebrafish embryos revealed that Notch is responsible for increased production of OPCs from ventral spinal cord precursors and that the increased OPC number is not due to increased OPC proliferation (Snyder et al., 2012). Moreover, contactin/F3-dependent Notch signaling promoted OPC differentiation from the rat oligodendroglial OLN-93 cell line and further increased the expression of myelin-associated glycoprotein (*MAG*; Hu et al., 2003). In line with that, differentiation of hNPCs in presence of the Notch inhibitor DAPT concentration-dependently decreased the percentage of $O4^+$ cells compared to the solvent control, indicating that Notch signaling is a prerequisite for hNPC differentiation into the OL lineage (**Figure 5D**). In addition, OL differentiation is negatively influenced by bone morphogenic protein (BMP) 7 (Baumann et al., 2015) and BMP2 (Masjosthusmann et al., 2018), proteins of the transforming growth factor β family. BMP 2 and 7 also negatively regulated oligodendrocyte differentiation of primary rat NPC generated from E17 and PND2 brains (Zhu et al., 1999) and reduced myelin gene expression in Schwann cells (Liu et al., 2016). These data demonstrate that two major developmental pathways, i.e. Notch and BMP, are functional in these hNPCs.

Several studies — including observations in humans — confirmed that pre-OLs are especially susceptible to oxidative stress and that pre-OL damage by reactive oxygen species (ROS) is a potential underlying factor for the emergence of the cerebral white matter injury termed periventricular leukomalacia (PVL) (reviewed in Volpe et al. (2011)). In accordance, Guo et al. (2018) reported that the ROS scavenger vitamin C (ascorbic acid, Asc, 150 μM) enhanced the differentiation of primary mouse NPC-derived OPCs into OLs and further promoted expression of OL lineage markers O4, CNPase and MBP concentration-dependently (Guo et al., 2018). We also observed that ascorbic acid enhanced hNPC-derived OL maturation (Dach et al., 2017). However, in contrast to Guo et al. (2018), we did not observe this enhanced maturation in OLs derived from PND1 mouse neurospheres. This might be due to different developmental stages of animals as Guo et al. (2018) used cortices of E14.5 mouse embryos for NPC generation. Likewise, differentiation of hNPCs in presence of 100 μM ascorbic acid increased the percentage of $O4^+$ oligodendrocytes within the NPC5 assay by approximately 60% (**Figure 5E**). This is in contrast to our previously published data where ascorbic acid solely induced maturity but not the number of OLs (Dach et al., 2017), which might be explained by inter-individual differences of the one individual used in the Dach et al. (2017) compared to the three individuals in this study.

Due to the particular sensitivity of OPCs and pre-OLs towards multiple stressors including ROS, excitotoxic damage, thyroid hormone disruption, or inflammatory cues (Volpe et al., 2011; Barateiro et al., 2016; Chesnut et al., 2021a), it is hypothesized that they might also be highly sensitive towards a variety of chemical noxae (Chesnut et al., 2021a). Within the NPC5 assay, we here show as two examples that both the insecticide deltamethrin (DM, **Figure 5F** adapted from Masjosthusmann et al. (2020)) and the organophosphate flame retardant tetrabromobisphenol A (TBBPA, **Figure 5G** adapted from Klose et al. (2021a)) diminished the number of $O4^+$ oligodendrocytes concentration-dependently. Childhood exposure to pyrethroids like DM correlates with neurodevelopmental disorders including autism, attention deficit hyperactivity disorder (ADHD) and developmental delays reviewed in Pitzer et al. (2021). Likewise, developmental and early-life exposure to DM in rodents is associated with ADHD-like and anxiety-like behavior as well as deficits in working memory and spatial learning, often depending on the developmental stage of exposure (reviewed in Pitzer et al. (2019), (2021); Richardson et al. (2015)). The primary mode-of-action (MoA) of DM for its anti-pest action in mature neurons is the prolonged opening of voltage-gated sodium channels (VGSC). OPCs express active VGSC rendering this MoA highly likely for DM action on this immature OL state (comprehensively summarized in Hernández-Jerez et al. (2021)). In addition, DM induces oxidative stress and lipid peroxidation, which most likely also

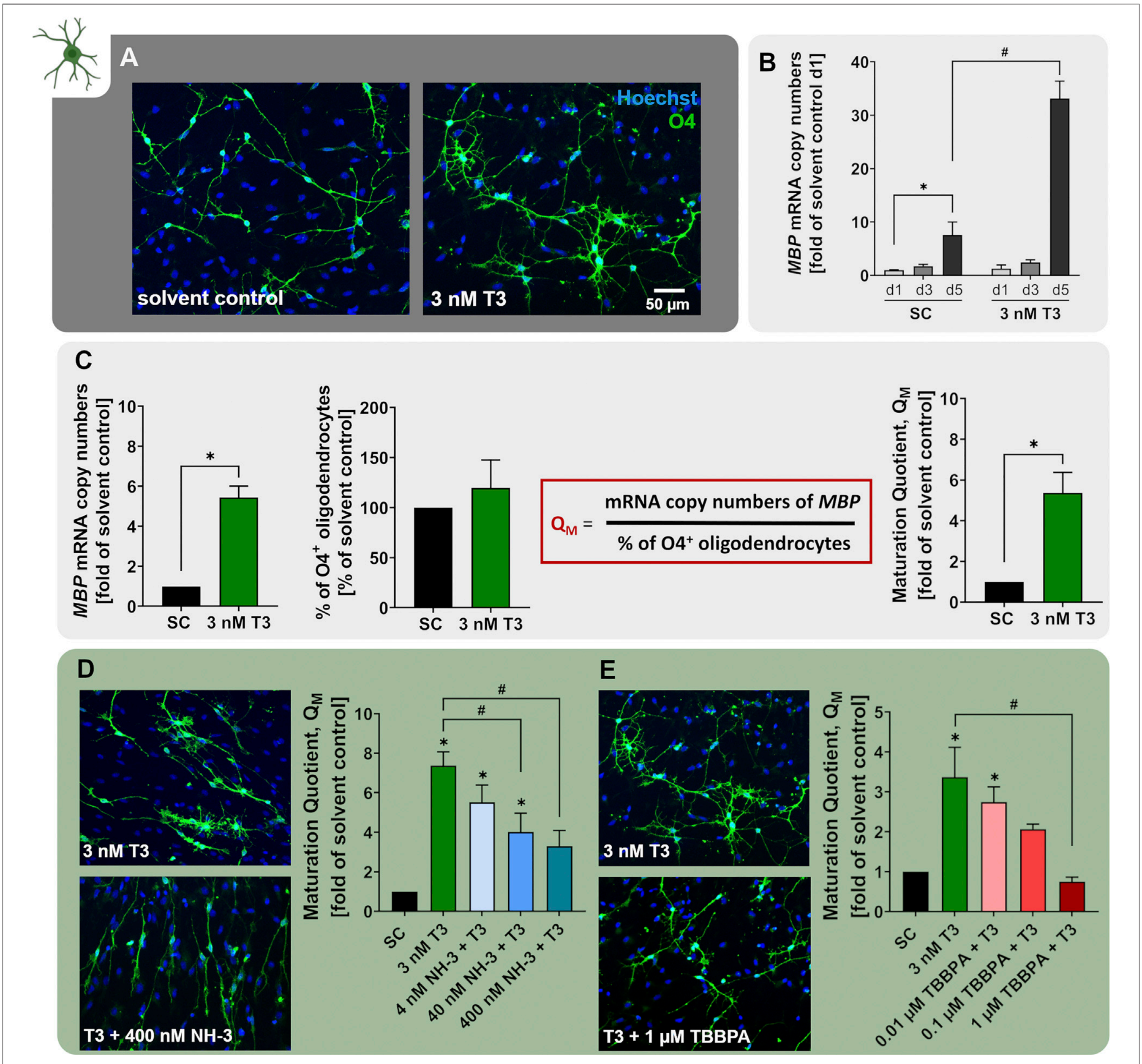

FIGURE 6 | The NPC6 assay identifies disruptors of TH-dependent oligodendrocyte maturation. **(A)** Primary hNPCs were differentiated for 5 days in differentiation medium without growth factors on PDL-laminin coated plates either in presence of 3 nM T3 or solvent control. Immunocytochemical stainings were performed to identify cells of the OL lineage (O4) and cell nuclei (Hoechst33258). **(B)** mRNA expression of the OL lineage marker *MBP* was assessed in hNPCs differentiated in the presence of 3 nM T3 or solvent for 24, 72 or 120 h using quantitative real-time PCR. Expression is displayed as *MBP* mRNA copy numbers (CN) per CN of the reference gene ACTB multiplied by 10.000. The expression is displayed as fold of expression after 24 h (adapted from Dach et al., 2017). **(C)** OL maturation was quantified using the maturation quotient (Q_M), which is calculated by dividing the *MPB* mRNA CN ((copy number *MBP*/ copy number *ACTB*) *10.000) by the percentage of $O4^+$ cells. Exposure to 3 nM T3 significantly increased the Q_M compared to the solvent control (SC). **(D+E)** The Q_M was calculated for hNPCs differentiating for 5 days in presence of solvent (SC), 3 nM T3 alone or T3 in combination with increasing concentrations of the TH receptor antagonist NH-3 (4–400 nM) or the flame retardant TBBPA (0.01–1 µM). Both NH-3 and TBBPA concentration-dependently decreased the Q_M compared to 3 nM T3 and thus impaired T3-depedent OL maturation (adapted from Klose et al. (2021b)). Data are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA **(B, D, E)** and two-tailed Student's t-tests **(C)**. A *p*-value below 0.05 was termed significant. *significant compared to the respective solvent control. $^{\#}$significant compared to 3 nM T3.

contribute to its neurotoxicity (reviewed in Pitzer et al. (2021)). The flame retardant (FR) TBBPA interferes with brain development in rodents (Hendriks et al., 2015; Rock et al., 2019) and its DNT-relevance for humans is supported by studies reporting bioaccumulation in maternal serum, cord blood and breast milk (Cariou et al., 2008; Kim and Oh, 2014). The adverse effects of TBBPA on OL differentiation in the NPC5 assay were accompanied by deregulation of a gene

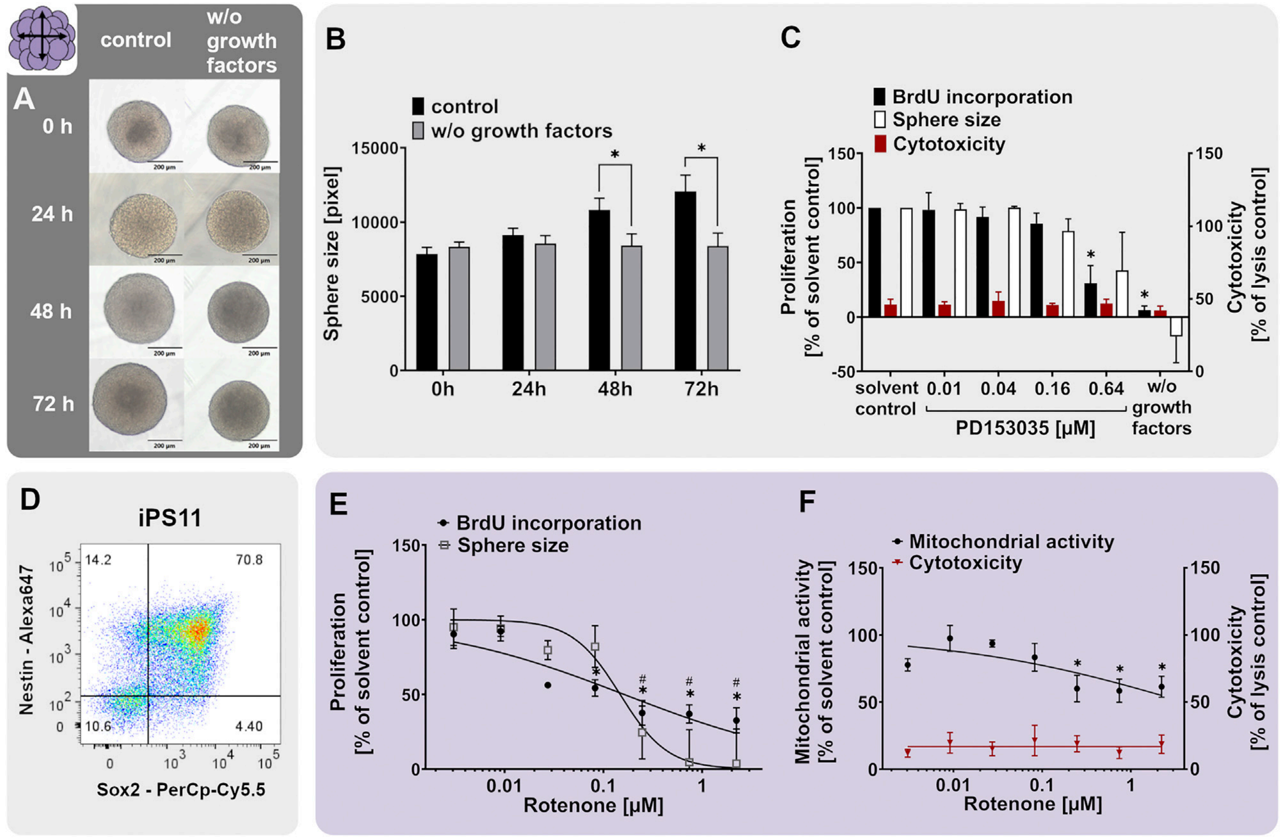

FIGURE 7 | The proliferation of hiNPCs is assessed with the iNPC1ab assay. **(A,B)** Human iPSC-derived hiNPCs were cultivated for 3 days in proliferation medium containing 20 ng/ml of the growth factors EGF and FGF (control) or in medium without growth factors (w/o growth factors). Representative pictures **(A)** and quantifications of the sphere size **(B)**, as assessed within the iNPC1a assay, showed that growth factors are necessary for hiNPC proliferation. **(D)** Proliferating hiNPC neurospheres issued from an iPS11 neural induction were analyzed using flow cytometry analysis, confirming high expression of the neural stem/progenitor markers nestin and SOX2. The percentage of double-positive cells is indicated in the upper right quartile. **(C, E, F)** Exposure of proliferating hiNPCs for 3 days to increasing concentrations of the EGFR inhibitor PD153035 **(C)** or the mitochondrial complex I inhibitor rotenone **(E,F)** concentration-dependently decreased hiNPC proliferation compared to the respective solvent controls. Proliferation was assessed by sphere size increase (iNPC1a) and BrdU incorporation into the DNA (iNPC1b). The values of the chemical-treated conditions are expressed as % of the respective solvent controls. Cytotoxicity (LDH release) was assessed in parallel and is depicted as % of a lysis control (spheres treated with 0.2% Triton-X100). For rotenone-treatment, mitochondrial activity **(F)** was assessed in parallel and is depicted as % of solvent control. Data are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA **(C, E, F)** and two-tailed Student's t-tests **(B)**. A *p*-value below 0.05 was termed significant. Symbols * and # show statistical differences in comparison to the solvent control of the respective endpoint if not marked otherwise.

cluster involved in cholesterol metabolism suggesting lipotoxicity as TBBPA's MoA (Klose et al., 2021b). Since myelinating OLs exhibit an exceptionally high rate of cholesterol metabolism, disturbances are particularly problematic in this cell type (Haq et al., 2003; Bezine et al., 2017).

In the past, the NPC5 assay has been identifying compounds of various substance classes as disruptors of OL differentiation including brominated as well as alternative organophosphate FRs (Dach et al., 2017; Klose et al., 2021a), sodium arsenite (Masjosthusmann et al., 2019), and a variety of substances within a recent screening project where the NPC5 assay was the most frequently positive assay across the Neurosphere Assay battery (Masjosthusmann et al., 2020). In neurotoxicological studies, oligodendrocytes are an understudied, yet highly relevant cell type that just recently received more attention (Chesnut et al., 2021a).

3.5 Thyroid Hormone (TH)-dependent Oligodendrocyte Maturation (NPC6)

In order to develop into myelinating OLs, OPCs and pre-OLs have to mature and express myelin-associated genes including myelin basic protein (MBP) and myelin proteolipid protein (PLP1). This maturation processes and the proper development of white matter tracts in humans depend on thyroid hormones (TH), such as the thyroxine metabolite triiodothyronine (T3; Annunziata et al., 1983; Baas et al., 1997; Murray and Dubois-Dalcq, 1997). In line with that, *in vivo* studies on hypothyroid rats reported reduced numbers of mature OLs and impaired expression of PLP1 and MBP (Ibarrola and Rodríguez-Peña, 1997; Schoonover et al., 2004). The devastating effects of TH disruption for human neurodevelopment are illustrated by clinical pathologies describing hypomyelination as a result of TH insufficiencies, including congenital hypothyroidism, maternal hypothyroidism, or the

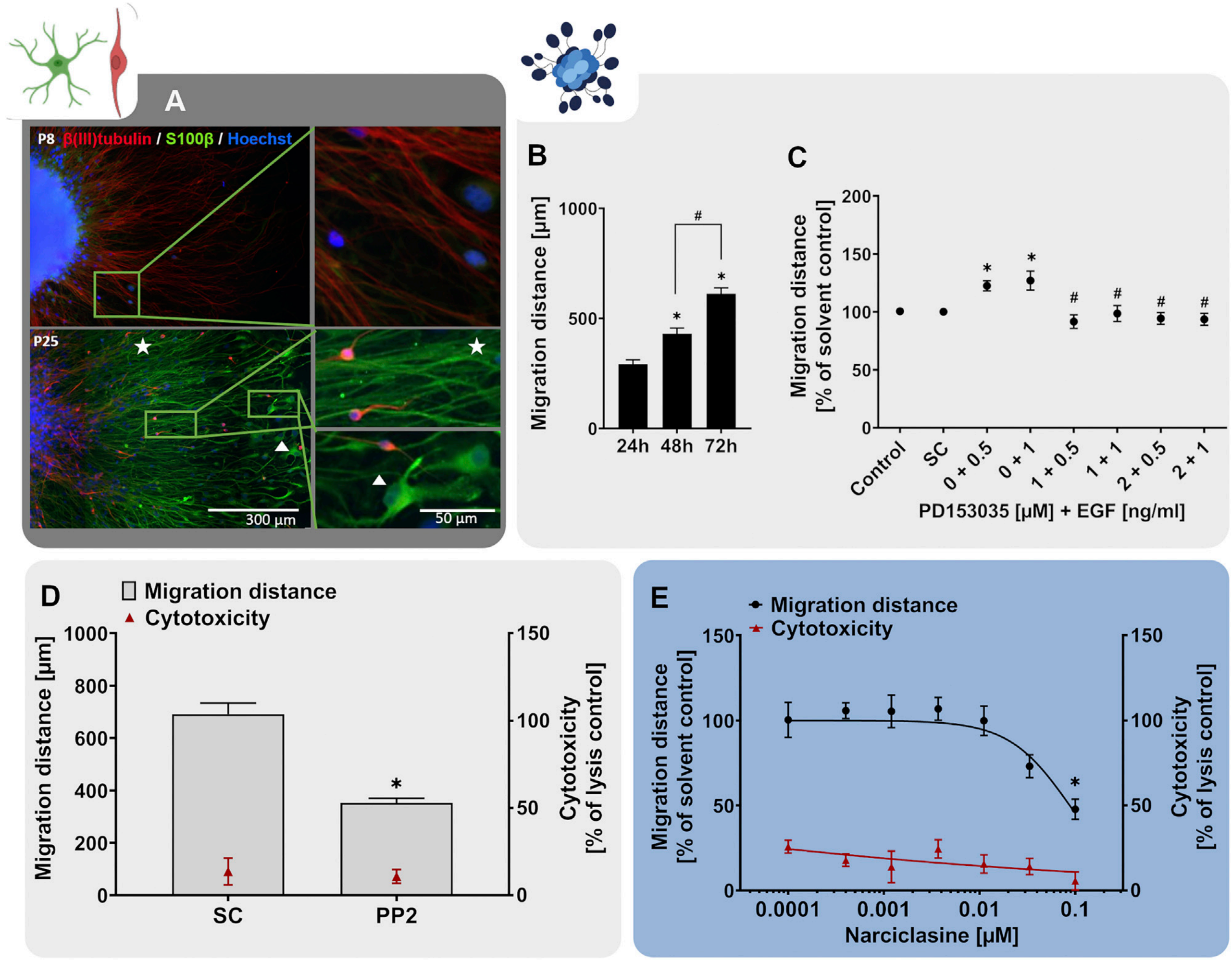

FIGURE 8 | The hiNPC2 assay identifies disturbances of neural migration. Human hiNPCs were differentiated on PDL-laminin-coated 96-well plates in the absence of growth factors. **(A)** After 3 days of differentiation, immunocytochemical stainings of hiNPCs in early (p8) and later (p25) passages confirmed expression of the neuronal marker β(III)tubulin and the glial marker S100β. Nuclei were counterstained with Hoechst33258. Stars indicate RG-like cells and triangles indicate astrocyte-like morphology. **(B)** The migration distance of hiNPCs increased gradually over time, as assessed by determining the distance from the sphere core to the furthest migrated cells at four opposite positions in brightfield images every 24 h **(C)** hiNPC migration over 3 days was assessed in presence of EGF (0.5–1 ng/ml) alone, in combination with the EGFR inhibitor PD153035 (1–2 µM), or the respective solvent. While EGF increased hiNPC migration, PD153035 inhibited the EGF-induced effect. **(D)** A negative effect of the SRC-family kinase inhibitor PP2 on hiNPC migration was confirmed by differentiating hiNPCs for 3 days in presence of 10 µM PP2 or the respective solvent (SC). **(E)** hiNPCs differentiation in presence of increasing concentrations of narciclasine (MeHg, 0.0001–0.1 µM) for 3 days concentration-dependently reduced the migration distance. For **(D,E)**, cytotoxicity (LDH release) was assessed in parallel and is depicted as % of a lysis control (differentiated hiNPCs treated with 0.2% Triton-X100). Data are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA **(B, E)** and two-tailed Student's t-tests **(C, D)**. A *p*-value below 0.05 was termed significant. *significant compared to the respective solvent control; #significant compared to the respective EGF concentration.

Allan-Herndon-Dudley syndrome (AHDS). These conditions feature clinical symptoms ranging from mild cognitive deficits to severe intellectual disabilities (Haddow et al., 1999; Rovet and Daneman, 2003; Sarret et al., 2010). ADHS is caused by inactivating mutations in the monocarboxylate transporter 8 (MCT8), a TH transporter, which is responsible for TH transport into the brain, and thus required for OL maturation (Vatine et al., 2021).

Maturation of pre-OLs can be induced *in vitro* by differentiation of hNPCs in presence of 3 nM T3 (NPC6). Exposure to T3 clearly caused the O4$^+$ cells to develop a more mature morphology with more branched processes (**Figure 6A**). Moreover, *MBP* mRNA expression increases over the time-course of differentiation already under control conditions, and even further in the presence of 3 nM T3 (**Figure 6B** adapted from Dach et al. (2017)). Since MBP is one of the major components of myelin, hNPC-derived pre-OLs differentiating in presence of T3 are on the path to myelinating OLs. In order to quantify the degree of OL maturation within the NPC6 assay, we calculated the maturation quotient (Q_M), which is defined as the mRNA copy numbers of *MBP* per percentage of O4$^+$ cells within the migration area. In line with the multitude of studies reporting that TH favor OL maturation (Baas et al., 1997; Dach et al., 2017; Klose et al., 2021b), we observed an increase of the Q_M upon exposure to 3 nM T3 (**Figure 6C**). The TH-dependent maturation of hNPC-derived OLs within the NPC6 assay further reacts to the synthetic antagonist NH-3 (Nguyen et al., 2002; Singh et al., 2016) since NH-3 concentration-dependently reduced the Q_M indicating that disruptors of TH receptor signaling can be identified with the NPC6 assay (**Figure 6D** adapted from Klose et al. (2021b), Dach et al. (2017)). A human-relevant disruptor of OL maturation identified within the NPC6 assay is TBBPA. At low concentrations, not yet affecting OL differentiation, TBBPA disturbs TH-dependent OL maturation, hence concentration-dependently reducing the Q_M in hNPCs differentiated in presence of 3 nM T3 (**Figure 6E** adapted from Klose et al. (2021b)). Impaired OL maturation is accompanied by alteration of TH-dependent genes, including *EGR1*, *IGFBP4*, *IL33* and *KLF9* (Klose et al., 2021b). These data provide the scientific basis for studying the disruption of TH-dependent oligodendrocyte maturation in differentiating hNPC. In the past, the NPC6 assay identified both BDE-99 and perfluorooctanoic acid (PFOA) not to be disruptors of human TH-dependent OL maturation, although BDE-99 reduced OL numbers (Dach et al., 2017; Klose et al., 2021b).

3.6 Human iPSC-derived hiNPC proliferation, migration and differentiation (hiNPC1+2a+3)

For 21st-century toxicity evaluation, hiPSCs have been strongly promoted as the basis for diverse test systems since they are of human origin, have unlimited availability and resemble different features of the desired target tissues very well (Wobus and Löser, 2011; Jennings, 2015; Csöbönyeiová et al., 2016; Fritsche et al., 2021). For brain tissues, one can generate hiPSC-derived neural progenitor cell (hiNPC) neurospheres, a relatively simple and easy to generate cell system (Sareen et al., 2014; Hofrichter et al., 2017; Kobolak et al., 2020). The hiNPCs have the ability to differentiate into neurons and astrocytes in secondary 3D (Sareen et al., 2014; Paşca et al., 2015; Zhou S. et al., 2016; Hofrichter et al., 2017; Nimtz et al., 2020; Soubannier et al., 2020) and 3D cultures (Pamies et al., 2017; Sloan et al., 2017; Marton et al., 2019; Chesnut et al., 2021b). For the generation of OLs, however, hiNPC differentiation time takes at least 8 weeks (Pamies et al., 2017) and is therefore not directly comparable to the primary neurospheres which produce OLs within 5 days of differentiation. Here we present data on hiNPC proliferation, migration and the differentiation potential.

When relating hiNPC (**Figure 7A**) to hNPC neurospheres (**Figure 2A**), they display the same neurosphere morphology and cannot be distinguished from each other with the bare eye. Moreover, hiNPCs express the neural stem/progenitor markers nestin and SOX2 (**Figure 7D**). The percentage of Nestin/SOX2 double-positive hiNPCs (70.8%) was in the same ballpark as that of hNPC (76.6%). Human iNPCs contained 10.6% cells not expressing any of the two markers, which is higher compared to the average of 1.9% of the three hNPC individuals (**Figure 2**). The proliferative capacity of hiNPCs was confirmed by measuring the sphere size increase over 3 days (iNPC1a) as shown in **Figure 7B**. hiNPC neurospheres increased their size by 53.6% during the 3 days in a proliferation medium containing EGF and FGF basic (control), whereas hiNPCs cultivated in proliferation media without growth factors (w/o growth factors) did not increase in size. In comparison, primary hNPC spheres only increased by approximately 30% in size over the 3 days (**Figure 2B**). EGF-dependent hiNPC proliferation is also EGFR-dependent since the EGFR inhibitor PD153035 decreased hiNPC proliferation (**Figure 7C**) similar to the primary hNPCs. These data demonstrate that the EGFR, as a crucial molecule for NPC proliferation, is also functioning in proliferating hiNPCs.

The proliferation of hiNPCs was also effectively inhibited by rotenone, an anti-proliferative compound with a known mode of action i.e. inhibition of the mitochondrial complex I of the electron transport chain (Saravanan et al., 2005). Rotenone produced oxidative stress in iPSC-derived neural stem cells (Pistollato et al., 2017) and mitochondrial dysfunction in human neural progenitor cells (Mahajan et al., 2019). In the present study, rotenone inhibited the proliferation of hiNPCs in a concentration-dependent manner with the lowest observed effect concentration of 30 nM (**Figure 7E**). In comparison, the proliferation of primary hNPCs was not affected by rotenone in the same concentration range (Masjosthusmann et al., 2020). Rotenone did not cause cytotoxicity in hiNPCs, however as expected from a mitochondrial complex I inhibitor, significantly reduced the mitochondrial activity (**Figure 7F**). Overall, the iNPC1ab assay behaves similar to the NPC1ab assay, however, hiNPCs proliferate faster.

Plating hiNPC neurospheres onto a PDL-laminin-coated matrix-initiated cell migration out of the sphere core accompanied by neuronal and astrocyte differentiation (**Figure 8A**). Importantly, the migration and neuron-glia cell differentiation patterns, as well as their respective cell morphologies highly depended on the sphere culture passage number. Early passages (P8) first and primarily differentiated into β(III)tubulin-positive neurons with elongated neurites that form dense neuronal networks followed by the appearance of S100β-positive astrocytes. Differentiation of hiNPCs from higher passages (P25) first led to the emergence of S100β-positive cells with RG-like morphology and subsequently of β(III) tubulin-positive neurons (**Figure 8A**). Regarding the S100β-positive cells, one could distinguish between elongated RG-like cells (**Figure 8A**, stars) and more star-shaped astrocytes (**Figure 8A**, triangle), the first being overrepresented in differentiating hiNPCs from higher passages.

Next, we inquired whether hiNPC neurospheres can also be used for studying neural migration. Therefore, we assessed the migratory capacity of hiNPC-derived cells (passages >17). After plating on PDL-laminin-coated matrices, the hiNPC-derived cells formed a circular migration area comparable to the primary hNPCs. Moreover, as observed for the hNPCs (**Figure 3B**), the migration distance of cells emerging from hiNPCs increased over time (**Figure 8B**). After 72 h, hiNPC migration was shorter (~600–750 μm) than hNPC migration (~950 μm, **Figure 2**), yet reproducible and fully sufficient for analyses.

As discussed above, EGFR-dependent signaling guides radial migration *in vivo* (Craig et al., 1996; Kornblum et al., 1998). To study migratory responses to EGF in hiNPCs, we measured the migration distances of hiNPC-derived cells in presence and absence of EGF and the EGFR inhibitor PD153035. Similar to hNPCs, the migration of plated hiNPCs was increased by both 0.5 and 1 ng/ml EGF, which was antagonized by co-treatment with PD153035 at concentrations of 1 and 2 µM (**Figure 8C**). Yet the EGF effects on hiNPC migration were weaker than in primary hNPCs (122% compared to 130% (0.5 ng/ml EGF) and 127% compared to 143% (1 ng/ml EGF)). This might be due to the developmental timing since the density of EGFR increases processivity through brain development (Burrows et al., 1997; Lillien and Raphael, 2000). Hence, the reduced responsiveness of hiNPCs to EGFR signaling could indicate that hiNPCs represent an earlier developmental time point compared to fetal hNPCs. However, since the observed differences were minor, additional studies are needed to thoroughly compare EGF function on hNPC and hiNPC migration. Further important regulators of neurodevelopmental migratory processes are SRC-kinases (Jossin et al., 2003; Kuo, 2005; Moors et al., 2007; Wang et al., 2015). Treatment of differentiating hiNPCs with the SRC-kinase inhibitor PP2 for 72 h reduced the migration distance to 51% of the respective solvent control without inducing any signs of cytotoxicity (**Figure 8D**). This is comparable to the hNPC response to PP2 exposure (**Figure 3D**). Last, we studied the effects of the RhoA GTPase activator narciclasine on hiNPC migration. RhoA activation reduced hiNPC migration in a concentration-dependent manner (**Figure 8E**). Comparing the hiNPC results (**Figure 8E**) to the response of primary hNPCs to narciclasine exposure (Masjosthusmann et al., 2020), the two cell systems did not differ in sensitivity (BMR_{10} 0.010 and 0.018 µM, respectively). In line with our observations, knockout of RhoA destabilized the actin and tubulin cytoskeleton in neurons and especially in radial glia cells, resulting in accelerated migration *in vitro* and *in vivo* (Cappello et al., 2012). Hence, narciclasine-mediated activation of RhoA could cause a hyperstabilization of the cytoskeleton and thus impair migration. This was observed in PARK2 knockout hiPSC-derived neurons, where migration was reduced by RhoA upregulation and rescued by RhoA inhibition (Bogetofte et al., 2019). Likewise, methylmercury, a metal disturbing neural migration in humans, affected hiNPC and hNPC migration at similar concentrations (Hofrichter et al., 2017).

Taken together, hiNPC proliferation and migration (iNPC1/2) work similarly as in primary hNPCs (NPC1/2). More work is needed to understand if these two test systems have also distinct applicability domains, i.e. concerning developmental timing, or if these are redundant assays. Nevertheless, primary NPCs produce oligodendrocytes within a very short time of 5 days, whereas hiPSCs need several weeks to produce oligodendrocytes. In addition, the convolutional neuronal networks were trained to quantify neuron and oligodendrocyte differentiation in the primary NPC assays. This has not been established for differentiating hiNPC, hence objective quantification methods for cell differentiation are lacking for this test system. Therefore, the primary neurosphere assay possesses its unique selling points.

3.7 Applications of the Neurosphere Assay

The Neurosphere test methods, which allow studying a large variety of neurodevelopmental KE, are suitable for many different applications ranging from basic scientific to different regulatory questions. The Neurosphere Assay can be applied in low to medium throughput formats by manual pipetting up to larger-scale applications for screening purposes using liquid handling systems. In the past, we studied the contribution of a variety of signaling pathways including interleukin-7 (Moors et al., 2009), the extracellular related kinase Erk1/2 (Moors et al., 2007), NO signaling (Tegenge et al., 2011), BMP2, the EGFR in intrinsic signaling, Notch1 (Masjosthusmann et al., 2018) and TH signaling (Dach et al., 2017) on neurodevelopmental KE using the Neurosphere assay. Moreover, we assessed the effects of a large variety of compounds on the Neurosphere Assay KEs and studied their MoA for some of them (Fritsche et al., 2005; Moors et al., 2007; Schreiber et al., 2010; Gassmann et al., 2010, 2014; Baumann et al., 2016; Barenys et al., 2017; Masjosthusmann et al., 2019, 2020; Klose et al., 2021b, 2021a). In addition, species aspects were investigated using time-matched (Clancy et al., 2007) rat, mouse or rabbit neurospheres (Gassmann et al., 2010; Baumann et al., 2016; Barenys et al., 2017, 2021; Dach et al., 2017; Masjosthusmann et al., 2018, 2019; Ali et al., 2019; Kühne et al., 2019; Klose et al., 2021b). On the regulatory side, data from the Neurosphere Assay was used for hazard characterization of deltamethrin and flufenacet building an IATA (Hernández-Jerez et al., 2021). Also, the application of screening and prioritization was served by the neurospheres studying banned and currently in use flame retardants (Klose et al., 2021a). Last, data from the Neurosphere Assay contributed to the establishment of putative AOPs (Bal-Price et al., 2015b; Barenys et al., 2020; Klose et al., 2021b) demonstrating the usefulness for helping to frame the regulatory landscape.

Currently, we are further expanding the future regulatory application of this promising test system. Firstly, we have been studying the contribution of 14 hormone receptors, i.e. AhR, RAR, RXR, GR, LXR, PPARα,δ/γ, TH, and the consequences of their disruption to hNPC development (Koch et al., in preparation) within the H2020 ENDpoiNTs project (Lupu et al., 2020). This work shall bring about new test methods for studying endocrine disruption-related DNT (ED-DNT) for regulatory application. Secondly, the Neurosphere Assays are used for feeding and substantiating ontologies for risk assessment purposes concerning cognitive function defects within the H2020 ONTOX project (Vinken et al., 2021). Moreover, more radial and astroglia-related endpoints are currently established with the hNPC test system, since these cell types are not entirely covered in the current assay setup. Besides these applications, we are currently enlarging the data basis for signaling pathways known to be crucial for human brain development (Fritsche, 2017; Sachana et al., 2021b). Altogether these data will continue to define the biological and toxicological applicability domains of the Neurosphere Assay and hence increase confidence in this valuable assay.

To use the results from the Neurosphere Assay in a risk assessment context, the calculated Point of Departure (PoD) values, in our case a benchmark concentration (BMC), need to be translated to an internal dose within the fetal brain. Therefore, reverse physiology-based kinetic modeling (PBK) and quantitative *in vitro* to *in vivo* extrapolations (qIVIVE) can be applied (Basketter et al., 2012; Proença et al., 2021). One fundamental input to IVIVE is the determination of the free test compound concentration, which is defined as the concentration of the compound not bound to plastics, protein or lipid. If the nominal concentration is used instead, the uncertainty of the data analysis increases. The internal dose, determined by qIVIVE can then be translated into an external dose which highly depends on the exposure scenario (e.g. oral, dermal) and modeling of the barriers relevant for the respective type of exposure (e.g. oral bioavailability, dermal bioavailability). Especially in the DNT-context not only the classical parameters like uptake, distribution, metabolism and excretion are relevant, but additional modeling of the blood-placenta barrier and the developing blood-brain-barrier is of the highest relevance. The calculated external concentration can then be used to determine a toxic threshold dose and define an acceptable daily exposure/intake. As an alternative to the approach, known human exposures can be used as a starting point. As an example, they can be modeled from average food intake, dermal exposure or measured as plasma concentrations in epidemiological studies (Sexton et al., 1995). Moreover, exposure limits from animal studies that evidently caused a DNT phenotype can be used as a starting point for the setup of *in vitro* experiments. Another important point that needs to be considered is the metabolism of the test compound in the human body, which is only partially covered in a human cell-based assay. To some extent enzymes are present in the different cell types which metabolize the test compound, however, the complete liver metabolism is absent. Therefore, the metabolism and distribution of a test compound have to be modeled using PBK and *in vitro* screening of metabolites instead of parent compounds has to be considered depending on the distribution of the parent compound and metabolites *in vivo*. In a neurodevelopmental context, PBK modeling could be used in the future to calculate fetal brain concentrations from plasma concentrations measured *in vivo*. These values can then be compared to the PoD values determined with the Neurosphere Assay.

4 CONCLUSION

The scientific validation of the Neurosphere Assay demonstrates that the neurodevelopmental processes, i.e. NPC proliferation, migration, neuronal differentiation, neurite outgrowth, oligodendrocyte differentiation and maturation, are well represented by the test methods. However, they denote a certain developmental time, the fetal period, and during this time especially early neurodevelopmental processes, like the switch from proliferation to initial migration and differentiation. However, how the assays are set up at the moment using an *in vitro* time of only 5 days, the developmental KEs are not followed to full cell maturity. Neurons stay in a mostly bipolar state and also oligodendrocytes do not reach the full myelinating condition. For studying earlier time-points during development, i.e. the embryonic period, or more mature endpoints, i.e. neuronal network formation and function or neuronal myelination, other assays are necessary. The current DNT IVB is evolving to close such biological gaps, yet thorough scientific validation has to be a prerequisite for each novel test system/method to proceed from hazard characterization finally to contributing to risk assessment for DNT using NAMs. Our data provide the rationale for the scientific validity of the endpoints depicted with the Neurosphere Assays. Hence, the DNT IVB, with the Neurosphere Assay as an integral part, is on a solid way to regulatory acceptance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request.

AUTHOR CONTRIBUTIONS

KK: Validation, formal analysis, investigation, writing—original draft, writing—review and editing, supervision, project administration; KB: Formal analysis, investigation, writing—original draft; JH: Formal analysis, investigation, writing—review and editing; JKa: Writing—review and editing, visualization; JKl: Formal analysis, investigation, writing—original draft; EK: Formal analysis, investigation, writing—original draft, writing—review and editing; MP: Formal analysis, investigation, writing—original draft; KS: Formal analysis, investigation, writing—original draft; EZ: Formal analysis, investigation, writing—original draft; EF: Conceptualization, validation, writing—original draft, writing—review and editing, supervision, project administration, funding acquisition.

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2.2 Nuclear Hormone Receptors Control Fundamental Processes of Human Fetal Neurodevelopment: Basis for Endocrine Disruption Assessment

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Despite growing awareness and extensive research on endocrine disrupting chemicals (EDCs), a profound knowledge gap remains regarding their adverse effects on the developing human brain. Risk assessment of EDCs focuses primarily on thyroid hormone (THR), androgen (AR), estrogen (ER), and retinoic acid (RAR) receptors, but comprehensive testing for the wide range of hormone receptors (HRs) expressed during development and beyond is lacking. Testing for developmental neurotoxicity (DNT) is only performed upon triggers and is based solely on *in vivo* rodent studies, which do not link DNT effects to endocrine modes of action (MoA). Furthermore, the extent to which species influence the sensitivity of KNDPs to exogenous noxae in both endocrine and non-endocrine contexts is largely enigmatic. Therefore, DNT *in vitro* test systems have been established that allow cross-species comparisons, such as the Neurosphere Assay, a multiplexed high-content assay based on primary NPCs. This assay was used to identify the impact of 14 different HRs on seven KNDPs, i.e., NPC proliferation, radial glia migration as well as differentiation and migration of neurons and oligodendrocytes, by employing respective HR agonists and antagonists in time-matched human (h) and rat (r)NPCs. In addition, the question of sex-specificity in HR modulation was addressed by including male and female hNPCs in the study. Co-exposure of hNPCs to the respective HR antagonists provided information on the specificities of the agonists and RNA sequencing on the underlying MoAs. Different KNDPs were mostly affected by HR activation at physiologically relevant hormone concentrations in the nanomolar range. The phenotypic effects on KNDPs elicited by activation of the glucocorticoid receptor (GR), liver X receptor (LXR), peroxisome proliferator-activated receptor beta/delta (PPARβδ), RAR and retinoid X receptor (RXR) could be counteracted by co-exposure to the respective HR antagonists. Global gene expression analyses link HR stimulation to cellular processes and signaling pathways relevant to the respective KNDPs, including Wnt, Notch and MAPK/ERK signaling and lipid homeostasis. Strikingly, only five HRs of rNPC control KNDPs similar to those of hNPC. In conclusion, this study presents novel results on cellular and molecular hormone actions in primary fetal hNPCs and illustrates major differences in corresponding rat cells. Thus, we provide the basis for investigating human-relevant, ED-related DNT and challenge current hazard assessment procedures with the low concordance of cellular hormone responses between human and rat cells. In addition, we provide human-based *in vitro* assays

for evaluating compounds for their ED-DNT potential, which is of paramount importance to various stakeholders, including regulatory agencies, industry and the general public.

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Nuclear Hormone Receptors Control Fundamental Processes of Human Fetal Neurodevelopment: Basis for Endocrine Disruption Assessment

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Abstract

Despite growing awareness of endocrine disrupting chemicals (EDCs), a knowledge gap remains regarding their effects on the developing human brain. EDC risk assessment focuses primarily on EATS modalities (estrogens, androgens, thyroid hormones and steroidogenesis), while comprehensive testing for interference with the wide range of hormone receptors (HRs) expressed during brain development is lacking. This leads to a profound gap in the evaluation of chemicals for their potential to cause endocrine disruption-mediated developmental neurotoxicity (ED-DNT). This study provides the molecular and cellular basis to (i) decipher HRs that regulate human key neurodevelopmental processes (KNDP), (ii) establish human-based test methods for ED-DNT, and (iii) compare human and rat responses to HR modulation. Using the Neurosphere Assay (female and male neural progenitor cells, NPCs), we identified the impact and specificity of 14 HRs on seven KNDPs, i.e. NPC proliferation, radial glia migration, neurite outgrowth, as well as differentiation and migration of neurons and oligodendrocytes. Molecular responses to HR agonists were assessed by RNA sequencing. Most HR agonists modulated KNDPs at concentrations in the range of physiologically relevant hormone concentrations. Phenotypic effects on KNDPs induced by activation of the glucocorticoid receptor (GR), liver X receptor (LXR), peroxisome proliferator-activated receptor beta/delta (PPARβδ), retinoic acid receptor (RAR) and retinoid X receptor (RXR) were counteracted by co-exposure to HR antagonists. Transcriptomics highlighted the involvement of conserved developmental pathways (e.g. Notch and Wnt) and revealed HR crosstalk on the molecular level. Species comparisons identified limited concordance of HRs modulating the same KNDP in human and rat NPCs. This study presents novel findings on cellular and molecular hormone actions in human fetal NPCs, illustrates major differences in corresponding rat cells and provides human-based *in vitro* assays for evaluating compounds for their ED-DNT potential, which is of paramount importance to various stakeholders, including regulatory agencies, industry and the general public.

1 Introduction

Human brain development is orchestrated by a tightly regulated interplay of growth factors and conserved developmental pathways that constantly balance proliferative and differentiation processes. As a result, the developing brain is in a temporally unique state of high plasticity in which key neurodevelopmental processes (KNDPs) such as neural progenitor cell (NPC) proliferation, migration, neuro- and gliogenesis as well as circuit formation occur within defined time windows (Silbereis et al., 2016). This exalted plasticity renders the developing brain highly sensitive towards exogenous noxae causing developmental neurotoxicity (DNT) (Rice and Barone, 2000). There is increasing evidence that hormones such as thyroid hormones, sex steroids, and retinoids regulate KNDPs, with perturbations in fetal hormone levels and nuclear hormone receptor (HR) signaling leading to endocrine disruption (ED)-mediated DNT (Cediel-Ulloa et al., 2022; Gore et al., 2014; Jansen et al., 2019; McCaffery et al., 2003; Sauer et al., 2020). ED during early development has the potential to disrupt normal developmental patterns and might contribute to neurodevelopmental disorders such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), and intellectual disability (Caporale et al., 2022; Gore et al., 2014; Lupu et al., 2020; Rivollier et al., 2019; Vorhees et al., 2018).

Endocrine disrupting chemicals (EDCs) are exogenous substances that interfere with the synthesis, secretion, transport, binding, action, or elimination of physiological hormones, ultimately disrupting growth and development. Due to their ubiquitous and often persistent nature and diverse chemical properties, EDCs pose a major health concern by exerting adverse effects on various target tissues including the brain (Tanner et al., 2020; Waring and Harris, 2005). EDCs include natural compounds such as flavonoids and phytoestrogens as well as man-made chemicals such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), plasticizers (bisphenol A, BPA), phthalates, or brominated flame retardants. Due to their often highly lipophilic nature, EDCs cross the placental and blood-brain barriers and accumulate in breast milk, thus exposing the developing child at all critical stages of development. The brain seems to be especially sensitive to EDCs since developmental exposure to EDCs such as PCBs or phthalates has been associated with neurodevelopmental disorders including ADHD, learning and memory deficits and autistic-like behavior (Engel et al., 2009; Guo et al., 2004; Schantz et al., 2003; Schug et al., 2015). While PCBs and phthalates have been associated with disruption of thyroid hormone receptor (THR) signaling and alterations of androgen and glucocorticoid levels during pregnancy, the underlying mechanisms remain elusive (Araki et al., 2017; Derakhshan et al., 2021; Dickerson et al., 2011; Giera et al., 2011; Zoeller et al., 2000). There is a growing awareness of the adverse effects of ED during brain development. However, research is (i) mainly focused on EATS modalities (estrogens, androgens, thyroid hormones and steroidogenesis), (ii) mostly based on animal data, and (iii) insufficient in providing information on the mechanistic links between endocrine signaling and neurodevelopment (Lupu et al., 2020; Schug et al., 2015). This results in a knowledge gap and high uncertainties regarding the consequences of perinatal EDC exposure. This discrepancy is also reflected in the EDC risk assessment process: Although the OECD Conceptual Framework for Testing and Assessment of EDCs includes both mechanistic *in vitro* assays (i.e. the estrogen receptor binding or androgen receptor transactivation assays) and *in vivo* studies (i.e. the Uterotrophic assay and Hershberger assay), it is restricted to EATS modalities and neglects the plethora of HRs represented by the endocrine system (Grignard et al., 2020; LeBaron et al., 2014; OECD, 2018a). In some cases, a DNT concern is addressed by performing the Developmental Neurotoxicity Study (OECD TG 426) or by including the DNT cohort in the Extended One-Generation Reproductive Toxicity Study (EOGRTS), but these studies (i) are not designed to detect endocrine modes of action (MoAs), (ii) neglect species differences between humans and rodents regarding brain development and EDC metabolism, and (iii) do not provide mechanistic information about the tested chemical (OECD, 2018b, 2007). In view of the socio-economic implications of ED-DNT, the Horizon 2020 research and innovation action ENDpoiNTs was initiated to focus on the development of novel testing strategies for ED-DNT (Bellinger, 2012; Cediel-Ulloa et al., 2022; Lupu et al., 2020). A fundamental element of ENDpoiNTs is the generation of knowledge about the causal links between HR signaling and KNDPs. Due to the peculiarities of human brain development (Borrell, 2019; Borrell and Götz, 2014; Florio and Huttner, 2014; Rice and Barone, 2000) and species-specificities of the endocrine system (Cooke et al., 1998; Medvedev et al., 2020; Walter et al., 2019; Whitten and Patisaul, 2001) we strongly support the scientific and regulatory efforts to use human-based New Approach Methodologies (NAMs) for DNT assessment, including in the context of ED (Bal-Price et al., 2018b; Fritsche et al., 2018; NRC, 2007; Ramhøj et al., 2023; Sachana et al., 2021, 2019).

Endocrine signaling is mainly mediated by nuclear HRs whose transcriptional targets dynamically regulate the synthesis and degradation of their respective receptor ligands, a chemically diverse mixture of hormones and biologically active lipids. Thus, HRs actively contribute to the hormonal balance in the body (Evans and Mangelsdorf, 2014; Ribeiro et al., 1995). Of note, the retinoid X receptor (RXR) is a universal heterodimerization partner for several other HRs, with non-permissive partner receptors such as the retinoic acid receptor (RAR), THR, and vitamin D3 receptor (VDR) requiring ligand binding to the partner receptor (e.g., retinoids and vitamins), while binding of permissive partners such as liver X receptor (LXR) and peroxisome proliferator-activated receptors (PPARs) has synergistic effects and orchestrates dynamic responses to dietary substances such as fatty acids and steroids (Evans and Mangelsdorf, 2014; Ribeiro et al., 1995).

In the present study, we explored the causal links between HR signaling and human fetal brain development to use these findings as a basis for ED-DNT NAM development. Therefore, we used the Neurosphere Assay, a high content assay based on primary fetal human neural progenitor cells (hNPCs) growing as neurospheres that model KNDPs (assay names in brackets) such as NPC proliferation (NPC1), migration of radial glia, neurons and oligodendrocytes (NPC2a-c), neuronal differentiation (NPC3), neurite outgrowth (NPC4), and oligodendrocyte differentiation (NPC5) *in vitro* which represents an integral assay of the DNT in vitro battery (DNT IVB) (Koch et al., 2022; OECD, 2023). The Neurosphere Assay has been mechanistically validated (Baumann et al., 2016, 2014; Koch et al., 2022; Masjosthusmann et al., 2019, 2018) and applied to determine the DNT hazard potential of flame retardants (Klose et al., 2022b, 2021; Schreiber et al., 2010), pesticides (Blum et al., 2023; Masjosthusmann et al., 2020), Chinese herbal medicines (Klose et al., 2022a), and a set of 120 chemically and biologically diverse compounds compiled by the US Environmental Protection Agency (US EPA), the European Food Safety Association (EFSA), and several OECD working groups (Blum et al., 2023). Here, we analyzed the influence of specific agonists and antagonists of 14 HRs, the androgen receptor (AR), aryl hydrocarbon receptor (AhR), estrogen receptor (ER), glucocorticoid receptor (GR), LXR, PPARs, progesterone receptor (PR), prostaglandin E2 receptor (PGE2R), RAR, RXR, THR and VDR, covering the major receptors of the human endocrine system. By integrating hNPCs derived from male and female individuals into our testing strategy, we were able to dissect the sex-specificities of neurodevelopmental HR signaling. By comparing the HR expression pattern of hNPCs to fetal human cortical tissue and further linking concentration-response curve-derived benchmark concentrations (BMCs) to human cord blood hormone levels, we evaluated the physiological relevance of the observed effects. Using transcriptome analyses, we further linked physiologically relevant HR activation and associated neurodevelopmental phenotypes to the regulation of key developmental pathways (epidermal growth factor receptor (EGFR), Notch, Wnt and MAPK/ERK). Finally, the identified HR-regulated KNDPs were examined in time-matched rat (r)NPCs, revealing that most fetal KNDPs are regulated by HR in a species-specific manner.

2 Material and Methods

2.1 Reagents

Table 1: List of hormone receptor agonists and antagonist used for modulation of receptor activity, including their manufacturers, catalog numbers, solvents, and stock concentrations.

Chemical	Abbreviation	CAS-Nr.	Manufacturer	Catalog-Nr.	Solvent	Stock [mM]
3'-Methoxy-4'-nitroflavone	MNF	145370-39-4	Symrise AG	customized order	DMSO	10
17β-Estradiol	E2	50-28-2	Merck (Sigma)	E2758-250MG	2 % EtOH in DMEM	0.0735
AGN193109	AGN	171746-21-7	Merck (Sigma)	SML2034-5MG	DMSO	7.6
AL 082D06	AL08	256925-03-8	Biomol (Cayman)	Cay23455-1	DMSO	5
all-*trans* retinoic acid	*at*RA	302-79-4	Merck (Sigma)	R2625-50MG	DMSO	100
Benzo[*a*]pyrene	BaP	50-32-8	Merck (Sigma)	B1760-100MG	DMSO	2.5
Bexarotene	BEXA	153559-49-0	Merck (Sigma)	200499-50MG	DMSO	10
Calcitriol	Calci	32222-06-3	Biomol (Cayman)	Cay71820-5	DMSO	10
Dexamethasone	DEX	50-02-2	Merck (Sigma)	D4902-25MG	DMSO	50
Dihydrotestosterone	DHT	521-18-6	Selleckchem	S4757	DMSO	50
Fulvestrant	FU	129453-61-8	Biomol (Cayman)	Cay10011269-1	DMSO	5
GSK3787	GSK	188591-46-0	Biomol (Cayman)	Cay15219-5	DMSO	10
GW0742	GW0742	317318-84-6	Biomol (Cayman)	Cay10006798-5	DMSO	10
GW3965 hydrochloride	GW39	405911-17-3	Biomol (Cayman)	Cay10054-1	DMSO	10
GW6471	GW6471	880635-03-0	Biomol (Cayman)	Cay11697-1	DMSO	10
GW7647	GW7647	265129-71-3	Biomol (Cayman)	Cay10008613-1	DMSO	5
GW9662	GW9662	22978-25-2	Biomol (Cayman)	Cay70785-1	DMSO	10
HX531	HX	188844-34-0	Biomol (Cayman)	Cay20762-1	DMSO	10
Hydroxyflutamide	HFU	52806-53-8	Merck (Sigma)	H4166-5MG	DMSO	30
Mifepristone	MP	84371-65-3	Merck (Sigma)	M8046-100MG	DMSO	100
NH-375	NH-3	Nguyen 2002; Singh 2016 (provided by UC Davis)			DMSO	100
Progesterone	PG	57-83-0	Merck (Sigma)	P6149-1MG	DMSO	5
Prostaglandin E2	PGE2	363-24-6	Merck (Sigma)	P5640-1MG	H_2O	3
Rosiglitazone	Rosi	122320-73-4	Biomol (Cayman)	Cay71740-10	DMSO	10
SR9238	SR92	1416153-62-2	Biomol (Cayman)	Cay18771-1	DMSO	10
Triiodothyronine	T3	6893-02-3	Merck (Sigma)	T2877	96 %EtOH/1M HCl (1:1)	0.3
Ulipristal Acetate	UA	126784-99-4	Biomol (Cayman)	Cay23657-5	DMSO	10

2.2 Compound exposure

HR agonist and antagonist exposure included seven serial concentrations and a solvent control (SC) in 100 µl in 96-well plates with five technical replicates per condition. A compound predilution was prepared in solvent (Table 1) before dilution 1:999 (v:v) in cell culture medium (see 2.4) to generate the highest compound concentration. Six serial dilutions were prepared from the highest compound concentration using a dilution factor of three and medium spiked with 0.1% solvent. For co-exposure to HR agonists and antagonists, four serial dilutions of the HR antagonist (dilution factor of ten, 1:999 dilution) were prepared in cell culture medium containing the HR agonist (BMC_{30}, 1:999 dilution). As controls, cells were exposed to (i) solvent alone and (ii) HR agonist alone (BMC_{30}). Exposures were adjusted for the solvents of the agonists and antagonists and performed in 100 µl in 96-well plates with five technical replicates per condition. Notably, co-exposure experiments were performed only for the most sensitive endpoint (MSE) of agonist exposure for each HR.

2.3 Basic human and rat neurosphere culture

Primary hNPCs derived from cortex homogenates of male and female fetuses at 16-19 weeks of gestation (GW16-19) were purchased from Lonza Verviers SPRL, Belgium (#PT-2599). Time-matched rNPCs were prepared as described previously (Baumann et al., 2014; Klose et al., 2021; Masjosthusmann et al., 2019). Briefly, pregnant rat dams (Wistar) were obtained from Charles River, and on post-natal day one (PND1), whole brains of pups were dissected, enzymatically digested, and homogenized to obtain cell suspensions of rNPCs. Brain homogenates from male and female pups were pooled separately prior to rNPC culture. The process of rNPC isolation procedure is in accordance with the "Landesamt für Natur, Umwelt und Verbraucherschutz" (81-02.05.50.18.001) and complies with §4 Abs. 3 Tierschutzgesetz (TierSchG). The hNPCs and rNPCs were cultured as free-floating neurospheres in proliferation medium containing DMEM (#31966-021, Life Technologies) and Hams F12 (#31765-027, Life Technologies) in a 2:1 ratio (v:v) supplemented with 2 % B27 (#17504044, Life Technologies), 20 ng/ml EGF (#PHG0313, Thermo Fisher), 100U/mL penicillin and 100 µg/ml streptomycin (#P06-07100, Pan-Biotech) and either 20 ng/ml human FGF for hNPCs (#233-FB, R&D Systems) or 10 ng/ml rat FGF for rNPCs (#3339-FB-025, R&D Systems). Neurospheres were cultured under standard cell culture conditions at 37 °C and 5 % CO_2 in 10 cm diameter cell culture dishes coated with poly-2-hydroxyethyl methacrylate (poly-Hema; #P3932, Sigma-Aldrich). Once a week, neurospheres were passaged by mechanical dissociation into 200 µm edge length cubicles using a McIlwain tissue chopper (#TC752, Campden Instruments). Three times per week, half of the culture medium was replaced with fresh culture medium.

2.4 The Neurosphere Assay (NPC1-5)

NPC proliferation (NPC1)

The proliferative capacity of hNPCs and rNPCs (NPC1 assay) was measured by incorporation of bromodeoxyuridine (BrdU, #11669915001, Roche) into newly synthesized DNA as described previously (Koch et al., 2022). Briefly, proliferating hNPCs of 300 µm diameter were cultured in 100 µl proliferation medium in poly-Hema-coated 96-well plates for 3 days (5 technical replicates with one sphere per well for each experimental condition). As an endpoint specific positive control for proliferation arrest, NPCs were cultured in medium without growth factors (w/o growth factors). Exposure to the HR agonists and antagonists (Table 1) was performed for the entire duration of the assay. After three days of exposure, the Cell Proliferation ELISA (#11669915001, Roche) was performed according to the manufacturer's protocol. A detailed description of the NPC1 assay can be found in (Masjosthusmann et al., 2020).

NPC differentiation and immunocytochemical staining

Spontaneous differentiation of hNPCs and rNPCs was achieved by transferring 300 µm neurospheres into 96-well plates coated with 0.1 mg/mL poly-D-lysine (PDL, #P0899-50MG, Sigma-Aldrich) and 12.5 µg/ml laminin (#L2020-1MG, Sigma-Aldrich) followed by culturing for five days in the following differentiation medium: DMEM (#31966-021, Life Technologies) and Ham's F12 (#31765-027, Life Technologies) at a 2:1 ratio (v:v) supplemented with 1 % N2 (Thermo Fisher, #17502-048) and 100 U/mL penicillin and 100 µg/ml streptomycin (#P06-07100, Pan-Biotech). During the five days of differentiation, progenitors migrate radially out of the sphere core, form a circular migration area, and differentiate into neurons, astrocytes, and oligodendrocytes. After five days, cells were fixed with 4 % paraformaldehyde for 30 min at 37 °C and stained with antibodies against β(III)tubulin (neurons) and O4 (oligodendrocytes) as described previously (Klose et al., 2022b; Koch et al., 2022). Briefly, unspecific binding sides were blocked with 10 % goat serum (GS, #G9023-10mL, Sigma Aldrich) in PBS for 30 min at 37 °C. Primary antibodies against β(III)tubulin (1:400, rabbit anti-β(III)tubulin monoclonal antibody [EP1569Y]-Alexa Fluor® 647, #ab190575, Abcam) and O4 (1:400, mouse anti-O4 IgM, #MAB1326, R&D Systems) were incubated overnight at 4 °C in PBS containing 0.01 % Triton-X and 2 % GS. After three washes with PBS, the secondary antibody for the O4-staining (1:400, goat anti-mouse IgM-Alexa Fluor® 488, #A-21042, Thermo Fisher) and Hoechst33258 (1:100, #94403-1ML, Merck) were diluted in PBS containing 2 % GS and incubated at 37 °C for 60 min. All images of immunocytochemical staining were acquired using the Cellomics ArrayScan high content analysis (HCA) platform at 200× magnification, 552×552 pixel resolution, and the provided HCS Studio Cellomics software (version 6.6.3; Thermo Fisher Scientific).

Assessment of radial glia, neuronal and oligodendrocyte migration (NPC2)

The migration distance of radial glia cells (NPC2a) that migrated out of the sphere core was manually assessed at 72 h using brightfield microscopy and automatically at 120 h by analyzing immunocytochemical stainings with the Omnisphero software as described previously (Schmuck et al., 2017). After 72 h, brightfield images of the entire well were taken with the Cellomics ArrayScan at 50x magnification. The radial distance of the furthest migrated cells to the sphere core was measured as the number of pixels for all four directions before conversion to µm using the Fiji Image J software (Schneider et al., 2012). After 120 h, radial glia migration was automatically determined by measuring the area of Hoechst33258-stained nuclei (migration area) using Omnisphero software. In addition, the migratory capacity of neurons (NPC2b) and oligodendrocytes (NPC2c) at 120 h was calculated by dividing the mean distance of all neurons (β(III)tubulin-positive cells) or oligodendrocytes (O4-positive cells) by the 120 h radial glia migration distance using Omnisphero software.

Assessment of neuronal differentiation, neuronal morphology, and oligodendrocyte differentiation (NPC3-5)

Multiplexed with the NPC2a-c endpoints, neuronal differentiation (NPC3), neurite outgrowth (NPC4), and oligodendrocyte differentiation (NPC5) were assessed using two convolutional neuronal networks (CNNs) and the Omnisphero software as described previously (Förster et al., 2022; Schmuck et al., 2017). Briefly, after immunochemical staining of differentiated cells, a series of images were acquired with the Cellomics ArrayScan and merged into one image per well using the Omnisphero software, including all three channels (nuclei (Hoechst33258), neurons (Alexa647®) and oligodendrocytes (Alexa488®)). The total number of nuclei was calculated using the SpotDetector (V4.1) bio-application of the HCS Studio Cellomics software (version 6.6.0, Thermo Fisher Scientific). Stained neurons and oligodendrocytes were identified using two CNNs implemented in Python 3 based on the open-source library Keras (Chollet, 2017), trained on historical expert-annotated data (Förster et al., 2022). Neuronal (NPC3) and oligodendrocyte (NPC5) differentiation was calculated as the number of cells stained for β(III)tubulin and O4, respectively, as a percentage of the total nuclei count within the migration area. The morphology of all neurons identified by the CNN was analyzed using Omnisphero software by determining their mean neurite area (NPC4a) and total subneurite length (NPC4b), as described in detail in (Schmuck et al., 2017). For the rNPCs, neuronal numbers were quantified using the co-localization tool of the HCS Studio Cellomics software (version 6.6.0, Thermo Fisher Scientific). To assess oligodendrocyte differentiation in rNPCs, manual image analysis was performed with the software Omnisphero. In brief, for two defined areas (1098 mm × 823 mm size; once placed above and once below the sphere core) per migration area, the number of $O4^+$ cells was normalized to the total number of nuclei. The resulting percentages of the two areas from the same sphere were averaged and the median was calculated for the five neurospheres of every treatment condition.

Mitochondrial activity and cytotoxicity assays

To discriminate specific effects on KNDPs from nonspecific effects due to necrosis or reduced cell viability, mitochondrial activity and cytotoxicity were assessed within the NPC1 and NPC2-5 assays after the respective days of chemical exposure by performing Alamar blue assays (CellTiter-Blue Assay (CTB), #G8081, Promega) and measuring the leakage of lactate dehydrogenase (LDH) from cells with damaged cell membranes (CytoTox-ONE Homogeneous Membrane Integrity Assay; #G7891, Promega). As a positive control on each plate, neurospheres were exposed to 0.2 % Triton X-100 for 45 min. Fluorescence was measured using a Tecan Infinite M200 Pro reader (ex: 540 nm; em: 590 nm). Relative fluorescence unit (RFU) values of replicates were averaged (median) and medium without cells was used to correct for background fluorescence. It should be noted that reduced NPC proliferation and radial glia migration correlates with lower CTB signal as a result of fewer cells within the neurospheres and the migration area. Therefore, the Alamar blue assay is an inadequate measure of cell viability for compounds that inhibit NPC proliferation or radial glia migration (Klose et al., 2022b).

RNA sequencing of hNPCs exposed to HR agonists

A total of 850 proliferating (GR and RAR) or differentiating (other HRs) hNPC spheres of 100 μm diameter were exposed to HR agonists (BMC_{30} derived for the MSE) or solvent (0.1 % DMSO) for 60 h in poly-Hema-coated or PDL-laminin-coated 6-well plates. Total RNA was isolated using the RNeasy Mini Kit (#74104, Qiagen) according to the manufacturer's instructions. On-column DNAse digests were performed using a commercial kit (#79254, Qiagen). The RNA was sent to BGI Genomics Co., Ltd. (Hong Kong) for RNA sequencing, and reads were mapped to the human reference genome hg38. Four biological replicates were performed for each exposure condition. Library Preparation: Quality control (QC) of total RNA samples was performed using the Agilent 2100 Bio Analyzer (Agilent RNA 6000 Nano Kit). All samples in this study showed high-quality RINs ≥ 7. Subsequently, mRNA was purified and fragmented using oligo(dT)-labeled magnetic beads. After synthesis of the first and second cDNA strands, end repair and "A" base was added to the 3'-end. Adaptor ligands were added and polymerase chain reaction (PCR) was performed. PCR products were purified using XP beads. QC was again performed on the Agilent 2100 Bio analyzer. Double-stranded PCR products were denatured and circularized by splint oligo-sequencing. The resulting single-stranded circular DNA was formatted as a final library. The library was amplified with phi29 to produce DNA nanoballs (DNB) having more than 300 copies of a molecule. The DNBs were loaded into the patterned nanoarray and single end 50 (pair end 100/150) base reads were generated in the way of combinatorial probe anchor synthesis (cPAS). Sequence data analysis: First, reads mapping to rRNAs were removed. Next, low quality reads (>40 % of base qualities <20), reads with adaptors, and reads with unknown bases (N bases >0.1 %) were removed to obtain the clean reads (20M clean reads per sample, BGI software

SOAPnuke v1.5.2). These clean reads were saved as FASTQ files. The reads were then mapped to the reference genome (GCF_000001405.39_GRCh38.p13) using the Hierarchical Indexing for Spliced Alignment of Transcripts software (HISAT2, v2.0.4). In addition, novel transcript prediction (StringTie v1.0.4; Cuffcompare v2.2.1; CPC v0.9-r2), SNP & INDEL calling (GATK), and gene-splicing detection (rMATS v4.0.2) were performed. Gene expression analysis was performed by mapping clean reads to the reference genome (Bowtie2 v2.2.5) and calculating expression levels using RSEM (v1.2.12). Differentially expressed genes (DEGs) between the HR agonist exposure conditions and the respective solvent were identified using DEseq2 (Kong et al., 2007). DEG criteria of $|\log2(FC)| > 0.486$, FPKM ≥ 1 and a q-value of < 0.05 were used for target gene and GO-term analysis. For GO-term analyses, terms were sorted by the number of DEG included and only significantly enriched GO-terms (p-value < 0.01) were selected. Analyses and visualization of transcriptome data were performed using Dr. Tom software (BGI Genomics Co., Ltd.).

RNA sequencing data from human cortical tissue and microarrays data of human and rat NPCs

HR expression in fetal cortex tissue (Fig. 1A) was derived from a publicly available data set (https://www.brainspan.org/). The BrainSpan atlas is a foundational resource for studying transcriptional mechanisms involved in human brain development (Miller et al., 2014). Expression was averaged for different cortical regions. Expression was further averaged for the different donors per gestational week if applicable (GW8 – 1 donor; GW12 – three donors; GW16 – three donors). Further information including raw RPKM values, donor information and stratification by brain region can be found in Sup. File 1. Microarray datasets (Fig. 8A) for proliferating and differentiating hNPCs (60h) and rNPCs (72h) were previously published and described in detail in (Klose et al., 2021; Masjosthusmann et al., 2018). Data generation and analysis was performed as described in the aforementioned publications.

Statistics

For all hNPC and rNPC experiments, at least three independent biological replicates with at least five technical replicates each were performed. Experiments using different individuals (hNPC donors) or different rNPC preparations served as biological replicates. In addition, biological replicates included experiments with at least one hNPC individual/rNPC preparation from each genetic sex. If sex differences became apparent after the first three biological replicates, three additional biological replicates were performed to obtain data from three male and three female donors/preparations each. Experiments were defined as independent if they were performed with cells from different hNPC individuals/rNPC preparations or at a different passage number. Statistical computing was based on the R package CRStats (https://crstats.github.io/; github.com/iuf-duesseldorf/koch-lab-CRStats) a framework for the analysis of *in vitro* data that relies on the established R packages drc (Ritz et al., 2015) and mvtnorm (Genz et al., 2020) paired with the step-down multiple test procedure of Dunnett and Tamhane (Dunnett and Tamhane, 1991). All data are presented as the mean of at least 3 biological replicates ± standard error of the mean (SEM), and a p-value ≤ 0.05 was considered significant unless otherwise noted. In addition, concentration-response data analysis R packages were customized to calculate the benchmark response (BMR) and to estimate the BMC, including the lower and upper limits of its confidence interval (BMCL, BMCU). The appropriate BMR definition for each Neurosphere Assay endpoint (NPC1-5) was based on the variation of the lowest compound concentration over the entire data set of $n = 244$ experiments: Here, for all test compounds, the endpoint responses of the lowest concentrations of all independent experiments were normalized to the respective solvent control. The inter-experimental variability was then expressed as the coefficient of variation (CV) of these collected data points across all test compounds. The BMR for each individual assay endpoint was defined as the CV*1.5 (rounded up to the nearest multiple of 5). The best curve-fitting model for the respective data set was chosen from a large pool of 13 mathematical concentration-response functions according to robust statistical criteria. Fitted curves can follow linear, sigmoidal, monotonic, and non-monotonic trends. The estimation method assumes continuous response data.

Prediction model: For the calculation of BMCs for the specific Neurosphere Assay endpoints NPC1-5, only HR modulator concentrations where less than 10 % cytotoxicity was observed were included (Sup. Fig. 1+2). Therefore, BMC_{10} was calculated for the non-specific endpoint of cytotoxicity, and all exposure concentrations above this BMC_{10} (cytotoxicity) were excluded from further analysis of the NPC1-5 endpoints. An HR modulator was considered a "KNDP hit" if at least the highest non-cytotoxic concentration was significantly different from the lowest concentration, as assessed by the step-down multiple test procedure of Dunnett and Tamhane, and/or if both the upper and lower limits of the 95 % percentile of the concentration-response curve intersected the BMR line.

3 Results

3.1 Comprehensive characterization of HR expression and neurodevelopmental effects of HR modulation in hNPCs

RNA sequencing revealed highest expression of *THRA, RARA, PPARD, LXRB, RXRA*, *RXRB, LXRA, RARG* and *GR* in proliferating (Prol) hNPCs (Fig. 1A). Moreover, expression of the *THRA* further increased (2.03-fold, $p < 0.0001$) during hNPC differentiation (Diff). On the contrary, the *ER*, *PR*, *PPARG*, *PGE2R1-4*, *RXRG, THRB* and *VDR* were barely expressed independent of the hNPC differentiation state. Comparison of the hNPC *in vitro* model (gestational week, GW 16) with primary fetal cortex tissues (GW 8, 12 and 16) consistently confirmed highest expression levels for *THRA, RXRB, LXR, GR*, *PPARD* and *RARA* (Fig. 1A and Sup. File 1). Analysis of fetal cortex tissue further confirmed the negligible expression of *ER*, *PR*, *PPARG*, *PGE2R1-4*, *RXRG, THRB* and *VDR* even in an earlier neurodevelopmental context (GW8 and 12). To determine which fetal KNDPs are regulated by HRs and therefore may be sensitive to EDC exposure, we exposed proliferating and differentiating hNPCs to agonists and antagonists of 14 HRs in the human Neurosphere Assay (Fig. 1B). The derived KNDP-specific assay endpoints were hNPC proliferation (NPC1), radial glia (NPC2a), neuronal (NPC2b) and oligodendrocyte (NPC2c) migration distance, neuronal differentiation (NPC3), neurite outgrowth (NPC4), and oligodendrocyte differentiation (NPC5). Cytotoxicity and mitochondrial activity were assessed in parallel (Sup. Fig. 1+2), and only non-cytotoxic (< 10 % cytotoxicity) concentrations were included in the concentration-response modeling of the KNDP-specific endpoints. All curves and BMCs, including upper and lower limits, and compiled in Sup. Files 2-4. For each HR, we defined the KNDP with the highest sensitivity for HR activation (lowest BMC), the most sensitive endpoint (MSE). We identified hNPC proliferation (NPC1) as the MSE for GR (BMC_{20} = 6.1 nM, male hNPCs) and RAR (BMC_{20} = 31.9 nM) activation, neuronal differentiation (NPC3) for PGE2R (BMC_{25} = 13.69 μM), PPARγ (BMC_{25} = 127.5 nM), RXR (BMC_{25} = 3.4 nM) and THR (BMC_{25} = 0.26 nM) activation, neurite outgrowth (NPC4) for AhR (BMC_{20} = 1.07 μM) and RXR (BMC_{20} = 3.4 nM), radial glia migration (NPC2a) for RXR (BMC_{10} = 3.7 nM) and oligodendrocyte differentiation (NPC5) for AhR (BMC_{25} = 1.02 μM), LXR (BMC_{25} = 25.1 nM), PPARα (BMC_{25} = 371.7 nM), PPARβ/δ (BMC_{25} = 435.8 nM), PG (BMC_{25} = 1.22 μM) and VDR (BMC_{25} = 785.6 nM) activation (Fig. 1C). Of note, for AhR and RXR, no clear MSE could be defined since several endpoints were affected at similar concentrations, thus all were tested for receptor specificity. Notably, activation of highest expressed receptors (GR, RXR and THR) revealed the KNDPs with the highest sensitivity to endocrine modulation, with BMCs in the low nanomolar or picomolar range. In contrast, exposure to agonists of lowest expressed receptors regulated KNDPs in the micromolar range (PGR and PGE2R) or had no effect (AR and ER). Although expressed in hNPCs, AR modulation had no effect on any KNDP studied at concentrations of up to 5 µM dihydrotestosterone (DHT) and 10 µM hydroxyflutamide, which is well above the physiological range (cord blood DHT = 60-88 pg/ml, corresponding to 0.2-0.3 nM) (Anderson et al., 2010).

To determine the biological relevance of the phenotypic effects observed upon HR agonists exposures, BMCs (MSE for each HR) were compared to fetal cord blood concentrations of the natural receptor ligands (Table 2). For all HRs except PGE2R and VDR, the calculated BMCs were in the range of or below fetal cord blood concentrations of the natural receptor ligands indicating biological relevance of the observed effects on KNDPs. The low *PGE2R* and *VDR* expression and effects at non-physiological concentrations suggest PGE2R- and VDR-independent effects of PGE2 and calcitriol. As a result, and because AR and ER activation did not affect any KNDP studied, no further analyses were performed for PGE2R, VDR, AR and ER. For the remaining 10 HRs, either proliferating or differentiating hNPCs (based on the identified MSE) were exposed to HR agonists (BMC_{30}) for 60 h and subjected to RNA sequencing analyses to confirm known target gene regulation and elucidate human developing brain cell-specific mechanisms underlying the phenotypic observations on the KNDP. For GR activation, the anti-proliferative effect was sex-specific, therefore male hNPCs were used for RNA sequencing analyses. Figure 2 places the DEGs identified in HR agonist-exposed hNPC in the context of the current literature on HR-dependent gene regulation in the brain and beyond. Within the DEGs identified for each HR, we searched for reported HR targets using a comprehensive, non-systematic literature search (PubMed, https://pubmed.ncbi.nlm.nih.gov/, Sup. File 5) and compared the HR-dependent gene regulation in this study (Fig. 2A) with evidence from the published literature (Fig. 2B). Non-human *in vivo* and *in vitro* studies were also included. Genes previously identified as HR targets in brain tissue or neural cultures and confirmed as such in hNPCs were labeled as 'reported target in brain'. Genes previously identified as HR targets in non-brain tissues or non-neural cultures and newly identified as neural targets in hNPCs were labeled as 'newly identified target in brain'. While hNPCs largely recapitulate the published brain-specific HR target gene pattern, we identified many HR targets in fetal hNPCs that were previously reported to be under HR control only in organs other than the brain (e.g., liver, kidney, or adipose tissue). In addition, we were able to confirm several genes as HR targets in hNPCs that had not been previously reported as HR targets. We have labeled these as "newly identified target overall" (Fig. 2B).

Gene Ontology (GO) enrichment analyses were performed to identify the mechanisms underlying the phenotypic observations. Moreover, receptor-specificities of the phenotypic effects were investigated by challenging the receptor agonists by co-exposure to the receptor antagonists. Receptor specificity was successfully confirmed for the GR, RAR, LXR, PPARβδ and RXR (only NPC3). Phenotypic and transcriptomic results of HR modulations (MSE for each HR) that did not result in receptor-specific and thus antagonizable effects on KNDPs, i.e. AhR, PPARα, PPARγ, PR and THR, are presented in the supplementary material (Sup. Fig. 3+4). For the AhR, PPARα, PPARγ and PR, the results are discussed in the supplementary material (Sup. File 6).

Table 2: Comparison of fetal cord blood concentrations of physiological HR ligands (current literature) and benchmark concentrations (BMCs) of key neurodevelopmental process (KNDP)-related HR agonist effects (present study). References: (Grygiel-Górniak, 2014; Lagiou et al., 2011; Lütjohann et al., 2001; Manolescu et al., 2010; Pearson Murphy, 1983; Sakamoto et al., 2018; Schlagenhauf et al., 2015; Wang et al., 2005; Wong et al., 2022)

Receptor	natural receptor ligand	fetal cord blood concentration [ng/ml or µg/ml]	fetal cord blood concentration [nM]	agonist (present study)	BMC [nM] (present study)	BMC biologically relevant?	Reference
Aryl hydrocyrbon receptor (AhR)	benzo(a)pyrene chlorodibenzo-p-dioxin (TCDD)			benzo(a)pyrene	*1020.0*	*questionable*	
Glucocorticoid receptor (GR)	cortisol	65 - 92 ng/ml	179.0 - 254.0	dexamethasone	**6.1**	**yes**	Pearson Murphy (1983)
Liver X receptor (LXR)	oxysterols and cholestenoic acid	24S-hydroxycholesterol: 35 - 62 ng/ml 27-hydroxycholesterol: 21 - 39 ng/ml	86.9 - 153.9 52.2 - 96.9	GW3965	**25.1**	**yes**	Lütjohann et al. (2001)
Peroxisome proliferator-activated receptors (PPARs)	poly unsaturated fatty acids (PUFAs) - eicosatetraenoic acids (i.e. arachidonic acid and metabolites) - octadecadienoic acids (i.e. linoleic acid and metabolites) docosahexaenoic acid (DHA)	Total n-6 PUFAs: 199.9 - 536.1 µg/ml arachidonic acid: 86.6 - 145.4 µg/ml linoleic acid: 84 - 154 µg/ml DHA: 41.7 - 75.9 µg/ml	n.a. 284,400.0 - 477,551.2 299,521.6 - 549,123.0 126,944.5 - 231,057.3	GW7647 (PPARα) GW0742 (PPARβδ) rosiglitazone (PPARγ)	**371.6** **435.6** **127.8**	**yes** **yes** **yes**	Sakamoto et al. (2017) Grygiel-Górniak et al. (2014)
Progesterone receptor (PR)	progesterone	176.4 - 2818.0 ng/ml	560.9 - 8961.1	progesterone	**1220.0**	**yes**	Lagiou et al. (2011)
Prostaglandin E2 receptor (PGE2R)	prostaglandin E2	9.5 ng/ml	27	prostaglandin E2	*11845.0*	*no*	Schlagenhauf et al. (2015)
Retinoic acid receptor (RAR)	all-*trans* retinoic acid	30 - 150.2 ng/ml	100 - 550	all-*trans* retinoic acid	**31.6**	**yes**	Manolescu et al. (2010)
Retinoid X receptor (RXR)	docosahexaenoic acid (DHA) 9-*cis* retinoic acid (9cRA)	DHA: 41.7 - 75.9 µg/ml n.a.	126,944.5 - 231,057.3 n.a.	bexarotene	**3.4**	**yes**	Sakamoto et al. (2017)
Thyroid hormone receptor (THR)	triiodothyronine (T3)	0.543 - 0.606 ng/ml	0.77 – 0.92	triiodothyronine (T3)	**0.3**	**yes**	Wang et al. (2005)
Vitamin D receptor (VDR)	calcitriol (1,25-dihydroxyvitamin D, active form) calcidiol (25-hydroxyvitamin D, 25[OH]D, stable metabolite)	25[OH]D: 16.6 ng/ml (mean over all studies included in meta-analysis)	41.55	calcitriol	*785.7*	*no*	Wong et al. (2022)

3.2 Glucocorticoids regulate hNPC proliferation and terminal differentiation in a sex-specific manner

Glucocorticoids (GCs) and their receptor are indispensable for brain development, with over-stimulation of the latter leading to neurodevelopmental disorders (Buss et al., 2012; Miranda and Sousa, 2018). Although cortisol is the major GR ligand in humans, the synthetic GC dexamethasone (DEX) was used in this study due to cortisol's limited stability. Proliferation of hNPCs was sex-specifically reduced to 56 % of controls upon GR activation in male but not female hNPCs (MSE, BMC_{20} = 6.1 nM DEX, Fig. 3A+B). Given human cord blood cortisol levels of 179-254 nM (Table 2) and the higher potency of DEX to activate GR-dependent transcription compared with cortisol (He et al., 2014), the observed effects are of physiological relevance. Notably, proliferation of DEX-treated male hNPCs was concentration-dependently restored by co-exposure to the GR antagonist mifepristone (0.1 µM MP), underscoring the receptor-specificity (Fig. 3C). Co-exposure to the GR-specific but weaker GR antagonist AL082D06 (10 µM AL08) was less effective in antagonizing DEX-induced effects (data not shown). GR activation in proliferating male hNPCs with 60 nM DEX (BMC_{30}) differentially regulated 438 genes. We detected upregulation of several suppressors of cell proliferation (*FKBP5*, *MT2A*, *PER1*, *RASD1* and *ZBTB16*), which are reported GR-targets (Fig. 2). Ranking of the top ten up- and down-regulated GO-terms with the most DEGs included, revealed suppression of DNA and RNA transcription upon GR activation (Fig 3D, Sup. File 7). In addition, we provide evidence for Notch pathway activation, a negative regulator of neuronal differentiation. Consistent with the transcriptomics, neuronal differentiation of male but not female hNPCs was impaired to 72 % of controls upon GR activation (BMC_{25} = 32.5 nM DEX, Fig. 3E) and increased to 152 % of controls upon GR inhibition (BMC_{25} = 40.8 nM AL082D06, Fig. 3F). Due to the observed low-dose effects (90 % of control at 6.8 nM DEX), the results of DEX exposure were not statistically significant in the present study but in a previous study on hNPCs (Moors et al., 2012). GR inhibition further reduced oligodendrocyte differentiation to 50 % of controls (BMC_{25} = 38.3 nM AL082D06) in hNPCs of both sexes (Fig. 3G).

3.3 Dysregulation of cholesterol homeostasis by LXR activation affects hNPC lineage specification

The LXR is the master regulator of cholesterol homeostasis in the brain, with cholesterol derivatives such as oxysterols and cholestenoic acid being its major endogenous ligands (Courtney and Landreth, 2016). LXR activation with the synthetic agonist GW3965 concentration-dependently impaired the differentiation of hNPCs into oligodendrocytes to 12 % of controls (MSE, BMC_{25} = 25.1 nM, Fig. 4A+B), a cell type highly dependent on lipid metabolism. Given human cord blood levels of natural LXR ligands 24S-hydroxycholesterol (86.9 - 153.9 nM) and 27-hydroxycholesterol (52.2 - 96.9 nM) in the concentration range of the derived BMC (Table 2), the observed effects are of physiological relevance. To confirm the receptor specificity, we successfully antagonized the GW3965-induced effects with the specific LXR-antagonist SR9238 (20 nM SR9238, Fig. 4C). LXR activation with 37 nM GW3965 (BMC_{30}) differentially regulated 188 genes in differentiating hNPCs, among which we identified several reported LXR targets involved in cholesterol and lipid metabolism (Fig. 2). Midst the known LXR targets, we observed transcriptional induction of suppressors of lipid biosynthesis and promoters of cholesterol efflux (e.g. *ABCA1, ABCG1, APOE* and *LPL*). GO-term analysis upon LXR activation revealed downregulation of lipid metabolism and sterol biosynthesis (including *FAXDC2, FDFT1, HMGCS1, MSMO1, NSDHL, PTGDS, SC5D* and *SERAC1*), Wnt pathway activation, and MAPK suppression (Fig. 4D, Sup. File 7). LXR activation further repressed transcription of oligodendrocyte lineage determinants (*SOX10* and *CD38*). LXR activation impaired radial glia migration of female but not male hNPCs to 75 % of controls in a sex-dependent manner (BMC_{10} = 58.9 nM, Fig. 4E) and enhanced neuronal differentiation in hNPCs of both sexes to 133 % of controls at concentrations approximately 10-fold higher than the MSE (BMC_{25} = 380.8 nM, Fig. 4F). Furthermore, blocking basal LXR activity by SR9238 in the absence of GW3965 increased neurite outgrowth to 138 % of controls (BMC_{20} = 111.4 nM; Fig. 4G) and attenuated oligodendrocyte differentiation of hNPCs to 51 % of controls (BMC_{25} = 1.02 µM; Fig. 4H), but at concentrations higher than those used to antagonize the LXR ligand GW3965.

3.4 PPARβδ is the most abundant PPAR subtype in human NPCs, regulating multiple KNDPs similar to LXR and RXR

PPARs are hormone and lipid-activated nuclear receptors and transcription factors that regulate the metabolism of fatty acids, cholesterol and sphingolipids and maintain carbohydrate and glucose homeostasis (Strosznajder et al., 2021). Upon heterodimerization with RXR, they bind a wide range of lipophilic substances including fatty acids and their derivatives, such as prostaglandins (Aleshin and Reiser, 2013). Exposure of differentiating hNPCs to the specific PPARβδ agonist GW0742 concentration-dependently reduced oligodendrocyte differentiation to 18 % of controls (MSE, BMC_{25} = 436 nM, Fig. 5A+B) at agonist concentrations way below natural PPARβδ ligand fetal cord blood concentrations (total n-6 polyunsaturated fatty acids (PUFAs): 199.9 - 536.1 µg/ml, equals > 100 µM, Table 2). Effects were antagonized by concomitant exposure to the PPARβδ antagonist GSK3787 (100 nM GSK3787, Fig. 5C). PPARβδ activation with 621 nM GW0742 (BMC_{30}) differentially regulated 182 genes, among which we identified several reported PPARβδ target genes (Fig. 2). PPARβδ activation suppressed several genes involved in reactive oxygen species (ROS) scavenging (*TIMP3, WWTR1, APOE* and *KLF15,* Fig. 2). GO-term analysis revealed upregulation of fatty acid metabolic processes (*ACACB, ACSBG1, BRCA1, CPT1A, EM1*), Wnt signaling pathway activation, impairment of the inflammatory response (*CD44, CSF1, FOS, JMJD7-PLA2G4B, NAIP, SELENOP*), MAPK inhibition, and reduced cell adhesion and migration (Fig. 5D and Sup. File 7). At concentrations higher than those affecting the MSE, PPARβδ activation impaired radial glia migration to 79 % of controls (BMC_{10} = 2.02 µM) and oligodendrocyte migration to 82 % of controls (BMC_{10} = 2.73 µM), whereas it enhanced neuronal migration to 123 % of controls (BMC_{10} = 930.7 nM) and neuronal differentiation to 162 % of controls (BMC_{25} = 1.45 µM) (Fig. 5E+F). Further supporting the involvement of PPARβδ signaling in neurogenesis and neuronal maturation, PPARβδ activation impaired the subneurite length to 60 % of controls (BMC_{20} = 3.49 µM), whereas receptor antagonism increased the subneurite length to 136 % of controls (BMC_{20} = 40 nM) and the neurite area to 125 % of controls (BMC_{20} = 47.7 nM) (Fig. 5G+H).

3.5 Retinoic acid signaling impairs hNPC proliferation by interfering with the transcriptional regulation of cell cycle progression

All-*trans* retinoic acid (a*t*RA) and associated RAR signaling are critical for cell differentiation, molecular patterning of the prefrontal cortex (PFC), and neural tube closure (Cunningham and Duester, 2015; Janesick et al., 2015; Li et al., 2018; Shibata et al., 2021). RAR activation impaired hNPC proliferation to 58 % of controls (MSE, BMC_{20} = 31.9 nM) at physiologically relevant a*t*RA concentrations (fetal cord blood total RA concentration: 100-550 nM, Table 2) (Fig. 6 A+B). The anti-proliferative a*t*RA effect was antagonized by co-exposure to the RAR antagonist AGN193109 (10 µM

AGN, Fig. 6C) confirming receptor specificity. RAR activation upon exposure to 90 nM a*t*RA (BMC_{30}) differentially regulated 1083 genes including several previously-reported RAR target genes (Fig. 2). Moreover, GO-term enrichment analysis revealed activation of MAPK signaling, increased expression of genes involved in cell adhesion (46 genes, including cadherins, contactins, collagens, laminins, and integrins) and impairment of cell cycle progression (43 genes) and cell proliferation (20 genes) (Fig. 6D and Sup. File 7). Exposure of differentiating hNPCs to a*t*RA reduced the percentage of oligodendrocytes within the migration area to 55 % of controls (BMC_{25} = 52 nM), but this was due to premature differentiation and thus immobilization of oligodendrocytes within the sphere core instead of impaired oligodendrogenesis (Fig. 6E). Moreover, a*t*RA exposure impaired radial glia migration to 73 % of controls (BMC_{10} = 121.3 nM, Fig. 6F).

3.6 Retinoid X receptor activity controls several KNDPs with multiple parallels to other nuclear receptors, suggesting the involvement of receptor heterodimers

The RXR forms both homo- and heterodimers and binds multiple physiological ligands, including 9-*cis*-retinoic acid (9*c*RA) and dietary docosahexaenoic acid (DHA), a major structural component of the brain (De Urquiza et al., 2000; Gamoh et al., 1999). RXR activation with the synthetic RXR agonist bexarotene (BEXA) affected several KNDPs in differentiating hNPCs at low nanomolar concentrations far below fetal cord blood concentration of 127 - 231 µM DHA (Fig. 7A, Table 2). Exposure to BEXA concentration-dependently induced neuronal differentiation to 176 % of controls (MSE, BMC_{25} = 3.4 nM, Fig. 7B), increased the neurite area to 143 % of controls (MSE, BMC_{20} = 3.4 nM, Fig. 7C) and impaired radial glia migration to 79 % of controls (MSE, BMC_{10} = 3.7 nM, Fig. 7D). While the positive effect of RXR activation on neurogenesis could be antagonized by the RXR antagonist HX531 (1 µM HX531, Fig. 7E), the effects on neurite outgrowth (Fig. 7F) and radial glia migration (Fig. 7G) could not be completely reversed because the RXR antagonist itself impairs radial glia migration (not statistically significant) and increases the neurite area (Fig. 7I) at significantly higher concentrations than BEXA. RXR activation in differentiating hNPCs with 30 nM BEXA (BMC_{30}) differentially regulated 475 genes and induced several reported RXR targets involved in lipid metabolism that are also LXR targets and thus most likely activated by LXR-RXR heterodimers (*ABCA1, ACSL3, APOE, LPCAT3, LPL, SCD* and *SREBF1,* Fig. 2). Consistent with the phenotypic effects on neurogenesis, GO-term analysis revealed Notch pathway suppression and activation of genes involved in memory and neuronal synaptic plasticity (Fig. 7H, Sup. File 7). Indicative of LXR-RXR heterodimers, RXR activation induced genes involved in lipid metabolic processes and impaired expression of genes involved in oligodendrocyte development (e.g. *SOX10*) and MAPK signaling (Fig. 7H, Sup. File 7). Similar to LXR activation, RXR activation reduced oligodendrocyte differentiation to 65 % of controls (BMC_{25} = 19 nM, Sup. File 3+4), but the effect was not statistically significant.

3.7 Thyroid hormones enhance neurogenesis, presumably via RXR-THR heterodimers and Notch pathway suppression

Thyroid hormones are critical for the development of white matter tracts in humans, most likely through their involvement in oligodendrocyte maturation (Annunziata et al., 1983; Baas et al., 1997) . Although we have previously established a NAM that detects EDCs based on their ability to disrupt TH-dependent oligodendrocyte maturation (Dach et al., 2017; Klose et al., 2021), we elucidated further how thyroid hormones affect other KNDPs of fetal neurodevelopment. We observed a concentration-dependent increase in neuronal differentiation to 160 % of controls upon triiodothyronine (T3) exposure at picomolar concentrations (MSE, BMC_{25} = 262 pM, Sup. Fig. 3I), which is in the range of fetal cord blood T3 concentrations of 0.85 - 0.92 nM (Table 2). However, the effect could not be antagonized by the THR antagonist NH-3 (Sup. Fig. 3J+K). THR activation during hNPC differentiation with 3 nM T3 caused differential expression of 590 genes, including reported THR targets (e.g. *DBP*, *HR, KLF9* and *NMB*, Fig. 2) that we and others have previously correlated with oligodendrocyte maturation (Dugas et al., 2012; Klose et al., 2021). Since RXR activation elicits similar effects on developing neurons (Fig. 7B) and THR is a non-permissive partner receptor of the RXR, RXR-THR heterodimers could orchestrate the observed effects. This is supported by similar repressive effects of THR and RXR activation on the Notch pathway (11 genes) and broad similarities within GO term enrichment analyses (Fig. 7H, Sup. Fig. 3L and Sup. File 7). THR inhibition with NH-3 impaired several modeled KNDPs in differentiating hNPCs in the absence of thyroid hormones (Fig. 1C, Sup. File 3+4). Since 3 nM T3 is present in the hNPC proliferation medium, residual THR activity in differentiating hNPCs is conceivable.

3.8 KNDPs are regulated by hormone receptors in a species-specific manner *in vitro*

Although we have reported species-specific regulation of the thyroid hormone machinery in previous studies (Dach et al., 2017; Klose et al., 2021; Walter et al., 2019), a large data gap remains regarding the involvements of other HRs during neurodevelopment in humans and rodents. HR expression was compared between species using microarray data on human proliferating (Prol) and 3 days differentiated (3d Diff) fetal hNPCs and primary post-natal day 1 (PND1) rNPCs which have been extensively characterized using phenomics and transcriptomics in previous studies (Klose et al., 2022a; Masjosthusmann et al., 2019, 2018; Walter et al., 2019). Both species show high expression of *GR*, *LXRB*, *PPARD*, *RARA* and *THRA* and low expression of *ER, PPARG, PGR, PTGER1-4, RXRG, THRB* and *VDR* (Fig. 8A). Based on the MSEs of HR activation identified in hNPCs (Fig.1C), we investigated putative species-specificities. For the exposure of rNPCs, we chose three concentrations that covered the entire range of effects identified in hNPCs using a higher dilution factor. Since we focused on hNPC MSEs, proliferating rNPCs were exposed to GR and RAR agonists, while differentiating rNPCs were exposed to agonists of the other HRs (Fig. 8B). All curves and BMCs can be found in Sup. Files 4, 8 and 9. Since neuronal and oligodendrocyte differentiation are multiplexed within the Neurosphere Assay, we evaluated both endpoints in exposed rNPCs. Morphologically, unexposed proliferating human and rat NPCs are indistinguishable (Walter et al., 2019) while differentiating NPCs show species-specific morphological differences (Fig. 8C). hNPC-derived neurons produce long neurites and are evenly distributed throughout the migration area, whereas rNPC-derived neurons have short neurites and tend to cluster together. In addition, rNPC-derived oligodendrocytes have more processes, which tend to be shorter. Phenotypically, for the activation of four HRs (LXR, PGE2R, PR and RAR) hormonal dependencies of KNDPs identified in hNPC (MSE) were confirmed using rNPCs (Fig. 8D). In terms of sensitivity, while both species were equally sensitive to PR activation (BMC_{25} (OL diff): 1.22 µM (h) vs. 0.58 µM (r, not statistically significant)), hNPCs were more sensitive than rNPCs to activation of the LXR (BMC_{25} (OL diff): 25.1 nM GW39 (h) vs. 387.1 nM GW39 (r)) and RAR (BMC_{20} (Prol): 31.6 nM a*t*RA (h) vs. 242 nM a*t*RA (r, not statistically significant). rNPCs were more sensitive to PGE2R activation than hNPCs (BMC_{25} (Neuro diff): 13.69 µM PGE2 (h) vs. 1.11 µM PGE2 (r)). Not the hNPC MSE, but a less sensitive endpoint, was equally affected in both species by receptor activation of the PPARβ/δ (BMC_{25} (Neuro diff): 1.45 µM GW0742 (h) vs. 1.07 µM GW0742 (r, not statistically significant)) and the RXR (BMC_{25} (OL diff): 18.8 nM BEXA (h, not statistically significant) vs. 15.5 nM BEXA (r)). For the THR, which is the highest expressed HR in both species, hNPCs and rNPCs responded to activation with phenotypic effects observed at picomolar T3 concentrations, but while neuronal differentiation (BMC_{25} = 262 pM T3) was promoted in hNPCs, oligodendrocyte differentiation (BMC_{25} = 106 pM T3) was promoted in rNPCs. For the GR, PPARα, PPARγ and VDR, we observed no effects on the KNDPs assessed in rNPCs. Since several rodent studies have reported that GR activation affects neurogenesis, we cannot exclude that differentiating rNPCs would respond to GR activation; however, the purpose of this study was to investigate whether human-relevant hormonal dependencies could be recapitulated in rat-based systems. In both species, oligodendrocytes were the most sensitive cell type to HR activation (Fig. 8E).

4 Discussion

A complete picture of how HR regulate neurodevelopment is essential to evaluate the hazard potential of EDCs. Sex steroid receptor (i.e. AR and ER), RAR and THR signaling are crucial for the sexual differentiation of the brain, neural tube closure, and white matter development, respectively, with perturbations in HR activities associated with deficits in learning, memory, sexually dimorphic behavior, as well as cognitive deficits, including severe intellectual disability (Gika et al., 2010; Gupta et al., 1995; López-Espíndola et al., 2014). The magnitude of these consequences not only illustrates the danger posed by EDC exposure of the developing fetus, but highlights the need to fill the data gaps on the neurodevelopmental effects of the wide range of HRs expressed in the brain. Here we show, that fetal hNPC neurospheres recapitulate the HR expression pattern during human corticogenesis (cortical tissue from GW8, 12 and 16) with highest expression of *THRA*, *RXRA*, *RARB*, *RARA*, *PPARD*, *NR1H2* (LXRβ) and *NR3C1* (GR), thus representing an excellent model to study hormonal dependencies of fetal brain development. These highly expressed HRs control fetal KNDPs at low nanomolar agonist concentrations which were in the range of reported cord blood concentrations of the natural HR agonist. Of note, biomonitoring data on fetal brain hormone levels are scarce, which is why we used fetal cord blood concentrations as reference. By comparing DEGs of HR agonist-exposed hNPCs to reported HR targets in the current literature, we identified several novel HR target genes (Hunter, 1996; Liu and Brent, 2010), suggesting that HR-mediated transcription is highly dependent on the investigated species and developmental stage, thus highlighting the need for human-based test systems for studying ED-DNT.

Fetal hNPCs are unable to synthesize GR, RAR or THR ligands *de novo*, since they neither express cortisol (e.g. *CYP11B1*, *CYP11A1*, *CYP17A1*, *HSD3B*) nor a*t*RA (e.g. *ALDH1A1-3*) synthesizing enzymes, while thyroid hormones are solely produced in the thyroid gland. However, the NPC proliferation medium contains 58 nM corticosterone and 3 nM T3, indicating basal GR and THR activity in proliferating NPCs (Brewer et al., 1993). Residual HR activity during differentiation could explain the phenotypic effects of GR and THR antagonists in absence of the HR agonists. Moreover, NH-3 has been described as a mixed agonist/antagonist, both repressing co-activator binding (antagonist activity) but also promoting co-repressor release (agonist activity) from the THR (Shah et al., 2008; Webb et al., 2002). It is thus conceivable that NH-3 is able to modify THR transcription in the absence of T3. In the case of the LXR, PPARs and RXR, we hypothesize that they are all intrinsically active in hNPCs, which allows the study of both HR overactivation and suppression. While the fetal brain is efficient in cholesterol and fatty acid *de novo* synthesis, it is unable to synthesize RXR ligands (e.g., DHA, phytanic acid, lithocholic acid and 9*c*RA) and relies on placental supply. However, due to the formation of heterodimers with permissive partners that most likely exhibit intrinsic activity in hNPCs (LXR and PPARs), also the RXR should exhibit a basal activity. This is supported by the multitude of KNDPs affected LXR, PPARs or RXR antagonists (Fig. 1C).

Considering the sex-specific effects of hormones in development and beyond, we analyzed all HR agonist and antagonist effects in hNPCs derived from female and male donors. We observed sex-specificities for both the impaired hNPC proliferation and reduced neuronal differentiation upon GR activation, with male hNPCs being more responsive to DEX exposure. Analyses of fetal cortisol and corticosterone levels immediately after cesarean delivery revealed higher GC levels in female infants, indicating that also female fetuses have a higher tolerance to GCs (Giesbrecht et al., 2016). In addition, *in vivo* rodent studies reported that perinatal BPA exposure affected GC levels, GR expression and offspring behavior in a sex-specific manner, with females exhibiting anti-anxiety-like behavior after *in utero* BPA exposure, whereas males exhibited depression-like behavior (Chen et al., 2014). Mood and anxiety disorders are caused by altered circuits of the limbic, brain stem, and higher cortical brain areas (Martin et al., 2009), endpoints that cannot be assessed with the Neurosphere Assay. This limitation of applicability domain towards neurotransmission and circuit formation is covered by neural network formation assays in the DNT *in vitro* battery (Bartmann et al., 2023; Brown et al., 2016; OECD, 2023). As a second sex-specific effect, LXR activation impaired radial glia migration exclusively in female hNPCs. To the best of our knowledge, this is the first mention of sex-specific effects of the LXR in the developing brain. However, the importance of the LXR for radial glia migration during cortical layer formation and subsequent radial glia -derived oligodendrogenesis was reported previously in mice (Fan et al., 2008; Xu et al., 2014). We therefore hypothesize that excessive LXR activation negatively affects radial glia migration in a sex-specific manner. The underlying mechanisms remain to be investigated.

Perturbations in progenitor cell proliferation cause pathologies such as microcephaly or megalencephaly, which are pathognomonic for various neurological disorders (Guerrini and Dobyns, 2014). We confirmed previously that hNPCs respond to signaling pathways regulating proliferation (e.g. EGFR signaling) and detect DNT chemicals that reportedly disrupt proliferative processes (e.g., cadmium, arsenic or methyl mercury) (Baumann et al., 2016; Koch et al., 2022; Masjosthusmann et al., 2020). In the present study, we correlate GR activation with impaired hNPC proliferation and repression of RNA- and DNA-based transcriptional promoters. Our observations are consistent with studies reporting antiproliferative effects of GCs in human immortalized hippocampal progenitors, rodent neural stem cells and in the same hNPC model used in this study (Alnoud et al., 2021; Anacker et al., 2013; Moors et al., 2012; Sundberg et al., 2006). Mechanistically, others have linked the antiproliferative effects of DEX to impaired cell cycle progression in mice (Samarasinghe et al., 2011). Emphasizing the neurodevelopmental impact of GCs, fetal exposure to elevated GC levels due to prenatal GC treatment, placental dysfunction, or maternal stress correlates with learning and memory deficits, ADHD, lower IQ and anxiety disorders in children (LeWinn et al., 2009; Moisiadis and Matthews, 2014). In the context of neurodevelopmental RAR activation, the hNPC proliferation arrest was accompanied by suppression of several genes coding for cell division cycle proteins (*CDC20, CDC25B, CDC25C, CDCA3, CDCA8*), regulator of cell cycle (*RGCC*), tubulins (*TUBA1B, TUBA1C, TUBA4A, TUBB, TUBB4B* and *TUBG1*) and the EGFR. EGFR suppression was previously associated with impaired NPC proliferation and microcephaly (Carpentieri et al., 2022). Moreover, consistent with our results, a*t*RA impaired the proliferation of embryonic stem cells (ESCs) by prolonging the G1 phase of the cell cycle (Lin et al., 2017).

Oligodendrocytes are the cell type we identified to be most sensitive to HR interference (GR, LXR, PPARα+βδ, PR, RAR, RXR, THR and VDR). Impaired oligodendrogenesis causes white matter injury (WMI) with clinical phenotypes ranging from mild cognitive impairment (diffuse and punctate WMI) to severe motor deficits and intellectual disability (periventricular leukomalacia)(Back, 2017). In line with the phenotypic effects in hNPCs, LXR, PR and RXR activation repressed *SOX10* (all) and *CD38* (LXR) expression, two reported oligodendrocyte lineage determinants (Hattori et al., 2017; Stolt et al., 2002). Oligodendrogenesis is regulated by multiple signaling pathways including MAPK/ERK and Wnt signaling (Gaesser and Fyffe-Maricich, 2016; van Tilborg et al., 2016). While previous rodent *in vitro* studies reported that MAPK/ERK signaling promotes oligodendrogenesis (Dai et al., 2014; Fyffe-

Maricich et al., 2011; Guardiola-Diaz et al., 2012), we provide first evidence that this process is under control of the LXR, PPARβδ, RAR and RXR and thus susceptible to EDC exposure. In contrast to MAPK/ERK, prenatal Wnt pathway overactivation impaired oligodendrocyte precursor cell (OPC) differentiation from neural stem cells (NSCs), while DKK1 exposure (Wnt inhibitor) increased OPC numbers during neocorticogenesis (Fancy et al., 2009; Guo et al., 2015; Langseth et al., 2010). In fetal hNPCs, four HRs that impair oligodendrocyte differentiation suppress the expression of the Wnt pathway inhibitor *FRZB* (LXR (0.595-fold), PPARα (0.690-fold), PPARβδ (0.668-fold) and PG (0.541-fold)), illustrating an extensive involvement of HR in fetal Wnt pathway regulation. We further provide evidence that HR control oligodendrogenesis by regulating fatty acid and cholesterol homeostasis. Impaired oligodendrogenesis of hNPCs upon LXR, RXR and PPARα activation correlated with a global deregulation of enzymes involved in cholesterol biosynthesis, efflux and lipid metabolism (*ABCA1, ABCD1, ABCG1, ACSL3, APOE, CPT1A, DHCR24, DHCR7, ECH1, FASN, HMGCS1, LPCAT3, LPL, PNPLA3, SCD* and *SREBF1*). Given that oligodendrocytes synthesize more than 3-fold their weight of myelin per day, which is composed to 73 % of lipids including cholesterol, balanced lipid homeostasis is crucial for oligodendrocyte development and function (Mathews and Appel, 2016; Norton and Poduslo, 1973). While excessive cholesterol causes oligodendrocyte lipotoxicity, cholesterol shortage causes hypomyelination and reduced oligodendrocyte numbers that can be restored by dietary cholesterol in mouse models (Berghoff et al., 2017; Saher et al., 2012). Our observations are supported by multiple studies correlating PPARs to fatty acid oxidation and the prevention of lipid accumulation (Grygiel-Górniak, 2014). We reported previously that disturbed cholesterol metabolism correlated with impaired oligodendrocyte differentiation in hNPCs exposed to the flame retardant tetrabromobisphenol A (TBBPA), however, now we postulate a link between this phenomenon and the dysregulation of HR pathways (Klose et al., 2021). In our previous study, we identified THR disruption-dependent impaired oligodendrocyte maturation as a second TBBPA MoA, demonstrating the complexity of EDCs and highlighting the threat of EDCs disrupting multiple pathways that converge on the same KNDP thus fortifying the hazard. For identifying such converging MoA, the Adverse Outcome Pathway (AOP) concept is very helpful, that allows their visualization via AOP networks as was exemplified for oligodendrocytes previously (Klose et al., 2022a). Further studies will reveal the involvement of HR crosstalk and identify the driver receptors of the observed phenotype. However, the immense threat of fetal EDC exposure for developing oligodendrocytes and thus white matter development is obvious.

A balanced neurogenesis is a prerequisite for the functionality of neural networks and thus learning and memory functions (Berdugo-Vega et al., 2020). While deficient neurogenesis manifests in behavioral disorders such as intellectual disability, increased neurogenesis is a major driver of epileptogenesis (Guarnieri et al., 2018; Jessberger and Parent, 2015; Stagni et al., 2018). We provide evidence that neurogenesis is under control of the THR and RXR, two reported partner receptors forming RXR-THR heterodimers. For both receptors, enhanced neurogenesis correlated with suppression of Notch pathway genes, an evolutionarily highly conserved pathway regulating neurogenesis at multiple stages of development (Pierfelice et al., 2011). A recent publication confirmed that that pharmacological Notch pathway suppression accelerates neuronal differentiation of hNPCs *in vitro*, supporting previous studies that correlate Notch with the formation of long-term memory (Borghese et al., 2010; Costa et al., 2005; Koch et al., 2022). Strikingly, the THR target *KLF9* is a reported Notch suppressor in glioblastoma-initiating cells (Ying et al., 2011) and was induced more than 11-fold in T3-exposed and 3-fold in BEXA-exposed hNPCs. Our observations are supported by rodent *in vitro* studies reporting enhanced neuronal differentiation upon THR and RXR activation and human data associating TH deficiency with impaired neuronal differentiation and intellectual disability, however, to our knowledge, we are the first ones connecting this to Notch pathway suppression (Chen et al., 2012; Liu et al., 2008; López-Espíndola et al., 2014; Mounier et al., 2015;

Namba et al., 2008). RAR activation, another partner receptor of the RXR, also suppressed Notch pathway genes and activated *KLF9* expression, however, the increase in neuronal differentiation we observed was below the BMR cutoff. The reduced neurogenesis and increased Notch pathway gene expression in GR-activated NPCs is in line with a study reporting enhanced Notch signaling and reduced neurogenesis upon cortisol exposure of human hippocampal progenitors and a second study confirming impaired neurogenesis of hNPCs upon DEX exposure (Anacker et al., 2013; Moors et al., 2012). THR and RXR activation further upregulated mitochondrial complex I (MCI)-related genes (*NDUFA1, NDUFAF2, NDUFAF5, NDUFB1, NDUFB2, NDUFS5*), expressed during periods of intense neurogenesis and synaptogenesis. They are associated with higher cognitive function *in vivo* (Wirtz and Schuelke, 2011), while MCI dysfunction in a developmental mouse model (conditional *NDUFS2* knockout in radial glia and neural stem cells) impaired neuronal differentiation (Cabello-Rivera et al., 2019). Moreover, mitochondrial dysfunction during development manifests in pathologies such as Leigh syndrome (LS), which is associated with defective metabolic reprogramming and impaired neurogenesis from NPCs (Inak et al., 2021). Of note is the significant overlap between THR- and RXR-dependent gene regulation, suggesting the involvement of THR-RXR heterodimers. Enhanced Wnt signaling could be responsible for the increased neurogenesis we observed upon LXR and PPARβδ activation. WNT signaling orchestrates neurogenesis in the developing mouse neocortex and its disruption is associated with intellectual disability (Da Silva et al., 2021; Zhuang et al., 2023). Our results are supported by rodent *in vivo* studies reporting elevated neuronal numbers upon LXR activation and reduced neurogenesis in the dentate gyrus of LXRβ-knockdown mice, which was associated with autistic-like behavior (Cai et al., 2018; Theofilopoulos et al., 2013). PPARβδ activation was associated with the restoration of Wnt pathway activity in an *in vivo* rat model of liver regeneration after ethanol exposure (Xu et al., 2015). In addition, LXR and PPAR ligands DHA and other PUFAs are essential for synaptic function, neuronal differentiation and neurite outgrowth, acting as protective antioxidants in the brain (Basak and Duttaroy, 2023; Cao et al., 2009; Drolet et al., 2021; Katakura et al., 2009). Figure 9 summarizes the proposed signaling pathways and cellular processes by which HRs regulate human KNDPs *in vitro*.

The OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters relies heavily on the use of rodent *in vivo* studies. However, we and others have previously reported that the endocrine system affects development in species-specific ways, questioning the predictability of rodent-based test systems for human health (Medvedev et al., 2020; Walter et al., 2019; Whitten and Patisaul, 2001). Despite the similarities between the HR expression patterns of human fetal cortex tissue, hNPC and rNPC neurospheres and adult rat cortex tissue (highest expression of GR, LXRβ, PPARβδ, RARα and THRα) (Gofflot et al., 2007), the phenotypic effects on KNDPs observed in hNPCs for activation of the AhR, GR, PPARβ/δ, PPARγ, RXR, THR and VDR could not be reproduced in rNPCs. Moreover, when KNDPs were affected by HR activation in both species, hNPCs were mostly more sensitive than rNPCs (LXR, PPARα, PR and PGE2R). One reason for this may be that rNPCs isolated from PND1 pups do not represent the same developmental stage as hNPCs isolated from GW16-18 fetuses. Clancy, Darlington and Finlay 2001 used empirical data and a regression theory model to create a matrix of 95 neurodevelopmental events in nine species to facilitate literature comparison of studies from different species (Clancy et al., 2001). Based on this publication and the corresponding online tool (https://www.translatingtime.org/translate/), GW16-18 of human development was matched to gestational day 21-24 in rats, which corresponds to the days around birth (Workman et al., 2013).

Species differences in HR-regulated KNDPs are not the only limitation of *in vivo* (ED)-DNT studies, which support the integration of human-based NAMs into the (ED)-DNT risk assessment framework (Paparella et al., 2020). The insufficient testing throughout, the large number of laboratory animals and the lack of mechanistic information led to the formation of a consensus statement by different scientific stakeholders that supports the integration of NAMs in DNT testing (Fritsche et al., 2018). In 2018, Bal Price et al. introduced a scoring system for NAMs based on readiness criteria, which helps to identify the applicability domain (screening/prioritization or risk assessment) and evaluate the use in fit-for-purpose integrated approach to testing and assessment (IATA) (Bal-Price et al., 2018a; Crouzet et al., 2023). Given that *in vitro* NAMs recapitulate specific KNDPs or molecular initiating events (MIEs) and do not provide information on an organism level, test system development can be promoted using the aforementioned AOP concept (Klose et al., 2022a) and the integration of NAMs into testing batteries covering all relevant models and endpoints (Bal-Price et al., 2010). Moreover, the use of Physiologically Based Pharmacokinetic (PBPK) modeling enables the prediction of organ-specific concentrations, pharmacokinetics, translation between species and the construction of quantitative AOPs (qAOPs), thus making the use of human-based NAMs in EDC risk assessment easily conceivable (Deepika and Kumar, 2023; Maass et al., 2023).

5 Conclusion

Although the exact mechanisms of endocrine disruption during brain development are not entirely delineated, the consequences of EDC exposure on the cognitive abilities of the developing child can be severe (Caporale et al., 2022; Özel and Rüegg, 2023; Tanner et al., 2020). This study substantially contributes to closing the data gap regarding hormonal dependencies, and thus EDC sensitivities, of key neurodevelopmental processes as a first important step in developing human-relevant ED-DNT test methods to eventually improve EDC hazard assessment (Cediel-Ulloa et al., 2024, 2022; Lupu et al., 2020). We show that hormones and associated HRs exhibit KNDP-specific patterns indicating that also EDCs will disturb brain development in KNDP-specific ways depending on their molecular mode of action. This strongly implies the need for mechanistic, KNDP-based ED-DNT test methods not assessing apical endpoints but endophenotypes of toxicity. We further question the human relevance of the predominantly rodent-based EDC risk assessment, unravelling that rodent-based neural *in vitro* models fail to recapitulate the neurodevelopmental consequences of HR disruption observed in human-based counterparts. The results of the present study are a first step towards broadening the applicability domain of the DNT *in vitro* testing battery (OECD, 2023) towards ED-DNT by showing that KNDPs like hNPC proliferation and terminal differentiation are highly sensitive towards GR, LXR, PPAR, PR, RAR and RXR modulation. Further research will serve more in-depth elucidation of the receptor crosstalks underlying our phenotypic observations and study the chemical applicability domain of the assays.

6 Conflict of Interest

Katharina Koch, Jördis Klose, Arif Dönmez and Ellen Fritsche are shareholders of the company DNTOX GmbH which provides DNT-IVB assay services. The shareholders and Jördis Klose are partly (KK, AD, EF) or fully (JK) employed by DNTOX GmbH. The data was generated solely at the IUF prior to the foundation of DNTOX GmbH. The funders of DNTOX had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results. KS, SH, LMS, SM and NF declare no conflict of interest.

7 Author Contributions

Katharina Koch: Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration; **Kevin Schlüppmann:** Formal analysis, Investigation, Methodology, Data curation, Writing - Review & Editing; **Saskia Hüsken:** Formal analysis, Investigation, Methodology, Writing - Review & Editing; **Louisa Merit Stark:** Formal analysis, Investigation, Methodology, Writing - Review & Editing; **Stefan Masjosthusmann:** Formal analysis, Investigation, Methodology; Data curation, Writing - Review & Editing; **Jördis Klose:** Formal analysis, Investigation, Writing - Review & Editing; **Nils Förster:** Formal analysis, Investigation, Methodology, Data curation, Writing - Review & Editing; **Arif Dönmez:** Validation, Formal analysis, Data curation, Investigation, Writing - Review & Editing; **Ellen Fritsche:** Conceptualization, Validation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition;

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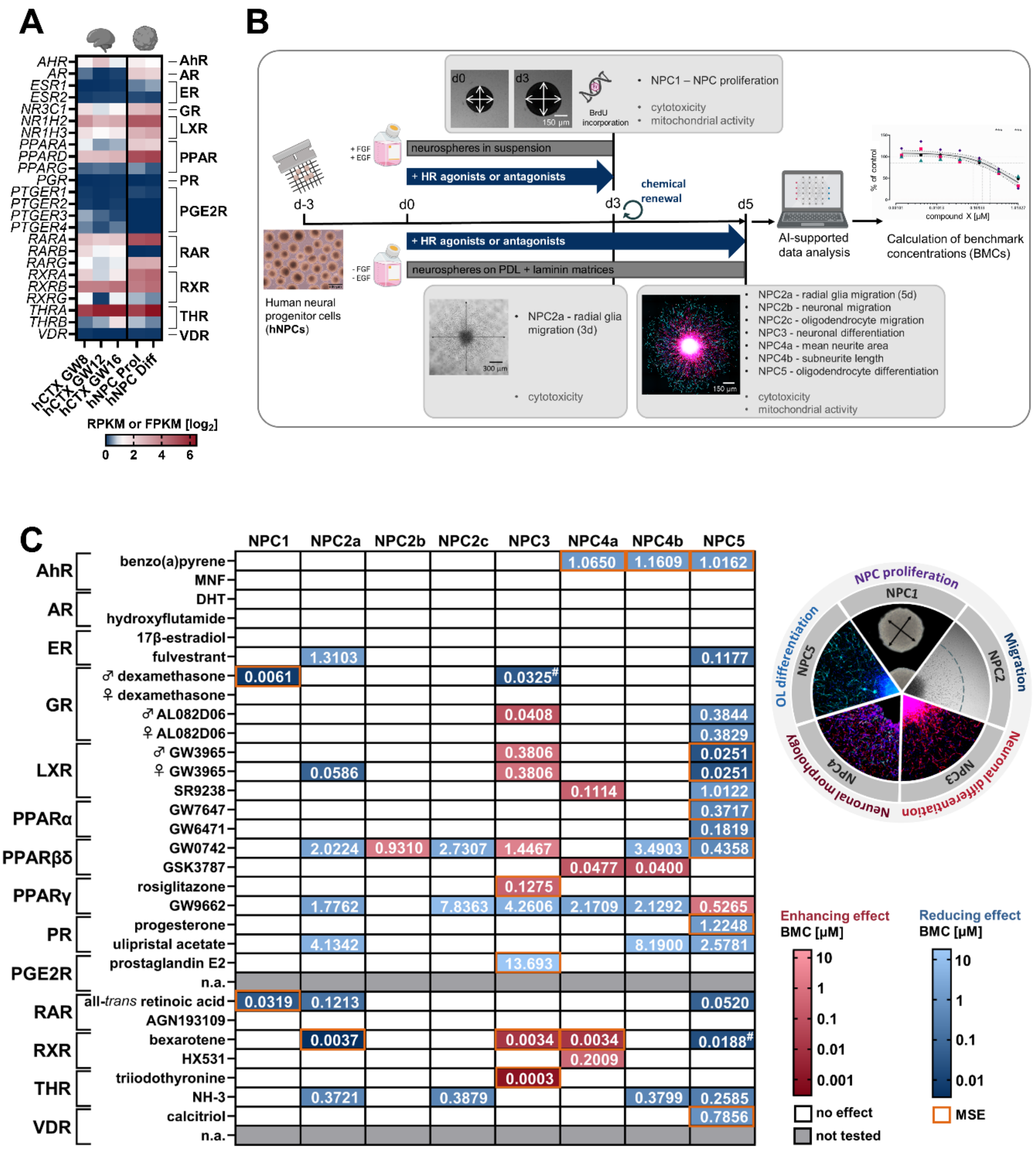
Captions of main and supplementary figures – Koch et al



		NPC1	NPC2a	NPC2b	NPC2c	NPC3	NPC4a	NPC4b	NPC5
AhR	benzo(a)pyrene						1.0650	1.1609	1.0162
	MNF								
AR	DHT								
	hydroxyflutamide								
ER	17β-estradiol								
	fulvestrant		1.3103						0.1177
GR	♂ dexamethasone	0.0061				0.0325#			
	♀ dexamethasone								
	♂ AL082D06					0.0408			0.3844
	♀ AL082D06								0.3829
LXR	♂ GW3965					0.3806			0.0251
	♀ GW3965		0.0586			0.3806			0.0251
	SR9238						0.1114		1.0122
PPARα	GW7647								0.3717
	GW6471								0.1819
PPARβδ	GW0742		2.0224	0.9310	2.7307	1.4467		3.4903	0.4358
	GSK3787						0.0477	0.0400	
PPARγ	rosiglitazone					0.1275			
	GW9662		1.7762		7.8363	4.2606	2.1709	2.1292	0.5265
PR	progesterone								1.2248
	ulipristal acetate		4.1342					8.1900	2.5781
PGE2R	prostaglandin E2					13.693			
	n.a.								
RAR	all-*trans* retinoic acid	0.0319	0.1213						0.0520
	AGN193109								
RXR	bexarotene		0.0037			0.0034	0.0034		0.0188#
	HX531						0.2009		
THR	triiodothyronine					0.0003			
	NH-3		0.3721		0.3879			0.3799	0.2585
VDR	calcitriol								0.7856
	n.a.								

Figure 1: Comparative HR expression analysis and phenotypic effects of HR modulation in hNPCs. **A.** HR expression in primary human fetal (GW 8, 12 and 16) cortical tissue (hCTX) and GW 16 proliferating (hNPC Prol) and 60h-differentiated hNPCs (hNPC Diff), presented as log2 RPKM (hCTX, downloaded from https://www.brainspan.org/) and log2 FPKM (hNPC, present study). **B.** Exposure setup: Proliferating hNPC neurospheres were mechanically passaged to reach a sphere sizes of 300 µm diameter on the plating day. In the presence of growth factors (EGF and FGF), hNPCs were exposed to HR agonists and antagonists for three days before hNPC proliferation (NPC1) was assessed by BrdU ELISA. In addition, hNPCs were exposed to HR agonists and antagonists during differentiation on poly-D-lysine (PDL)/laminin-coated plates in absence of growth factors. After 5 days, immunocytochemical stainings for neurons (β(III)tubulin), oligodendrocytes (O4) and nuclei (Hoechst33258) were analyzed using Omnisphero software and two convolutional neural networks (CNNs) to derive the Neurosphere Assay endpoints NPC2-5. Raw data were fed into an R-based data analysis pipeline to generate concentration-response curves and derive benchmark concentrations (BMCs). The BMCs correspond

to benchmark responses (BMRs) that were predefined for each assay endpoint (NPC1-5) as described in the Methods section. **C**. Summary of the phenotypic effects of HR agonists and antagonists on the Neurosphere Assay endpoints (NPC1-5), expressed as BMCs in micromolar (µM). Only effects at non-cytotoxic concentrations are shown. For HR activation, the most sensitive endpoint (MSE) is highlighted in orange (for AhR and RXR no clear MSE could be defined). $^{\#}$ not significant according to the prediction model, but BMR is reached. Abbreviations: AI, artificial intelligence; AhR, aryl hydrocarbon receptor; AR, androgen receptor; BrdU, bromodeoxyuridine; EGF, epidermal growth factor; DHT, dihydrotestosterone; ER, estrogen receptor; FGF, fibroblast growth factor; GR, glucocorticoid receptor; GW, gestational week; HR, hormone receptor; LXR, liver X receptor; MNF, 3'-methoxy-4'-nitroflavone; NPC, neural progenitor cell; OL, oligodendrocytes; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; PGE2R, prostaglandin E2 receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; THR, thyroid hormone receptor; VDR, vitamin D receptor.

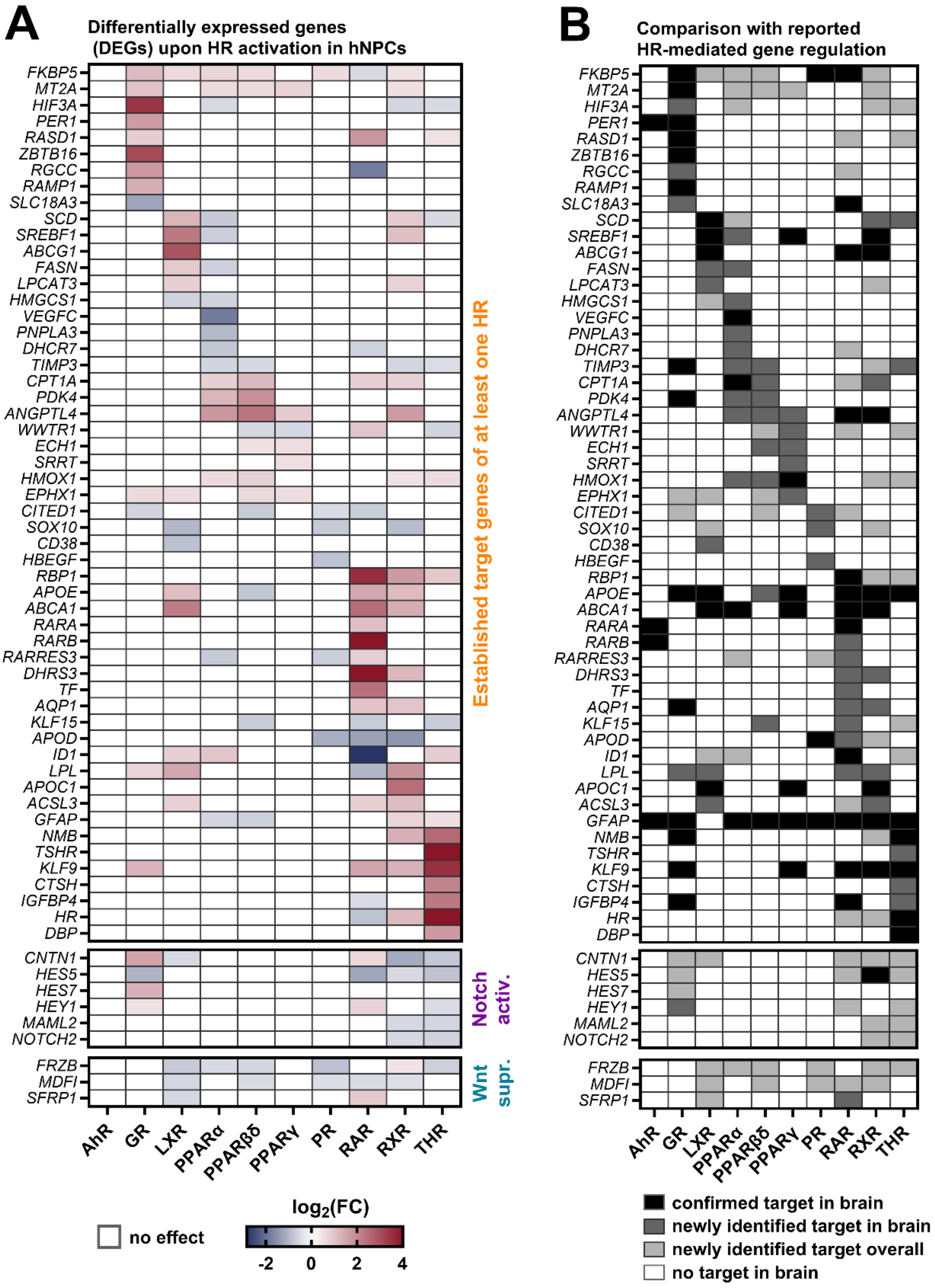


Figure 2: Transcriptomic analysis of selected HR target genes in fetal hNPCs and comparison to the current literature. A. Proliferating (GR and RAR) and differentiating (remaining HRs) hNPCs were exposed to HR agonists for 60 h (BMC_{30} calculated for the most sensitive endpoint (MSE)) before total RNA was extracted and subjected to RNA sequencing. Genes with a |log2(FC)| > 0.486 and a q-value < 0.05 were defined as DEGs. **B**. Evidence for HR-dependent gene regulation was based on a comprehensive literature search (PubMed). DEGs previously identified as HR targets in brain tissue or neuronal cultures (black) or in non-brain tissue or non-neuronal cultures (dark gray) are distinguished from genes without evidence of HR-mediated regulation in the literature but in the present study (light gray). Genes with no evidence of HR regulation in the literature or in the present study are highlighted in white. All references can be found in Sup. File 5. Abbreviations: AhR, aryl hydrocarbon receptor; AR, androgen receptor; DEGs, differentially expressed genes; ER, estrogen receptor; GR, glucocorticoid receptor;

HR, hormone receptor; LXR, liver X receptor; NPC, neural progenitor cell; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; THR, thyroid hormone receptor.

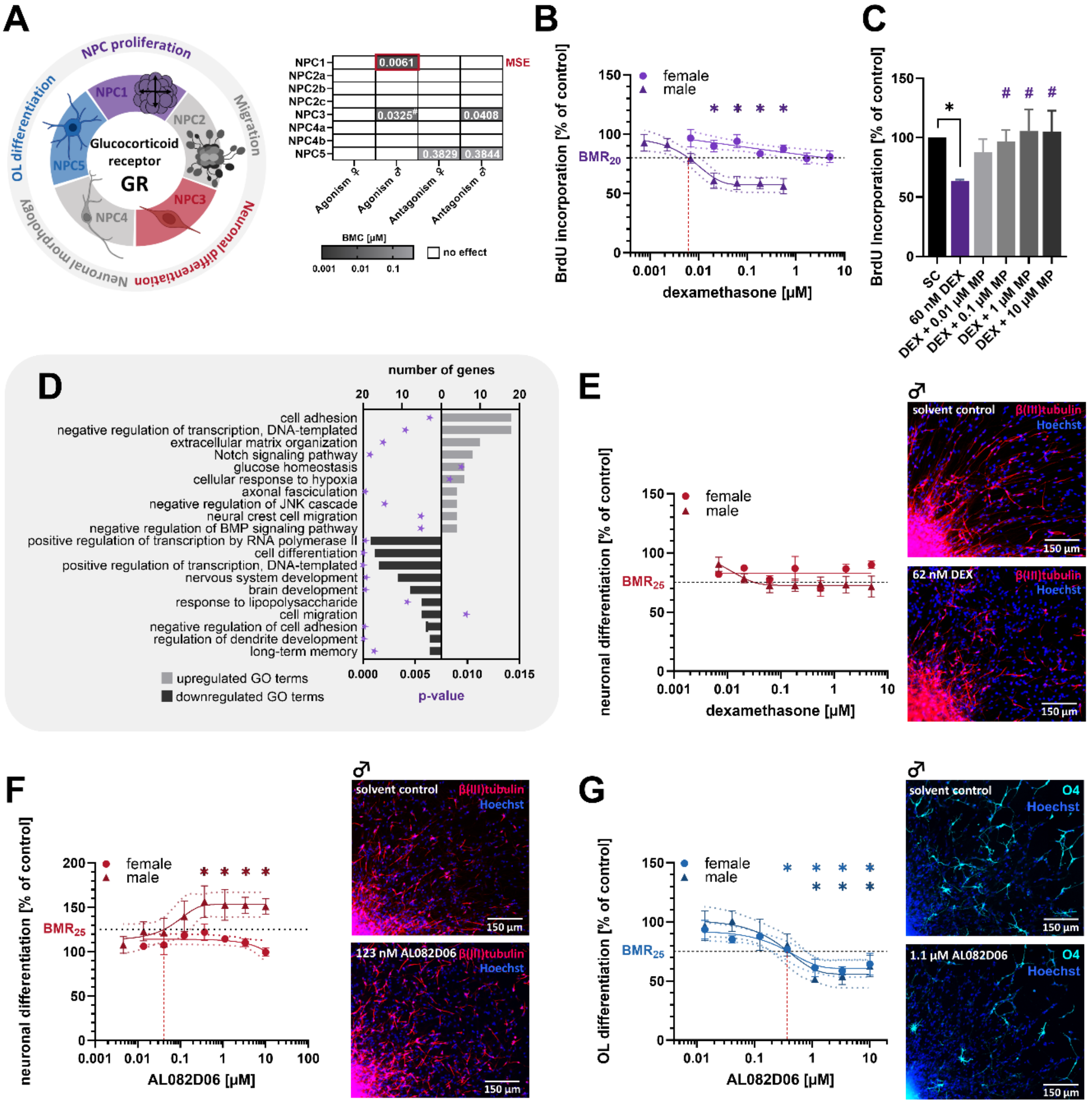


Figure 3: The glucocorticoid receptor regulates hNPC proliferation and terminal differentiation in a sex-specific manner. A. GR-sensitive key neurodevelopmental processes (KNDPs) are highlighted in color and respective BMCs (µM) derived from the agonist and antagonist screening described in Figure 1 are listed. **B**. Proliferating hNPCs were exposed to serial concentrations of the GR agonist dexamethasone (DEX) or solvent (0.1 % DMSO) for 3 days before the proliferative capacity was assessed by BrdU ELISA and presented as % of the solvent control (SC). Stratification of data by hNPC sex was performed. **C**. Male proliferating hNPCs were exposed to solvent (0.2 % DMSO), 60 nM DEX alone, or DEX in combination with increasing concentrations of the GR antagonist mifepristone (MP, 0.01 – 10 µM) for 3 days before the proliferative capacity was assessed by BrdU ELISA. *Significant ($p<0.05$, two-tailed Student t-test) compared to the solvent control. #Significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to 60 nM DEX. **D.** Top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in proliferating hNPCs exposed to 60 nM DEX for 60 h. Significant enrichment of DEGs in GO terms was defined by a p-value < 0.01 (purple star). A complete list of DEGs for each GO term can be found in Sup. File 7. **E-G**. Differentiating male and female hNPCs were exposed to solvent (0.1 % DMSO) and serial concentrations of DEX (E) or the GR-specific

antagonist AL082D06 (F+G) for 5 days. Neuronal differentiation (E+F) was assessed as the percentage of β(III)tubulin-positive cells (neurons) within the migration area, while oligodendrocyte differentiation (G) was assessed as the percentage of O4-positive cells (oligodendrocytes) within the migration area based on immunocytochemical stainings. For B, E, F and G: *significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to the lowest concentration. All data (B, C, E-G) are expressed as mean ± SEM. Abbreviations: BMC, benchmark concentration; BMR, benchmark response; BrdU, bromodeoxyuridine; DEG, differentially expressed genes; DMSO, dimethyl sulfoxide; GO, gene ontology; MSE, most sensitive endpoint; OL, oligodendrocyte.

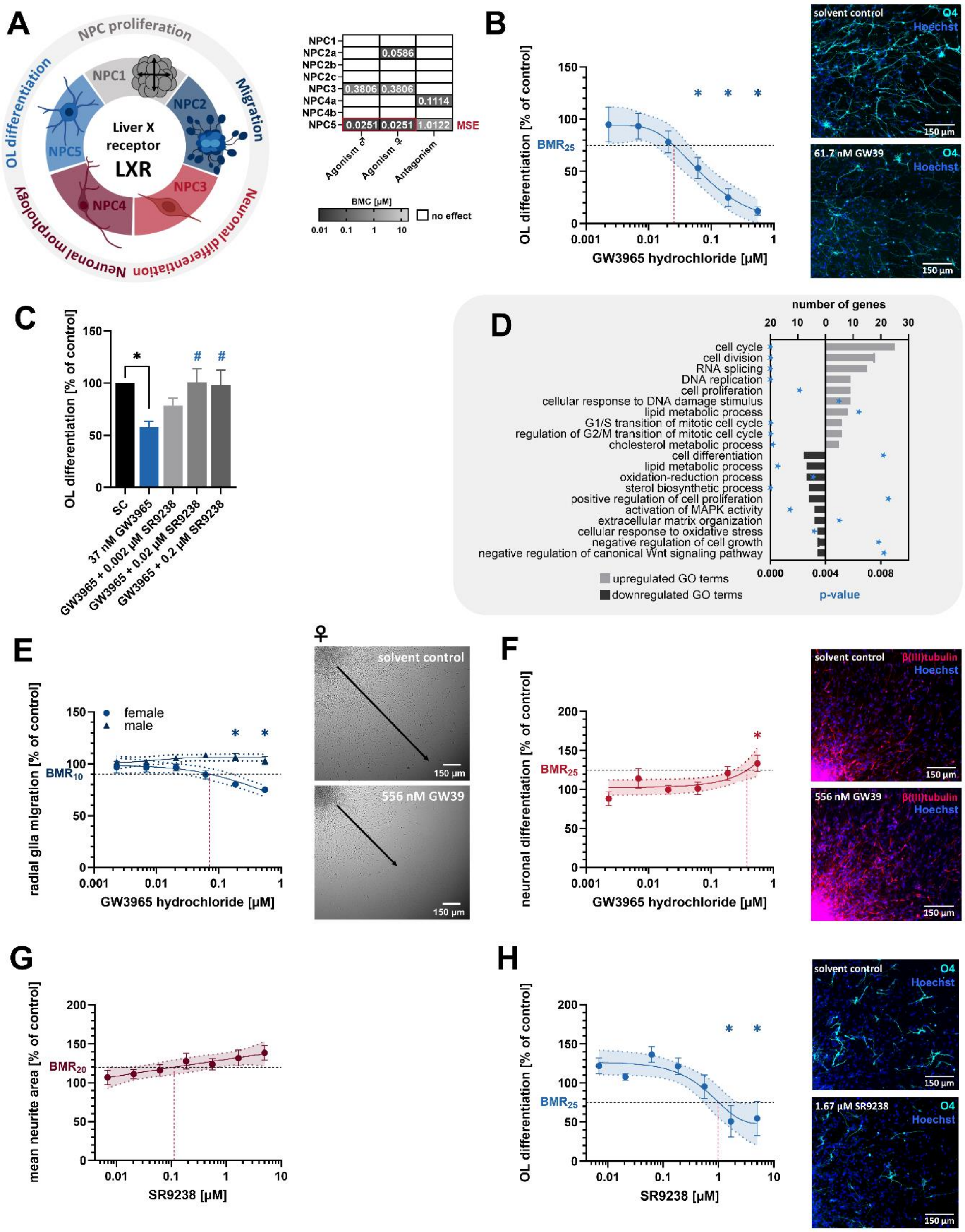


Figure 4: The LXR controls multiple KNDPs, presumably by interfering with cholesterol homeostasis and developmental signaling pathways. A. LXR-sensitive key neurodevelopmental processes (KNDPs) are highlighted in color and respective BMCs (µM) derived from the agonist and antagonist screening described in Figure 1 are listed. **B**. Differentiating hNPCs were exposed to serial concentrations of the LXR agonist GW3965 (GW39) or solvent (0.1 % DMSO) for 5 days before oligodendrocyte differentiation was assessed as the percentage of O4-positive cells (oligodendrocytes) within the migration area based on immunocytochemical stainings. **C**. Differentiating hNPCs were exposed to solvent (0.2 % DMSO), 37 nM GW39 alone, or GW39 in combination with increasing concentrations of the LXR antagonist SR9238 (SR92, 0.002 – 0.2 µM) for 5 days before oligodendrocyte differentiation was assessed as described in B. *Significant ($p<0.05$, two-tailed Student t-test) compared to solvent control (SC). #Significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to 37 nM GW39. **D**. Top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in differentiating hNPCs exposed to 37 nM GW39 for 60 h. Significant enrichment of DEGs in GO terms was defined by a p-value < 0.01 (blue star). A complete list of DEGs for each GO term can be found in Sup. File 7. **E**. Differentiating hNPCs were exposed to serial concentrations of the GW39 or solvent (0.1 % DMSO) for 5 days before radial glia migration was assessed by measuring the distance of the farthest migrated cells to the sphere core using the software Omnisphero. Stratification of data by hNPC sex was performed. **F-H**. Differentiating hNPCs were exposed to solvent (0.1 % DMSO) and serial concentrations of GW39 (F) or SR92 (G+H) for 5 days. Neuronal differentiation (F) was assessed as the percentage of β(III)tubulin-positive cells (neurons) within the migration area, the mean neurite area (G) was assessed based on the morphology of β(III)tubulin-positive neurons using Omnisphero software, while oligodendrocyte differentiation (H) was assessed as the percentage of O4-positive cells (oligodendrocytes) within the migration area. For B, E-H: *significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to the lowest concentration. All data (B, C, E-H) are expressed as mean ± SEM. Abbreviations: BMC, benchmark concentration; BMR, benchmark response; DEG, differentially expressed genes; DMSO, dimethyl sulfoxide; GO, gene ontology; MSE, most sensitive endpoint; OL, oligodendrocyte.

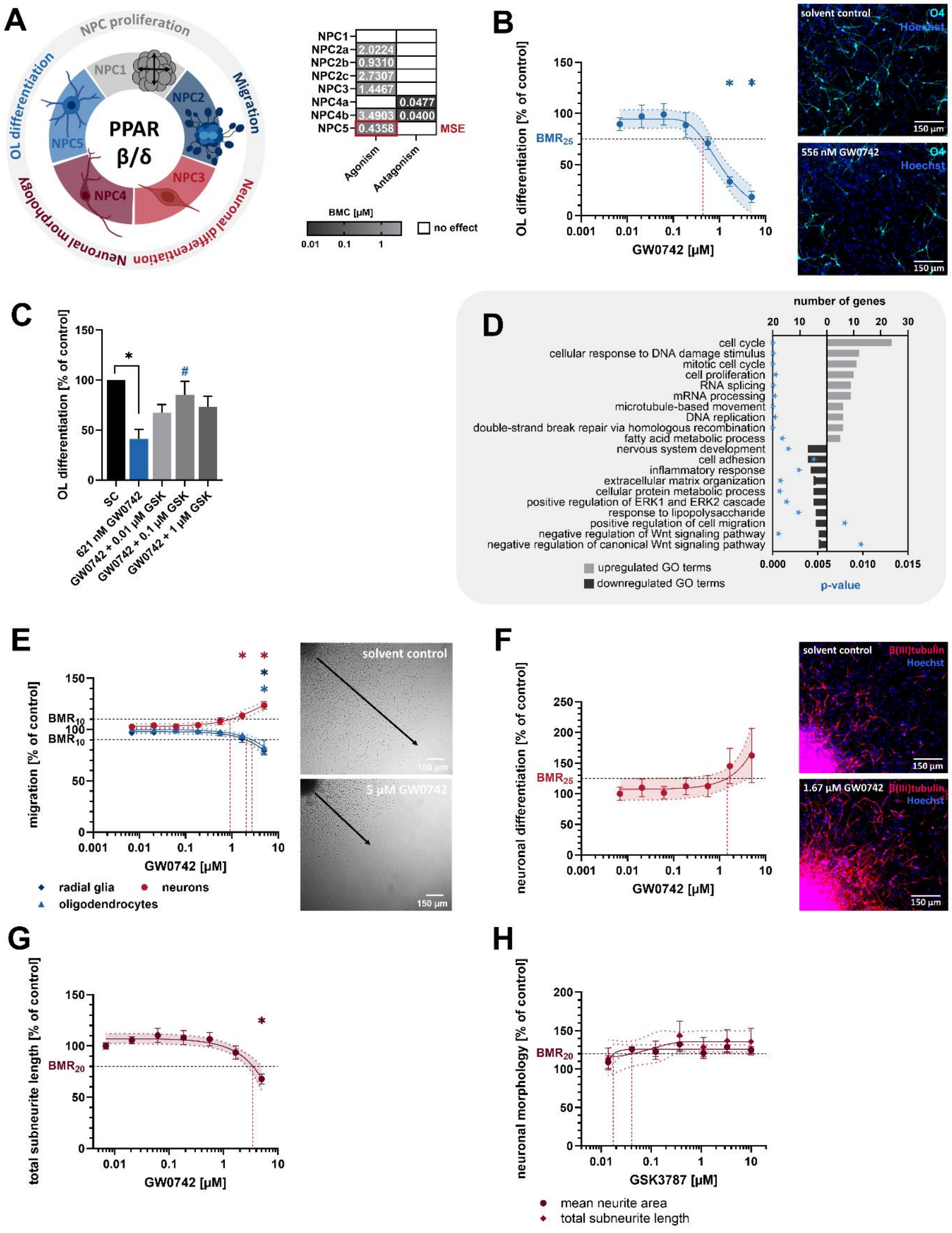


Figure 5: The influence of PPARβδ activity on hNPC lineage specification resembles the effects observed upon LXR and RXR modulation. A. PPARβδ-sensitive key neurodevelopmental processes (KNDPs) are highlighted in color and respective BMCs (µM) derived from the agonist and antagonist screening described in Figure 1 are listed. **B**. Differentiating hNPCs were exposed to serial concentrations of the PPARβδ agonist GW0742 or solvent (0.1 % DMSO) for 5 days before oligodendrocyte differentiation was assessed as the percentage of O4-positive cells (oligodendrocytes) within the migration area. **C**. Differentiating hNPCs were exposed to solvent (0.2 % DMSO), 621 nM GW0742 alone, or GW0742 in combination with increasing concentrations of the PPARβδ antagonist GSK3787 (GSK, 0.01 – 1 µM) for 5 days before oligodendrocyte differentiation was assessed as described in C. *Significant (p<0.05, two-tailed Student t-test) compared to the solvent control. #Significant

(p<0.05, step-down multiple test procedure of Dunnett and Tamhane) compared to 621 nM GW0742. **D**. Top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in differentiating hNPCs exposed to 621 nM GW0742 for 60 h. Significant enrichment of DEGs in GO terms was defined by a p-value < 0.01 (blue star). A complete list of DEGs for each GO term can be found in Sup. File 7. **E**. Differentiating hNPCs were exposed to serial concentrations of the PPARβδ agonist GW0742 or solvent (0.1 % DMSO) for 5 days before the migration of radial glia, neurons and oligodendrocytes was assessed by measuring the distance of the farthest migrated cells to the sphere core (radial glia) and automated image analysis (neurons and oligodendrocytes) using the software Omnisphero. **F-H.** Differentiating hNPCs were exposed to solvent (0.1 % DMSO) and serial concentrations of GW0742 (F+G) or GSK (H) for 5 days. Neuronal differentiation (F) was assessed as the percentage of β(III)tubulin-positive cells (neurons) within the migration area and the mean neurite area (G+H) and total subneurite length (H) were assessed based on the morphology of β(III)tubulin-positive neurons using Omnisphero software. For B, E-H: *significant (p<0.05, step-down multiple test procedure of Dunnett and Tamhane) compared to the lowest concentration. All data (B, C, E-H) are expressed as mean ± SEM. Abbreviations: BMC, benchmark concentration; BMR, benchmark response; DEG, differentially expressed genes; DMSO, dimethyl sulfoxide; GO, gene ontology; MSE, most sensitive endpoint; OL, oligodendrocyte differentiation.

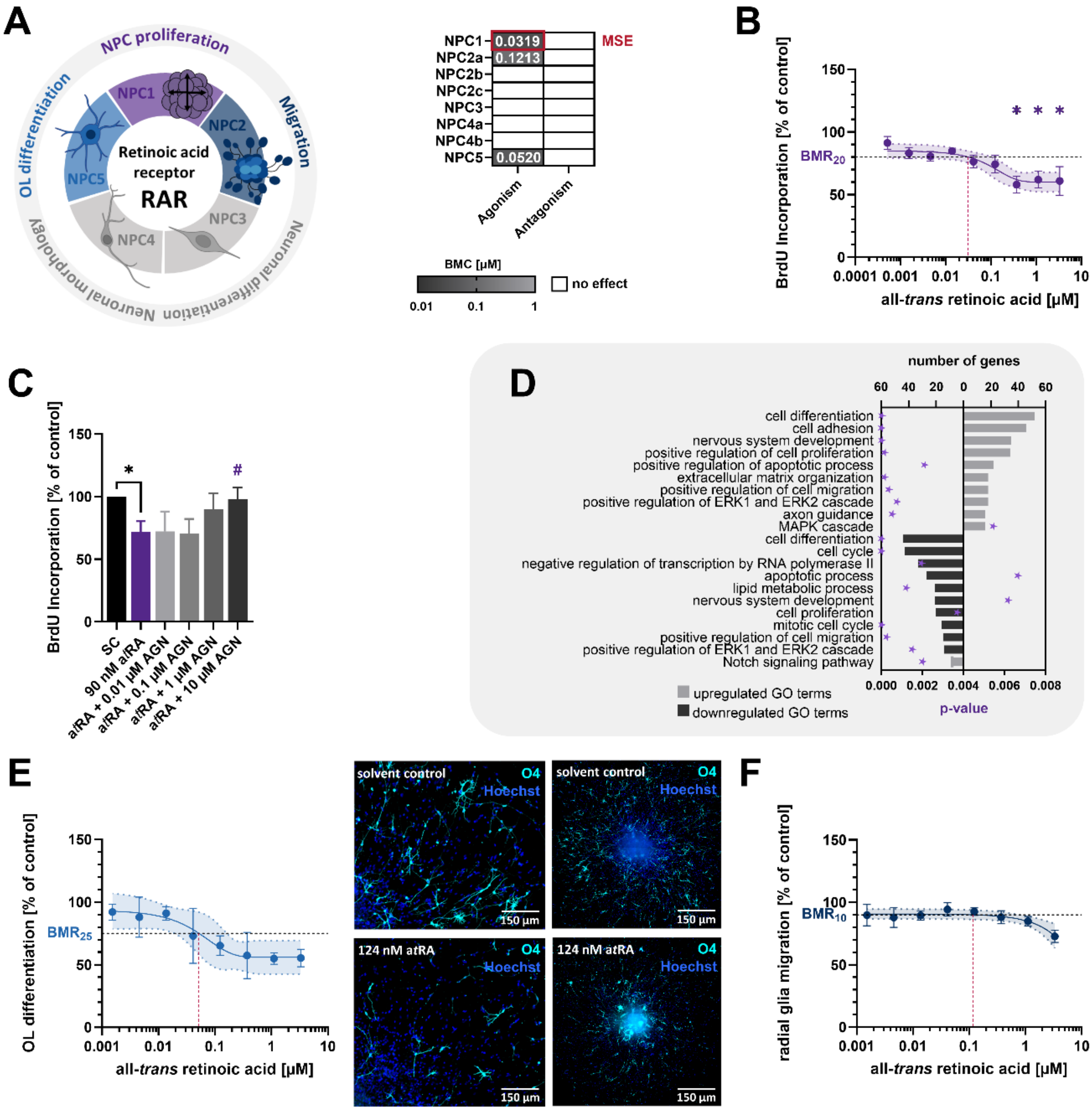


Figure 6: The RAR regulates hNPC proliferation and terminal differentiation into oligodendrocytes. A. RAR-sensitive key neurodevelopmental processes (KNDPs) are highlighted in color and respective BMCs (µM) derived

from the agonist and antagonist screening described in Figure 1 are listed. **B**. Proliferating hNPCs were exposed to serial concentrations of the RAR agonist all-*trans* retinoic acid (a*t*RA) or solvent (0.1 % DMSO) for 3 days before the proliferative capacity was assessed by BrdU ELISA. **C**. Proliferating hNPCs were exposed to solvent (0.2 % DMSO), 90 nM a*t*RA alone, or a*t*RA in combination with increasing concentrations of the RAR antagonist AGN193109 (AGN, 0.01 - 10 µM) for 3 days before the proliferative capacity was assessed by BrdU ELISA. *Significant (p<0.05, two-tailed Student t-test) compared to the solvent control. #Significant (p<0.05, step-down multiple test procedure of Dunnett and Tamhane) compared to 90 nM a*t*RA. **D. <t**op ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in proliferating hNPCs exposed to 90 nM a*t*RA for 60 h. Significant enrichment of DEGs in GO terms was defined by a p-value < 0.01 (purple star). A complete list of DEGs for each GO term can be found in Sup. File 7. **E+F**. Differentiating hNPCs were exposed to a*t*RA for five days before oligodendrocyte differentiation (E) was assessed as the percentage of O4-positive cells (oligodendrocytes) within the migration area. Representative pictures show a closeup of the migration area and an overview of the whole differentiated sphere including the core. Radial glia migration (F) was assessed by measuring the distance of the farthest migrated cells to the sphere core using the software Omnisphero. For B, E and F: *significant (p<0.05, step-down multiple test procedure of Dunnett and Tamhane) compared to the lowest concentration. All data (B, C, E and F) are expressed as mean ± SEM. Abbreviations: BMC, benchmark concentration; BMR, benchmark response; BrdU, bromodeoxyuridine; DEG, differentially expressed gene; DMSO, dimethyl sulfoxide; GO, gene ontology; MSE, most sensitive endpoint; OL, oligodendrocyte.

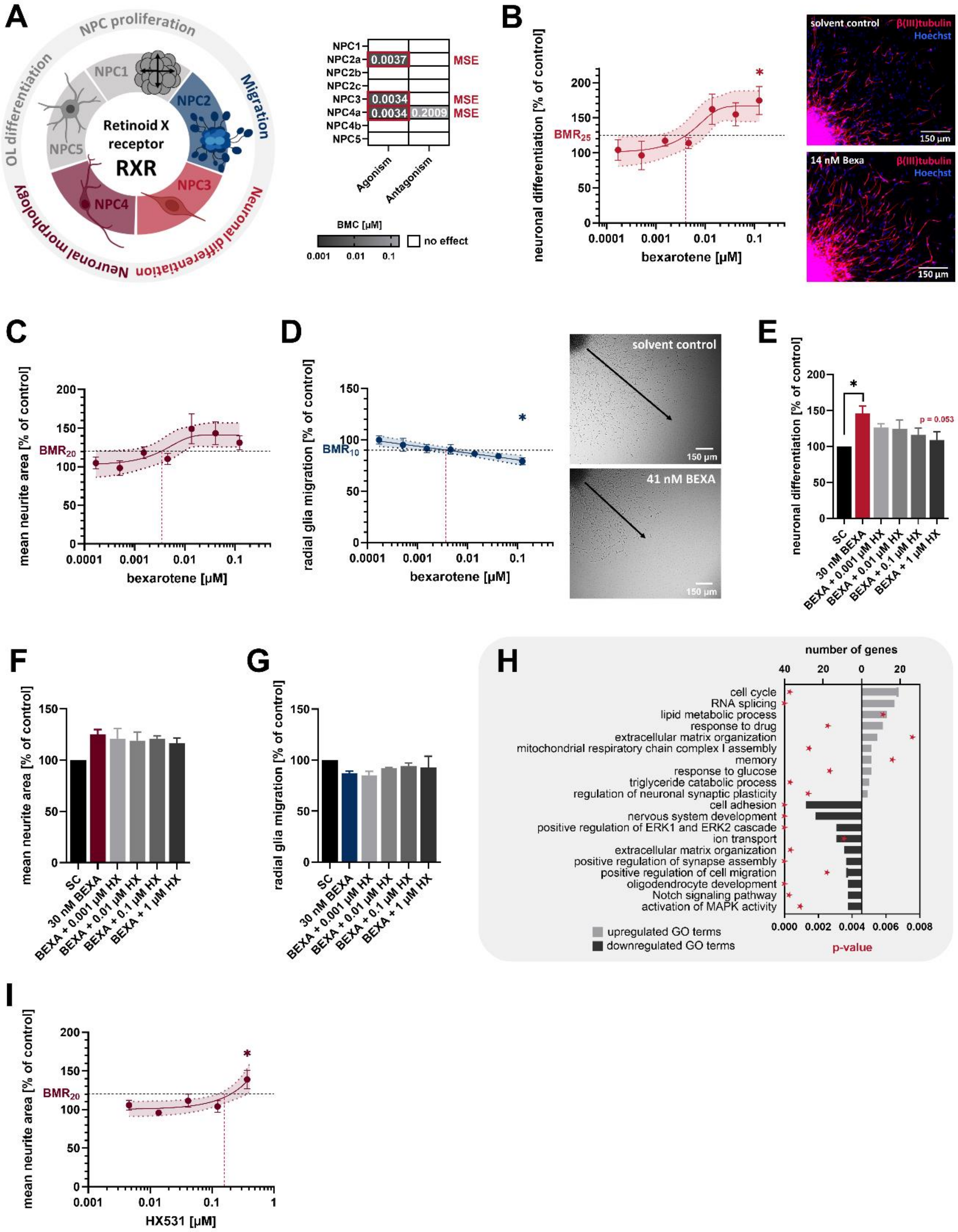


Figure 7: RXR signaling promotes neurogenesis and exhibits transcriptomic similarities to LXR activation. A. RXR-sensitive key neurodevelopmental processes (KNDPs) are highlighted in color and respective BMCs (µM) derived from the agonist and antagonist screening described in Figure 1 are listed. **B-D**. Differentiating hNPCs were exposed to serial concentrations of the RXR agonist bexarotene (BEXA) or solvent (0.1 % DMSO) for 5 days. Neuronal differentiation (B) was assessed as the percentage of β(III)tubulin-positive cells (neurons) within the migration area, the mean neurite area (C) was assessed by analyzing the morphology of β(III)tubulin-positive neurons using Omnisphero software and radial glia migration (D) was assessed by measuring the distance of the farthest migrated cells to the sphere core using Omnisphero software. *Significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to the lowest concentration. **E-G**. Differentiating hNPCs were exposed to solvent (0.2 % DMSO), 30 nM BEXA alone, or BEXA in combination with increasing concentrations of the RXR antagonist HX531 (HX, 0.001 – 1 µM) for 5 days before neuronal differentiation (E), the mean neurite

area (F) and radial glia migration (G) were assessed as described in B-D. *Significant ($p<0.05$, two-tailed Student t-test) compared to the solvent control (SC). #Significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to 30 nM BEXA. **H**. Top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in differentiating hNPCs exposed to 30 nM BEXA for 60 h. Significant enrichment of DEGs in GO terms was defined by a p-value < 0.01 (red star). A complete list of DEGs for each GO term can be found in Sup. File 7. **I**. Differentiating hNPCs were exposed to serial concentrations of the RXR antagonist HX531 or solvent (0.1 % DMSO) for 5 days before the mean neurite area was assessed as described in C. *Significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to the lowest concentration. All data (B-G, I) are expressed as mean ± SEM. Abbreviations: BMC, benchmark concentration; BMR, benchmark response; DEG, differentially expressed gene; DMSO, dimethyl sulfoxide; GO, gene ontology; MSE, most sensitive endpoint.

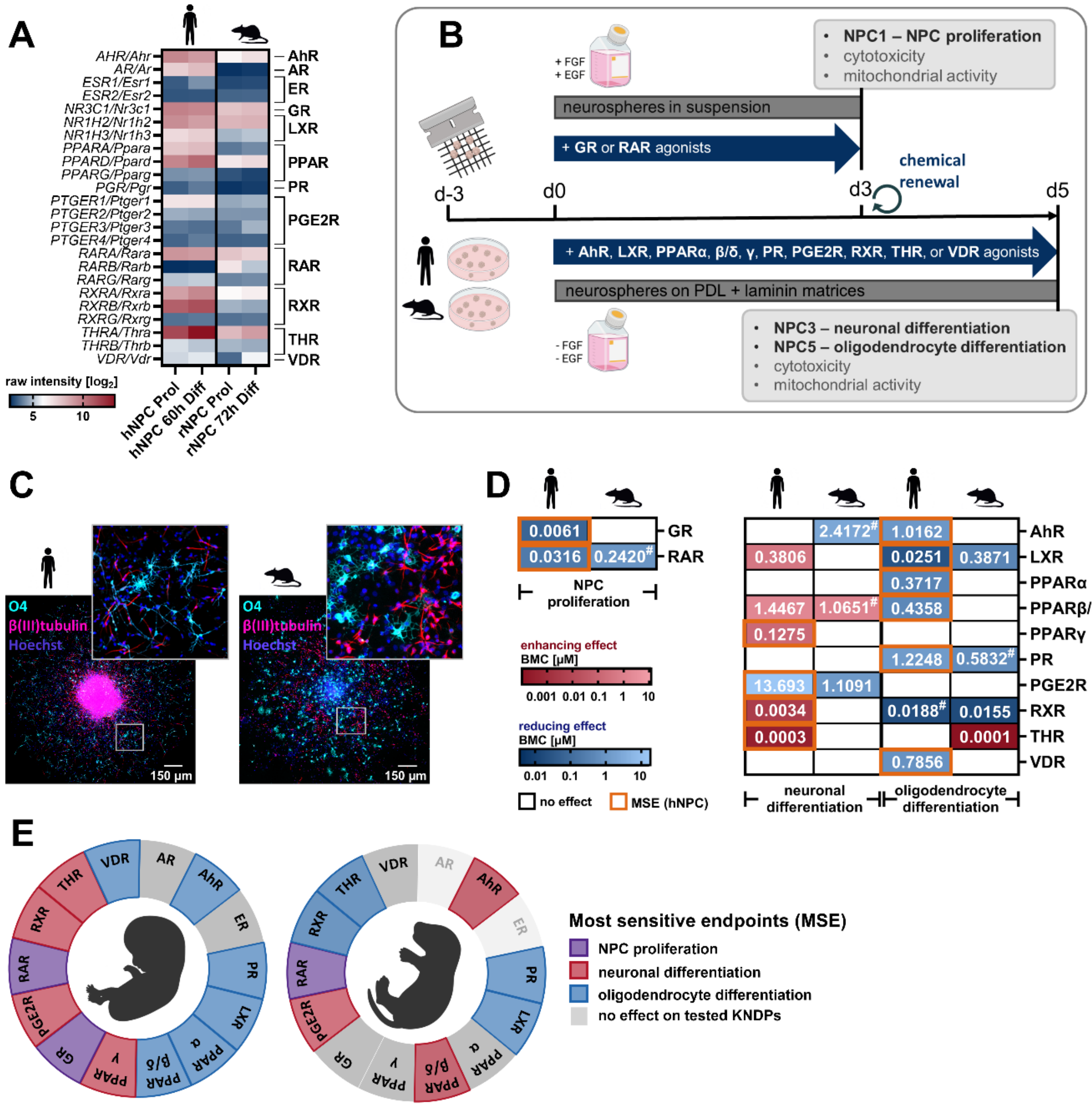


Figure 8: Comparative analysis of HR-sensitive KNDPs between human and rat NPCs. A. HR expression in proliferating (rNPC Prol) and 72 h differentiated rNPCs (rNPC 72h Diff) was compared with expression in proliferating (hNPC Prol) and 60 h differentiated (hNPC 60h Diff) hNPCs. The microarray datasets were previously published in Klose et al. 2021 (hNPCs), and Masjosthusmann et al. 2018 (rNPCs). **B**. Exposure setup: Proliferating post-natal day 1 (PND1) rNPC neurospheres (300 µm diameter) were exposed to 3 serial concentrations of GR and RAR agonists or solvent (0.1 % DMSO) in presence of growth factors for 3 days before the proliferative capacity was assessed by BrdU ELISA. Differentiating hNPCs were exposed to three serial concentrations of AhR,

LXR, PPAR, PR, PGE2, RXR, THR or VDR agonists or solvent (0.1 % DMSO) in absence of growth factors for 5 days. Neuronal differentiation was assessed as the percentage of β(III)tubulin-positive cells (neurons) and oligodendrocyte differentiation as the percentage of O4-positive cells (oligodendrocytes) within the migration area. All raw data were analyzed using the same R-based data analysis pipeline described for the analysis of hNPCs in Figure 1 and in the Methods section. **C**. Representative immunocytochemical stainings of human and rat NPCs differentiated on PDL-laminin matrices for 5 days. Neurons were stained with β(III)tubulin, oligodendrocytes with O4 and nuclei with Hoechst33258. **D**. Summary of the phenotypic effects of HR agonists on the Neurosphere Assay endpoints (NPC proliferation (NPC1), neuronal differentiation (NPC3) and oligodendrocyte differentiation (NPC5)) in hNPCs and rNPCs, expressed as BMCs in micromolar (µM). Only effects at non-cytotoxic concentrations are shown. **E**. Summary of identified MSEs in hNPCs and rNPCs. Agonism of AR and ER was not assessed in rNPCs. **Abbreviations:** AhR, aryl hydrocarbon receptor; AR, androgen receptor; BMC, benchmark concentration; BMR, benchmark response; BrdU, bromodeoxyuridine; EGF, epidermal growth factor; ER, estrogen receptor; FGF, fibroblast growth factor; GR, glucocorticoid receptor; HR, hormone receptor; KNDP, key neurodevelopmental process; LXR, liver X receptor; MSE, most sensitive endpoint; NPC, neural progenitor cell; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; PGE2R, prostaglandin E2 receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; THR, thyroid hormone receptor; VDR, vitamin D receptor.

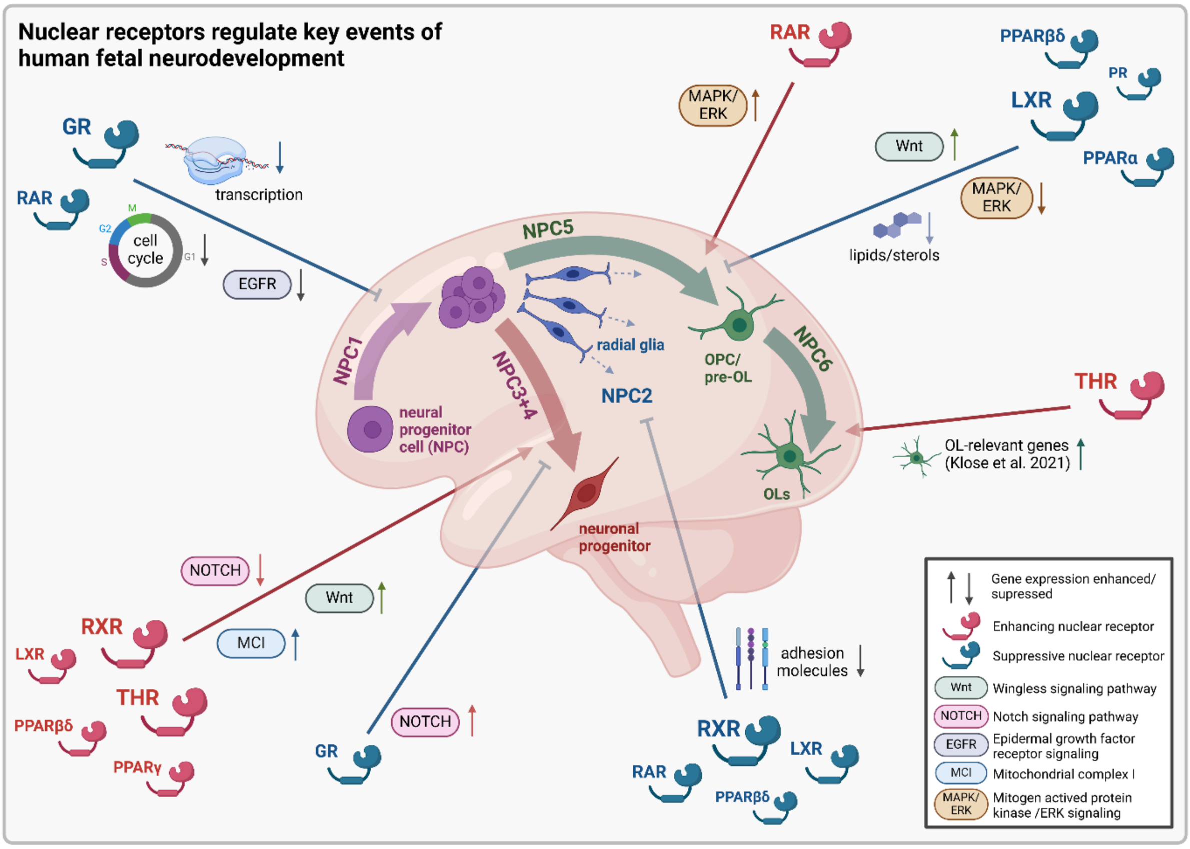
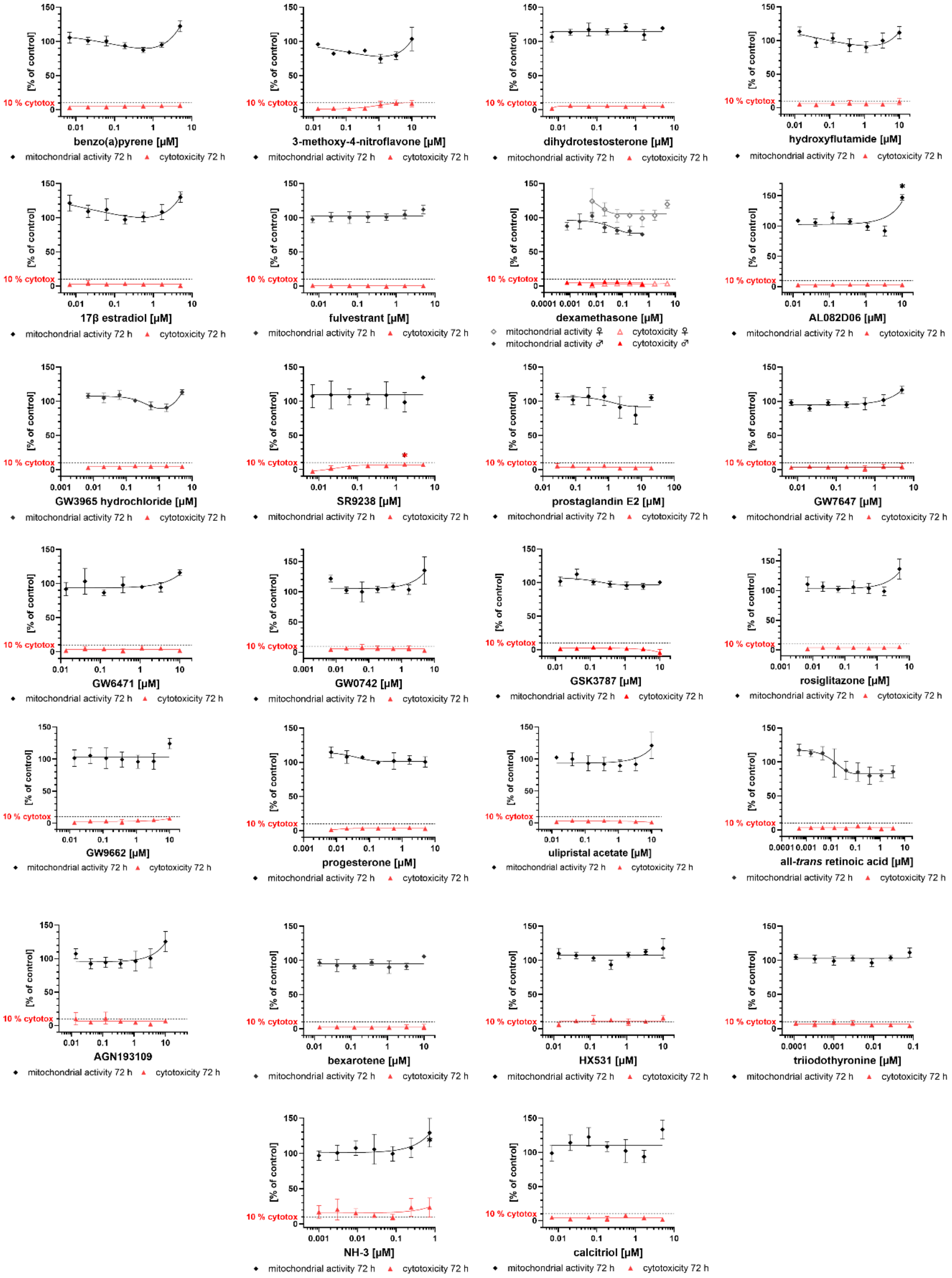
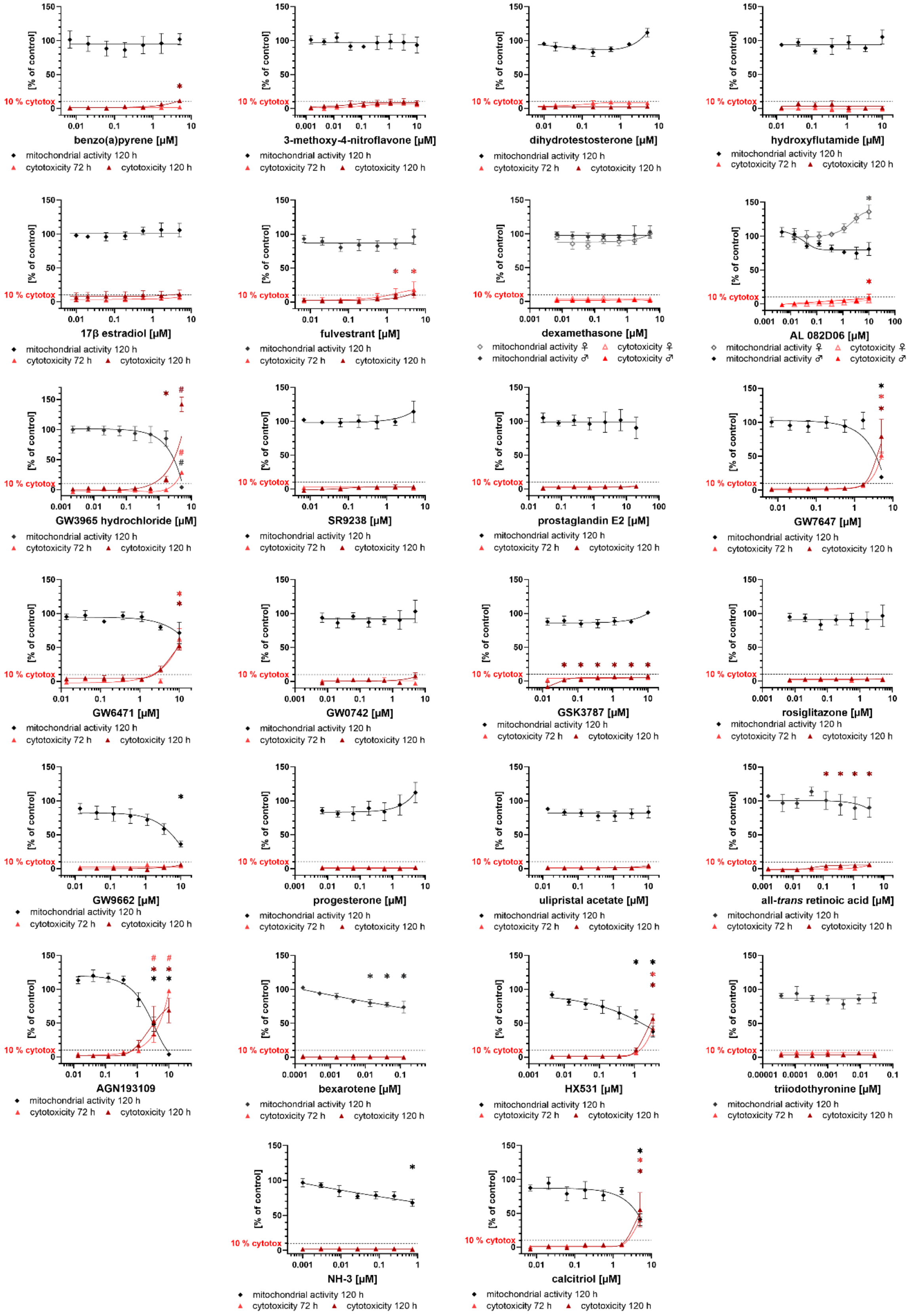


Figure 9: Overview of the putative mechanisms underlying the regulation of key neurodevelopmental processes by HR activation in proliferating and differentiating hNPCs. Hormone receptors whose activation causes an enhancing effect on the respective KNDP are highlighted in red while those whose activation suppresses the KNDP are highlighted in blue. The size of the receptor icon visualizes the sensitivity (lowest BMC) of the KNDP to activation of the respective receptor. Abbreviations: BMC, benchmark concentration; GR, glucocorticoid receptor; KNDP, key neurodevelopmental process; LXR, liver X receptor; NPC, neural progenitor cell; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; THR, thyroid hormone receptor. Created with biorender.com.

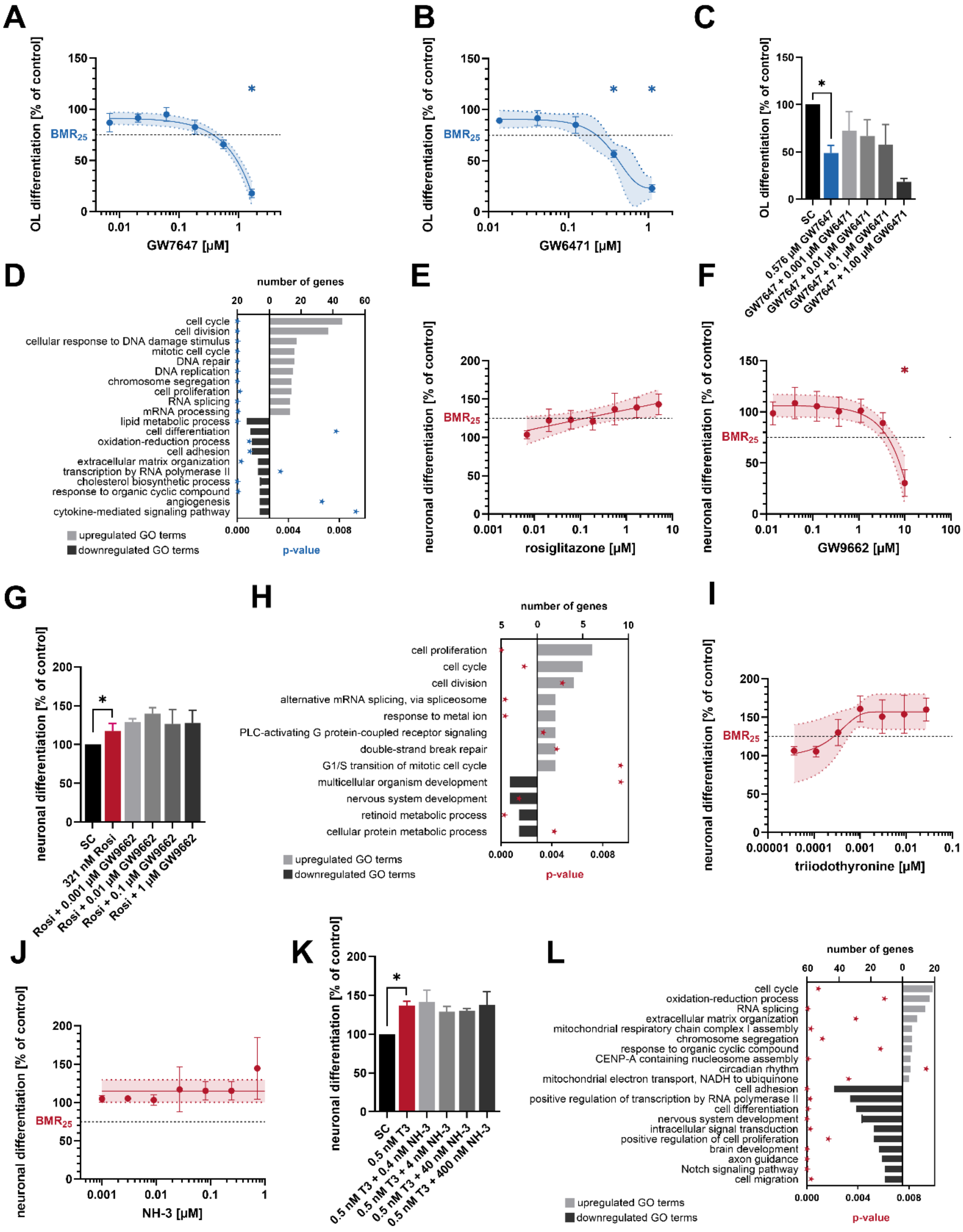


Supplementary Figure 1: HR agonist and antagonist effects in proliferating hNPCs on mitochondrial activity and cytotoxicity. Proliferating hNPCs were exposed to serial concentrations of the HR agonists, antagonists, or solvent (0.1 % DMSO) for 3 days before mitochondrial activity was assessed using the CellTiter-Blue Assay (Promega) and cytotoxicity was assessed using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega). Stratification of data by hNPC sex was performed for the glucocorticoid receptor agonist dexamethasone. *significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to the lowest concentration. All data are expressed as mean ± SEM. Abbreviations: DMSO, dimethyl sulfoxide; hNPCs, human neural progenitor cells; HR, hormone receptor.

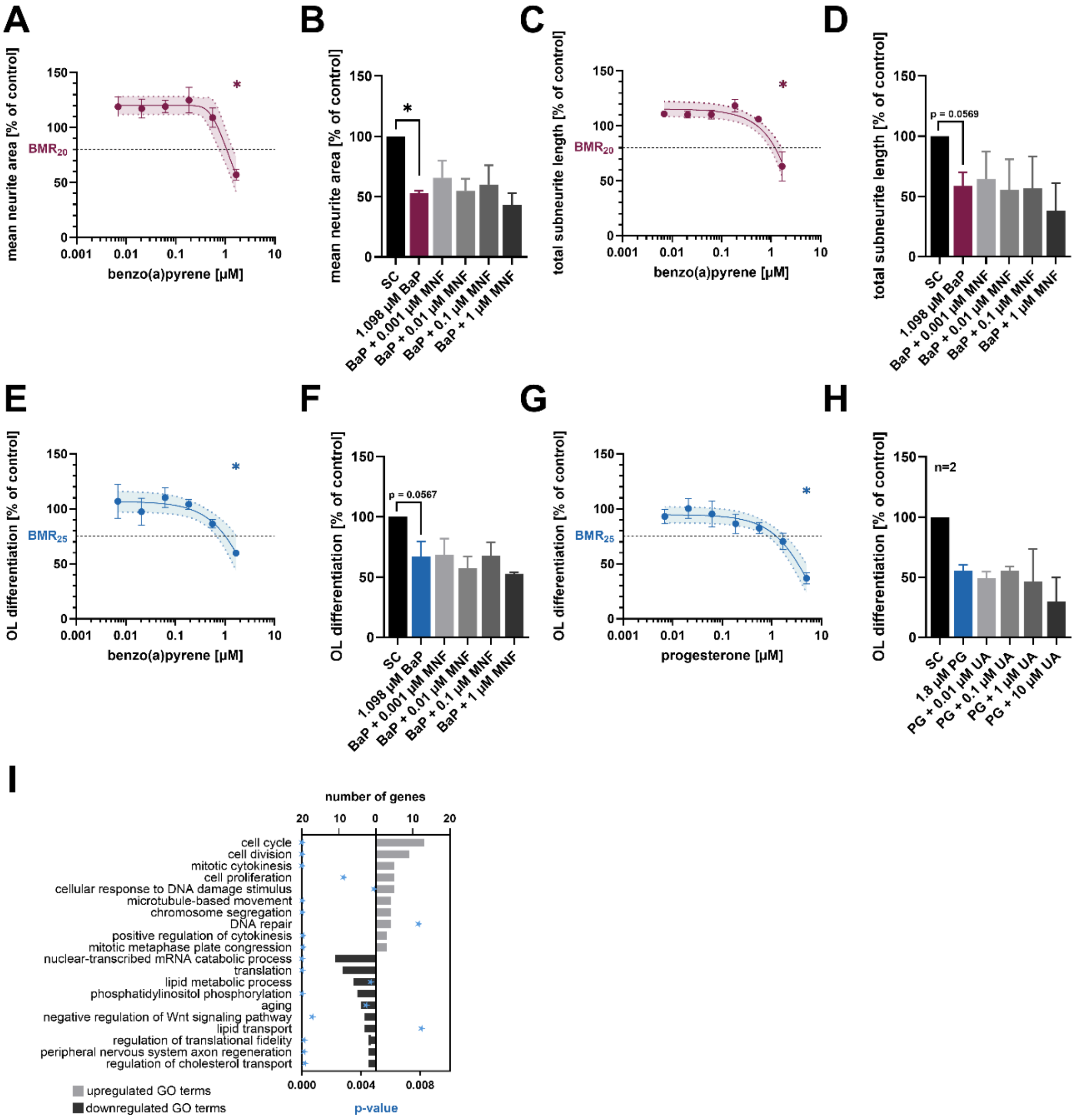


Supplementary Figure 2: HR agonist and antagonist effects in differentiating hNPCs on mitochondrial activity and cytotoxicity. Differentiating hNPCs were exposed to serial concentrations of the HR agonists, antagonists, or solvent (0.1 % DMSO) for before mitochondrial activity was assessed using the CellTiter-Blue

Assay (Promega) after 5 days of exposure and cytotoxicity was assessed using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) after 3 and 5 days of exposure. Stratification of data by hNPC sex was performed for the glucocorticoid receptor agonist dexamethasone and antagonist AL082D06. *significant ($p<0.05$, step-down multiple test procedure of Dunnett and Tamhane) compared to the lowest concentration. All data are expressed as mean ± SEM. Abbreviations: DMSO, dimethyl sulfoxide; hNPCs, human neural progenitor cells; HR, hormone receptor.



Supplementary Figure 3: Effects of PPARα, PPARγ and THR modulation on KNDPs modelled in hNPCs. Depicted are the most sensitive endpoints (MSE) for each of the three hormone receptors. Differentiating hNPCs were exposed to serial concentrations of the PPARα agonist GW7627 (**A**), antagonist GW6471 (**B**), or solvent (0.1 % DMSO) for 5 days before oligodendrocyte differentiation was assessed as the percentage of O4-positive cells (oligodendrocytes) within the migration area. **C**. Differentiating hNPCs were exposed to solvent (0.2 % DMSO), 576 nM GW7647 alone, or in combination with GW6471 (0.001 – 1 µM) for 5 days before oligodendrocyte differentiation was assessed as described for A+B. **D**. Top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in differentiating hNPCs exposed to 576 nM GW7647 for 60 h. Differentiating hNPCs were exposed to serial concentrations of the PPARγ agonist rosiglitazone (**E**), antagonist GW9662 (**F**), or solvent (0.1 % DMSO) for 5 days before neuronal differentiation was assessed as the percentage of TUBB3-positive cells (neurons) within the migration area. **G**. Differentiating hNPCs were exposed to solvent (0.2 % DMSO), 321 nM rosiglitazone alone, or in combination with GW9662 (0.001 – 1 µM) for 5 days before neuronal differentiation was assessed as described for E+F. **H**. Top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in differentiating hNPCs exposed to 321 nM rosiglitazone for 60 h. Differentiating hNPCs were exposed to serial concentrations of the THR agonist T3 (**I**), antagonist NH-3 (**J**), or solvent (0.1 % EtOH/HCl (T3) or DMSO (NH-3)) for 5 days before neuronal differentiation was assessed as described for E+F. **K**. Differentiating hNPCs were exposed to solvent (0.1 % EtOH/HCl (T3) and 0.1 % DMSO), 0.5 nM T3 alone, or in combination with NH-3 (0.4 – 400 µM) for 5 days before oligodendrocyte differentiation was assessed as described for E+F. **L**. Top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in differentiating hNPCs exposed to 0.5 nM T3 for 60 h. For D, H and L: Significant enrichment of DEGs in GO terms was defined by a p-value < 0.01 (colored stars). A complete list of DEGs for each GO term analysis can be found in Sup. File 7. Abbreviations: DEG, differentially expressed genes; DMSO, dimethyl sulfoxide; GO, gene ontology; hNPCs, human neural progenitor cells; KNDP, key neurodevelopmental process; T3, triiodothyronine.



Supplementary Figure 4: Effects of AhR and PR modulation on KNDPs modelled in hNPCs. Depicted are the most sensitive endpoints (MSE) for both hormone receptors. Differentiating hNPCs were exposed to serial concentrations of the AhR agonist benzo(*a*)pyrene (B(*a*)P) or solvent (0.1 % DMSO) for 5 days before neurite outgrowth, measured as the mean neurite area (**A**) and total subneurite length (**C**), or oligodendrocyte differentiation (**E**) were assessed. Differentiating hNPCs were exposed to solvent (0.2 % DMSO), 1.098 µM B(*a*)P alone, or in combination with AHR antagonist MNF (0.001 – 1 µM) for 5 days before neurite outgrowth, measured as the mean neurite area (**B**) and total subneurite length (**D**), or oligodendrocyte differentiation (**F**) were assessed. Differentiating hNPCs were exposed to serial concentrations of the PR agonist progesterone (**G**) or solvent (0.1 % DMSO) for 5 days before oligodendrocyte differentiation was assessed. **H**. Differentiating hNPCs were exposed to solvent (0.2 % DMSO), 1.8 µM progesterone alone, or in combination with the PR antagonist ulipristal acetate (0.01 – 10 µM) for 5 days before oligodendrocyte differentiation was assessed. **I**. Top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in differentiating hNPCs exposed to 1.8 µM progesterone for 60 h. Significant enrichment of DEGs in GO terms was defined by a p-value < 0.01 (blue stars). A complete list of DEGs for each GO term analysis can be found in Sup. File 7. Abbreviations: DEG, differentially expressed genes; DMSO, dimethyl sulfoxide; GO, gene ontology; hNPCs, human neural progenitor cells; KNDP, key neurodevelopmental process; MNF, 3'-methoxy-4'nitroflavone; PR, progesterone receptor.

2.3 The NPC1_RAR_GR assay identifies putative endocrine disrupting chemicals (EDCs) perturbing glucocorticoid receptor (GR) or retinoic acid receptor (RAR) signaling in developing human brain cells

Kevin Schlüppmann, Louisa Merit Stark, Saskia Hüsken, Arif Dönmez, Ellen Fritsche and Katharina Koch

Despite the growing evidence that prenatal exposure to endocrine disrupting chemicals (EDCs) disrupts brain development subsequently causing developmental neurotoxicity (DNT), current testing and assessment for EDCs focuses primarily on androgen, estrogen, thyroid and steroidogenic signaling. Moreover, DNT studies are based on *in vivo* rodent studies with limited predictivity for humans and do not relay information regarding endocrine modes of action. For this purpose, a DNT *in vitro* testing battery (DNT-IVB) has been compiled consisting of *in vitro* test methods modeling KDNPs. Using one of these methods, the Neurosphere assay, we identified retinoic acid receptor (RAR) and glucocorticoid receptor (GR) mediated proliferation arrest in hNPCs by treatment with specific RAR and GR agonists and antagonists. Based on this, we developed the two-tiered NPC1_RAR_GR assay to detect disruptors of RAR and GR signaling. Furthermore, we established synthetic RAR and GR agonists and antagonists as positive controls and Narciclasine and Carbaryl, compounds not interacting with RAR or GR signaling as negative controls. In order to assess the applicability of the NPC1_RAR_GR assay, a list of putative EDCs was compiled and the insecticides p,p'-DDT and Dieldrin as well as their metabolites p,p'-DDE and Aldrin were identified as RAR or GR antagonists whereas the pharmaceutical valproic acid was identified as an RAR or GR agonist. In addition, a component-based mixture approach using a combination of three of the identified antagonists, Dieldrin, Aldrin, and p,p'-DDE, was attempted and revealed lower individual concentrations to be necessary to antagonize RAR or GR signaling. Therefore, the NPC1_RAR_GR assay not only allows for the identification of disruptors of the hormone signaling pathways, but also for a potential mixture-based approach. With this human-based approach, we provide a test assay which not only allows the identification of a cellular response to RAR or GR disruption but also avoid inherent species differences. Thus, the NPC1_RAR_GR assay could prove as a first step into developing methods to reliably identify compounds for their endocrine disruption mediated-DNT potential.

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The NPC1_RAR_GR assay identifies putative endocrine disrupting chemicals (EDCs) perturbing glucocorticoid receptor (GR) or retinoic acid receptor (RAR) signaling in developing human brain cells

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Abstract

Despite the growing evidence that prenatal exposure to endocrine disrupting chemicals (EDCs) disrupts brain development subsequently causing developmental neurotoxicity (DNT), current testing and assessment for EDCs focuses primarily on androgen, estrogen, thyroid and steroidogenic signaling. Moreover, DNT studies are based on *in vivo* rodent studies with limited predictivity for humans and do not relay information regarding endocrine modes of action. For this purpose, a DNT *in vitro* testing battery (DNT-IVB) has been compiled consisting of *in vitro* test methods modeling key neurodevelopmental processes (KDNPs). Using one of these methods, the Neurosphere assay, we identified retinoic acid receptor (RAR) and glucocorticoid receptor (GR) mediated proliferation arrest in human neural progenitor cells (hNPCs) by treatment with specific RAR and GR agonists and antagonists. Based on this, we developed the two-tiered NPC1_RAR_GR assay to detect disruptors of RAR and GR signaling. Furthermore, we established synthetic RAR and GR agonists and antagonists as positive controls and Narciclasine and Carbaryl, compounds not interacting with RAR or GR signaling as negative controls. In order to assess the applicability of the NPC1_RAR_GR assay, a list of putative EDCs was compiled and the insecticides p,p’-DDT and Dieldrin as well as their metabolites p,p’-DDE and Aldrin were identified as RAR or GR antagonists whereas the pharmaceutical valproic acid was identified as an RAR or GR agonist. In addition, a component-based mixture approach using a combination of three of the identified antagonists, Dieldrin, Aldrin, and p,p’-DDE, was attempted and revealed lower individual concentrations to be necessary to antagonize RAR or GR signaling. Therefore, the NPC1_RAR_GR assay not only allows for the identification of disruptors of the hormone signaling pathways, but also for a potential mixture-based approach. With this human-based approach, we provide a test assay which not only allows the identification of a cellular response to RAR or GR disruption but also avoid inherent species differences. Thus, the NPC1_RAR_GR assay could prove as a first step into developing methods to reliably identify compounds for their endocrine disruption mediated-DNT potential.

Introduction

Human brain development is a highly complex and modular process comprising different neurodevelopmental key events, like neural progenitor cell proliferation or effector cell differentiation into neurons and oligodendrocytes (Stiles and Jernigan 2010; Stiles 2017). These key processes are interceded by endocrine signaling pathways and their intricate interplay. While the role of certain hormonal pathways is well understood such as retinoic acid signaling facilitating hindbrain patterning and neural tube formation or thyroid hormone signaling mediating various neurodevelopmental processes, the influence of other endocrine pathways is only insufficiently investigated (Wilson et al. 2003; Bernal 2005, Bernal 2007; Glover et al. 2006; Frank and Sela-Donenfeld 2019; Grignard et al. 2020). Furthermore, disruption of the fine-tuned processes of any endocrines signaling pathways involved in neurodevelopment are implicated in various different neurodevelopmental disorders such as congenital iodine deficiency syndrome, attention deficit hyperactivity disorder (ADHD) or other intellectual disabilities (Rivollier et al. 2019; LaFranchi 2021). Substances interfering with the hormonal systems and subsequently inducing adverse effects in an organism are defined as endocrine disrupting chemicals (EDCs) by the World Health Organization (WHO).

Identifying EDCs has been a rising concern due to severe setbacks through the registration of hazardous chemicals, most notably the approval of diethylstilbestrol (DES) in the 1940s and the subsequent adversities caused by the estrogen-like compound (Patisaul et al. 2018). Current ED assessment focuses primarily on estrogenic, androgenic, thyroid, and steroidogenic (EATS) modalities, mostly disregarding other endocrine signaling pathways such as the retinoid system (OECD 2018a; Browne et al. 2020; Grignard et al. 2020). Moreover, the conceptual framework for testing and assessment of endocrine disrupters covers test guidelines (TG) limited to reproductive effects for estrogen and androgen *in vivo* with the TG 440 (Uterotrophic Assay) or TG 441 (Hershberger Assay), while only TG 426 (Developmental toxicity*)* opens the potential to assess thyroid hormone-dependent perturbations of the developing brain (OECD 2007, OECD 2009, OECD 2018a, OECD 2018b; Andersson et al. 2018). Furthermore, *in vitro* TGs mainly identify receptor binding and activation without testing for effects on a cellular level, thus missing the molecular and cellular link between receptor activation and adverse effects caused by EDCs (Andersson et al. 2018; Grignard et al. 2020). Human health risk assessment through animal studies raises certain challenges due to distinct critical windows of susceptibility or metabolic differences potentially affecting the susceptibility to the tested chemical itself. Moreover, when covering tests for endocrine-disruption (ED), additional issues arise including interspecies variations among the endocrine signaling pathways themselves, which can affect the susceptibility to test compounds, depending on the animal model used (Patisaul et al. 2018). These characteristics become apparent in thyroid hormone signaling with longer T3 and T4 half-life in humans than in rodents or a higher thyroid hormone production in rodents compared to humans (Jahnke et al. 2004).

Similarly, testing and assessment of developmental neurotoxicity (DNT) has been undergoing drastic changes due to the high costs of current DNT guideline studies, a lack of mechanistic information of DNT-inducing chemicals, and severe species differences in brain development and composition in addition to the ethical concerns involved with animal studies (Sachana et al. 2019; Paparella et al. 2020). Therefore, efforts have been made to develop and select different *in vitro* methods covering selected neurodevelopmental key processes to combine them in a DNT-*in vitro* (IVB) battery (Fritsche et al. 2018a; Masjosthusmann et al. 2020; Blum et al. 2023). Based on a guidance document published by the Organization for Economic Co-operation and Development (OECD), the DNT-IVB is intended to facilitate the integration of alternative, new approach methodologies (NAM) into regulatory use to further increase human relevance in health and risk assessment as well as work in concordance with the 3R concept (Russel and Burch 1960; Crofton and Mundy 2021).

Nevertheless, while efforts to close the DNT data gap are advancing, investigation of ED and more precisely ED-mediated DNT (ED-DNT) is still very much in its infancy (Sachana et al. 2019; Lupu et al. 2020). As a consequence, the ENDpoiNTs project, which is part of the Horizon 2020 funded Eurion Cluster, aims to elucidate hormonal dependencies during brain development (Lupu et al. 2020). Moreover, by developing and validating novel test methods capable of identifying putative EDCs the data gap of ED-DNT in regulatory risk assessment ought to be closed. However, validation and especially interlaboratory validation of novel assays is often hindered by insufficient readiness and reproducibility of the test methods. It is therefore of utmost importance to assess the readiness by well-defined criteria and subsequently validate the robustness, reliability and relevance of new test methods (Bal-Price et al. 2018; Bas et al. 2021). Here, we attempt to validate a novel *in vitro* test method detecting RAR and GR disruption in cooperation with PEPPER, a public private platform for the pre-validation of testing methods on endocrine disruptors with the aim to facilitate integration of the assay into regulatory guidelines.

Using the Neurosphere Assay, a scientifically validated assay based on human neural progenitor cells (hNPCs) which allows the depiction of neurodevelopmental key events such as proliferation, migration and effector cell differentiation into neurons and oligodendrocytes, we first assessed hormonal dependencies of these processes (Koch et al. 2022; Koch et al. in preparation). We identified susceptibility of various neurodevelopmental key events to disruption of 12 hormonal pathways with RAR and GR activation reducing hNPC proliferation (Koch et al. in preparation). Based on this information, we developed the NPC1_RAR_GR assay which enables the identification of RAR and GR dependent proliferation arrest in hNPCs, and performed first steps of an interlaboratory validation. After initial setup using synthetic RAR and GR agonists and antagonists, the assay was evaluated for its potential to detect disrupters of both pathways subsequently identifying, amongst others, valproic acid as an RAR or GR agonist and p,p'-DDT (DDT) and Dieldrin as well as their metabolites p,p'-DDE (DDE) and Aldrin as RAR or GR antagonists in the process. Thus, the NPC1_RAR_GR assay will contribute to the emerging efforts of closing the ED-DNT data gap and increase chemical safety and risk assessment using human relevant NAMs.

Material & Methods

Chemicals

Table 1: List of compounds used for the NPC1_RAR_GR assay, including manufacturers, supplier, catalog numbers, solvents, and stock concentrations.

Chemicals	Abbreviation	Cas-Nr.	Manufacturer	Catalog-Nr.	Solvent	Stock [mM]
3,5,6-trichloro-2-pyridinol	TCP	6515-38-4	provided by Prof. Dr. Pim Leonards (VU)		DMSO	101.71
3-phenoxybenzoic acid	3-PBA	3739-38-6	provided by Prof. Dr. Pim Leonards (VU)		DMSO	102.45
4,4-Bisphenol F	BPF	620-92-8	provided by Prof. Dr. Pim Leonards (VU)		DMSO	101.26
AGN193109	AGN	191946-21-7	Merck (Sigma)	SML2034-5MG	DMSO	7.6
AL082D06	AL08	256925-03-8	Biomol (Cayman)	Cay23455-1	DMSO	5
Aldrin	Aldrin	309-00-2	Merck (Sigma)	36666-25MG	DMSO	50
all-*trans* retinoic acid	*at* RA	302-79-4	Merck (Sigma)	R2625-50MG	DMSO	100
Bisphenol A	BPA	1069-66-5	provided by Prof. Dr. Pim Leonards (VU)		DMSO	98.51
BMS493	BMS	215030-90-3	Biomol (Cayman)	Cay17418-1	DMSO	10
Carbaryl	CBR	63-25-2	EFSA (Masjosthusmann et al.2020)		DMSO	20
Chlordane	Chlordane	57-7-9	Merck (Sigma)	40089	Methanol	12.2
Dexamethasone	DEX	50-02-2	Merck (Sigma)	D4902-25MG	DMSO	50
Dieldrin	Dieldrin	60-57-1	Chem Service	RPN-11688-1G	DMSO	100.02
Dimoxystrobin	DMS	149961-52-4	Merck (Sigma)	33499	DMSO	99.975
Diphenylphosphate	DPHP	838-85-7	provided by Prof. Dr. Pim Leonards (VU)		DMSO	104.33
Endrin	Endrin	72-20-8	Merck (Sigma)	32014-100MG	DMSO	20
Fluticasone	Flu	80474-14-2	Biomol (Cayman)	Cay20703-1	DMSO	20
Methylmercury(II) chloride	MeHQ	115-09-3	Merck (Sigma)	442534	DMSO	100.001
Mifepristone	MP	84371-65-3	Merck (Sigma)	M8046-100MG	DMSO	100
Monobenzyl phtalate	MBzP	2528-16-7	provided by Prof. Dr. Pim Leonards (VU)		DMSO	95.77
Monoethyl phtalate	MEP	2306-33-4	provided by Prof. Dr. Pim Leonards (VU)		DMSO	99.11
Narciclasine	NCC	29477-83-6	EFSA (Masjosthusmann et al.2020)		DMSO	50
p,p'-DDE	DDE	72-55-9	Biomol (Cayman)	Cay24241-50	DMSO	50
p,p'-DDT	DDT	50-29-3	Biomol (Cayman)	Cay24243-1	DMSO	20
Tolylfluanid	Tolyl	731-27-1	Merck (Sigma)	32060-250MG	DMSO	20
TTNPB	TTNPB	71441-28-6	Biomol (Cayman)	Cay16144-1	DMSO	5
Tris(2,3-epoxypropyl) isocyanurate	TIC	2451-62-9	Merck (Sigma)	379506	DMSO	100.005
Valproic acid	VPA	1069-66-5	EFSA (Masjosthusmann et al.2020)		H_2O	100

hNPC cell culture

Primary hNPCs isolated from the cortices of gestational week 16-19 fetuses were purchased from Lonza Verviers SPRL, Belgium (#PT-2599). The hNPCs were cultivated as three-dimensional cell aggregates, so called neurospheres in proliferation medium containing a 2:1 ration of DMEM (#31966-021, Thermo Fisher, United States) to Hams F12-nutrient mix (#31765-027, Thermo Fisher, United States) supplemented with 2 % B27 (#17504044, Thermo Fisher, United States), 20 ng/ml epidermal growth factor (EGF) (#PHG0313, Thermo Fisher, United States), 20 ng/ml fibroblast growth factor (FGF) (#233-FB, R&D Systems, United States) and 100 U/ml penicillin and 100 µg/ml streptomycin (#P06-07100, Pan-Biotech, Germany). The Neurospheres were cultivated under standard cell culture conditions at 37 °C and 5 % CO_2 in 10 cm diameter cell culture dishes coated with poly-2-hydroxymethyl methacrylate (Poly-Hema, #P3932, Merck, United States) as described in Koch et al. 2022. Cell were passaged once per week by mechanically splitting the Neurospheres into 0.165 mm*0.165 mm cubicles using a McIlwain tissue chopper (#TC752, Campden Instruments, United Kingdom). Cells were supplied with fresh medium three times a week by replacing half of the cell culture medium (Koch et al. 2022).

Neurosphere based proliferation assay

Proliferative capabilities of hNPCs were measured via bromodeoxyuridine (BrdU) into newly synthesized DNA (Masjosthusmann et al. 2020; Koch et al. 2022). Neurospheres measuring 300 µm in diameter were cultivated for three days under proliferating conditions in 100 µl proliferation medium containing EGF and FGF in a well of a Poly-Hema coated 96-well U-bottom plate with four technical replicates per condition. As an assay specific positive control, neurospheres were cultivated in proliferation medium without EGF and FGF. The cells were treated with the different test compounds listed in table 1 over the whole duration of three days and, depending on the assay setup, in presence of the GR or RAR agonists dexamethasone (DEX) and all-*trans* retinoic acid (a*t*RA) or antagonists mifepristone (MP) and AGN193109 (AGN). The BrdU ELISA (#11669915001, Roche, Switzerland) was performed following the manufacturers' protocol. In addition, assays for mitochondrial activity (CellTiter-Blue Assay (CTB), #G8081, Promega, United States) and cytotoxicity CytoTox-ONE Homogenous Membrane Integrity Assay, #G7891, Promega, United States) were performed in order to distinguish compound specific and cytotoxic effects. A detailed description of the proliferation assay (NPC1) can be found in Masjosthusmann et al. 2020 and Koch et al. 2022.

NPC1_RAR_GR

Assessment of endocrine activity of the test compounds was performed in a two-tiered approach. For the tier 1 experiment, a classical NPC1 proliferation assay was performed, as described in Koch et al. 2022.

Compounds reducing hNPC proliferation in the tier 1 experiment were treated as potential RAR or GR agonists. In an ensuing tier 2 experiment co-treatments of increasing concentrations of the test substance in presence of fixed concentrations of the RAR and GR antagonists AGN (1 µM) and MP (1 µM) were performed. In parallel, a classical concentration-response experiment with the same concentrations of the test compound was performed. The benchmark-concentration 20 (BMC_{20}) of both C-R experiment in presence and absence of the HR antagonists were calculated. A significant increase of the BMC_{20} of the co-treatment compared to the C-R experiment indicates RAR or GR activation.

Compounds not affecting hNPC proliferation in the tier 1 experiment were treated as potential RAR or GR antagonists. In an ensuing tier 2 experiment co-treatments of increasing concentration of the test substance in presence of fixed concentrations of the RAR and GR agonists a*t*RA (0.09 µM) and DEX (0.06 µM) were performed and normalized to a hormone control containing 0.06 µM DEX and 0.09 µM a*t*RA. A significant increase of proliferation assessed by an increased BMC_{50} compared to the RAR and GR agonists indicates RAR or GR inhibition.

Mitochondrial Activity and Cytotoxicity Assays

Cytotoxicity and mitochondrial activity were assessed in parallel to BrdU incorporation in order to discriminate specific compound induced effects from unspecific effects caused by cytotoxicity or reduced mitochondrial activity. After three days cultivation in presence of a test compound, mitochondrial activity was assessed using the Alamar blue assay (CellTiter-Blue Assay (CTB), #G8081, Promega, United States). Cytotoxicity was determined simultaneously by measuring lactate dehydrogenase (LDH) release from cells with damaged membranes (CytoTox-One Homogeneous Membrane Integrity Assay, #G7891, Promega, United States). For the lysis control for both CTB and LDH assay, the lysis solution from the CytoTox-One Assay kit was used. Fluorescence was measured using a Tecan Spark… (ex: 540 nm; em: 590 nm). For the CTB assay, relative fluorescence unit (RFU) values of the replicates were averaged and background correction was performed using wells containing medium without cells. For the LDH assay, RFU values were averages and a dynamic range was calculated setting the solvent control as 0 % and the lysis control as 100 %.

RNA sequencing of human NCPs exposed to RAR and GR agonists

RNA sequencing was performed as described in Koch et al. in prep with a total of 850 proliferating (in presence and absence of GR and RAR agonists) or differentiating hNPC spheres were exposed to BMC_{30} (derived from the MSE) of HR agonists or solvent (0.1 % DMSO) for 60 hours in Poly-Hema-coated or PDL-laminin-coated 6-well plates, respectively. Total RNA was isolated using the RNeasy Mini Kit (#74104, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was sent to BGI Genomics Co., Ltd. (Hong Kong) for RNA sequencing, and reads were mapped to the human reference genome hg38. Four biological replicates were performed for each exposure condition.

Library Preparation: Quality control (QC) of total RNA samples was performed using the Agilent 2100 Bio Analyzer (Agilent RNA 6000 Nano Kit). Subsequently, mRNA was purified and fragmented using oligo(dT)-labeled magnetic beads. After synthesis of the first and second cDNA strands, end repair and "A" base was added to the 3'-end. Adaptor ligands were added and PCR was performed. PCR products were purified using XP beads. QC was again performed on the Agilent 2100 Bio analyzer. Double-stranded PCR products were denatured and circularized by splint oligo-sequencing. The resulting single-stranded circular DNA was formatted as a final library. The library was amplified with phi29 to produce DNA nanoballs (DNB) having more than 300 copies of a molecule. The DNBs were loaded into the patterned nanoarray and single end 50 (pair end 100/150) base reads were generated in the way of combinatorial probe anchor synthesis (cPAS).

Sequence data analysis: First, reads mapping to rRNAs were removed. Next, low quality reads (>40% of base qualities <20), reads with adaptors, and reads with unknown bases (N bases >0.1%) were removed to obtain the clean reads (20M clean reads per sample, BGI software SOAPnuke v1.5.2). These clean reads were saved as FASTQ files. The reads were then mapped to the reference genome (GCF_000001405.39_GRCh38.p13) using the Hierarchical Indexing for Spliced Alignment of Transcripts software (HISAT2, v2.0.4). In addition, novel transcript prediction (StringTie v1.0.4; Cuffcompare v2.2.1; CPC v0.9-r2), SNP & INDEL calling (GATK), and gene splicing detection (rMATS v4.0.2) were performed. Gene expression analysis was performed by mapping clean reads to the reference genome (Bowtie2 v2.2.5) and calculating expression levels using RSEM (v1.2.12). Differentially expressed genes (DEGs) between the HR agonist exposure conditions and the respective solvent were identified using DEseq2 (Kong, L. et al. CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. Nucleic Acids Res. 35, W345-W349 (2007)). DEG criteria of $|\log2(FC)| > 0.486$ and a q-value of < 0.05 were used for target gene and GO-term analysis. Genes with a minimum FPKM of 1 were defined as expressed. Analyses and visualization of transcriptome data were performed using Dr. Tom software (BGI Genomics Co., Ltd.).

Statistics

The experiments for testing and screening were performed in at least one male individual (hNPC donor) due to higher sensitivity of GR activation in male hNPCs (Koch et al. in prep). All experiments were performed in at least 3 independent biological replicates with at least 4 technical replicates. Experiments from different individuals or passage number were termed independent. Results are presented as mean ± SEM (Standard error of mean) unless otherwise indicated.

Benchmark responses (BMR) were defined based on the interpolate variability of the lowest concentration for the non-endocrine C-R experiments, for the co-exposure to the RAR and GR antagonists MP and AGN and for the co-exposure to the RAR and GR agonists a*t*RA and DEX of the whole dataset of $n > 116$ experiments, leading to a BMR_{20} for non-endocrine C-R experiments and Co-exposure treatment with the RAR and GR antagonists. For co-exposure with the RAR and GR agonists a BMR_{50} was determined.

Prediction model: For the calculations of the BMCs for the respective treatment schemes, only concentrations where less than 10 % cytotoxicity was observed were included. Therefore, the BMC_{10} for cytotoxicity was calculated for each compound and exposition scheme and concentrations inducing more than 10 % cytotoxicity were excluded from further analysis. Effects for the non-endocrine proliferation assay were termed significant if one concentration was significantly different from the lowest concentration, as assessed by post hoc Dunnett's test and/or if both the upper and lower limits of the 95 percentiles of C-R curve intersected the BMR_{20}. Receptor agonism was considered, if the upper limit of 95 percentile the non-endocrine C-R curve and the lower limit of the 95 percentile of the co-exposure C-R curve in presence of the RAR and GR antagonists did no intersect. Receptor antagonism was considered if one concentration was significantly different from the lowest concentration, as assessed by post hoc Dunnett's test and/or if both the upper and lower limits of the 95 percentile of the co-exposure C-R curve in presence of the RAR and GR agonists DEX and a*t*RA intersected the BMR_{50}.

Results

RAR and GR activation affect proliferation via differing gene expression patterns

With the emerging needs to close the scientific data gap concerning ED and more specifically ED-DNT, we investigated the effects of modulators of 14 different endocrine signaling pathways in a previous publication (Koch et al in prep). For an initial characterization, RNA sequencing was performed to compare the effects of differentiating hNPCs and proliferating hNPCs treated with RAR or GR agonists (Figure 1).

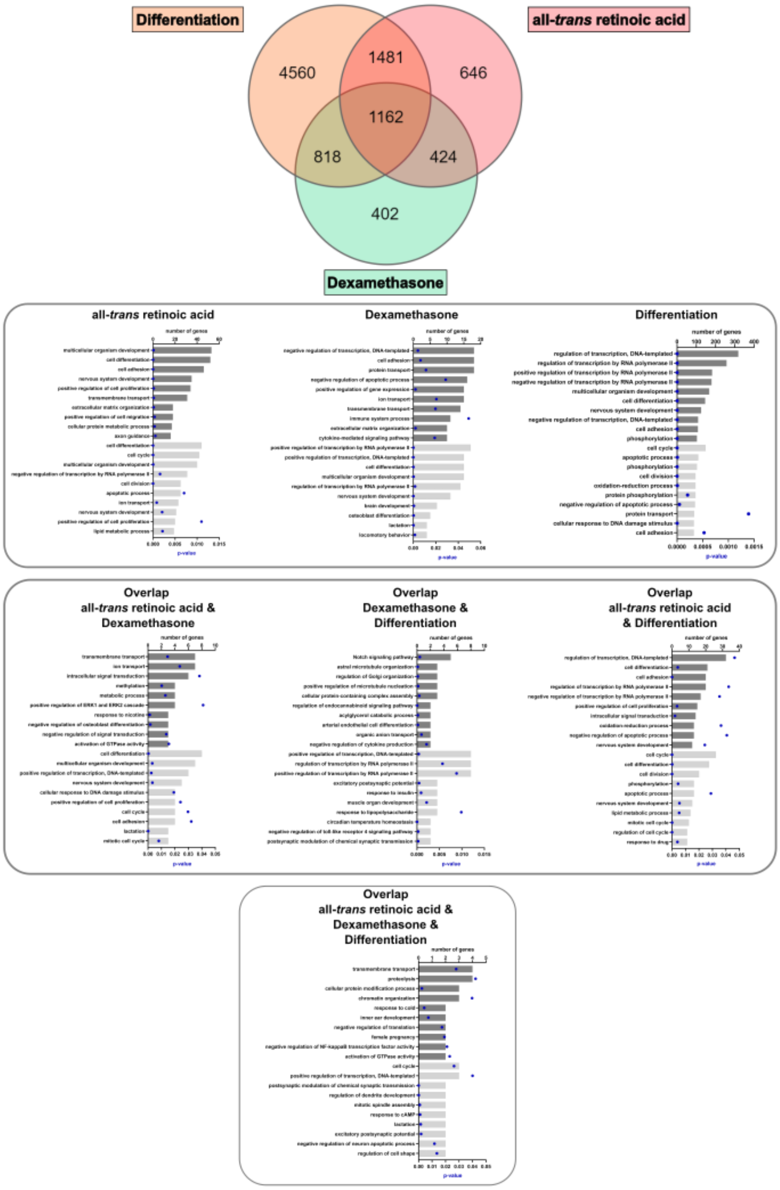


Figure 1: Comparative analysis of GO term enrichment of hNPCs under differentiating conditions and exposure to RAR and GR agonists. GO term mapping of DEGs is shown as the top ten GO terms with the most DEGs that were transcriptionally upregulated (light gray) and downregulated (dark gray) in differentiating hNPCs and proliferating hNPCs exposed to either 0.09 µM a*t*RA or 0.06 µM DEX for 60 hours individually and compared between the three different conditions. Significant enrichment of DEGs in GO terms was defined by a p-value < 0.01 (blue star). A complete list of DEGs for each GO term can be found in the supplemental material (Sup Tab. xyz). Abbreviations: GO, Gene Ontology; DEG, differentially expressed genes; hNPCs, human neural progenitor cells; a*t*RA, all-*trans* retinoic acid; DEX, dexamethasone)

By performing gene ontology (GO) enrichment analysis, we identified upregulation of 52 genes and simultaneous downregulation of 44 genes involved in cell differentiation (i.e., *RARB, RIPOR2, SOX3, PDGFRA, HES5*) after RAR activation by treatment of proliferating hNPCs with 0.09 µM a*t*RA. Moreover, RAR activation caused upregulation of 34 and downregulation of 42 genes mediating cell proliferation and cell cycle (i.e., *RARB, IGFBP6, RARA, FGFR2, CDKN3, CDCA8, FOXM1*) and upregulation of 35 genes regulating nervous system development (i.e., *EPHB2, EGS9)*. On the other hand, GR activation by treatment with 0.06 µM DEX induced gene expression of 18 genes negatively regulating transcription (i.e., *HES7, ZBTB16)* while also downregulating 17 and 15 genes positively associated with transcription (i.e., *HES5, EGR1)*. Lastly, by assessing differentiating hNPCs, we identified upregulation of 318 genes mediating DNA-templated transcription (i.e., *SOX10, FSTL5, HELT, DACH1)* and 257 genes mediating transcription by RNA polymerase II (i.e., *DLX6, DLX2, SOX10, SP9)*. In addition, differentiating conditions induced cell differentiation (i.e., *FGFR3, FRZB)* and nervous system development (i.e., *DCX, HES5, DLX5, DLX6)* by upregulation of 146 and 125 genes respectively and reduced differential expression of 148 cell cycle and 96 cell division-associated genes (i.e., *CDK2, CDKN3, CDKN1A, TACC3)*. Comparing GO terms for the three different conditions, similarities in differentiation and RAR activation become apparent in upregulation of 21 and 32 genes involved in cell differentiation (i.e., *FGFR2, FGFR3, SOX3)* and transcription (i.e., *HEY1, BACH2)* respectively with downregulation of 26, 22, and 16 genes involved in cell cycle (i.e., *CDKN3, FOXM1)*, cell division (i.e., *CDCA3, UBE2C)* and cell differentiation (i.e., *ELF4, CSRP2)*. On the other hand, less overlap between differentiation and GR activation was observed with the upregulation of 5 genes involved in Notch signaling (i.e., *HEY1, NOTCH2NLB)* and downregulation of 8 genes mediating transcription (i.e., *ELF4, EGR1, EGR2)*. Even fewer similarities were observed between RAR and GR activation regarding proliferative and differentiative processes with the downregulation of 8 genes regulation cell differentiation (i.e., *ELF4, HES5, PDGFRA)*. Therefore, while RAR and GR activation seem to be involved in differentiation, both signaling pathways only play a part in neuronal cell differentiation which also greatly differs between RAR and GR activation shown by the small number of genes being differentially regulated after a*t*RA and DEX treatment.

Characterization of RAR and GR disruption in proliferating primary hNPCs

Aside from investigating the effects on a genetic level, we investigated the effects of RAR and GR activation on hNPC proliferation using the Neurosphere assay (Koch et al in prep). For this, hNPCs were treated with different test compounds, including RAR and GR agonists and antagonists over a three-day duration in presence of medium containing growth factors. After three days proliferation was assessed by BrdU incorporation into the DNA. Moreover, in order to exclude unspecific, cytotoxic effects, cytotoxicity assays were performed simultaneously (Figure 2A). Here, we identified an RAR and a GR dependent proliferation arrest after activation of each of these receptors with the physiological RAR ligand a*t*RA reducing proliferation to 57 % of the solvent control (SC) at 0.37 µM (Figure 2B). Moreover, the synthetic glucocorticoid DEX elicited sex specificities where proliferation was reduced to 61 % of the SC at 0.02 µM in male hNPCs while proliferation was reduced to 81 % of the SC at 5 µM in female hNPCs(Figure 2E). Therefore, male individuals are more susceptible to GR activation than female individuals and ensuing experiments were performed solely in male hNPCs. In follow-up experiments to assess if this effect was receptor mediated or the receptor agonists reduce proliferation via a different mode of action, co-treatment experiments with 0.09 µM a*t*RA and increasing concentrations of the RAR antagonist AGN (Figure 2C) and with 0.06 µM DEX and increasing concentrations of the GR antagonist MP (Figure 2F). Both the DEX and the a*t*RA reduced proliferation to 63 % and 72 % of the SC respectively, an effect which was antagonized by MP or AGN shown by a significant increase of proliferation back to 98 % at 10 µM AGN (Figure 2C) and 97 % at 0.1 µM MP (Figure 2F) respectively. Moreover, in order to investigate potential receptor interactions co-treatments with the RAR agonist a*t*RA and increasing concentrations of the GR antagonists MP (Figure 2D) as well as with the GR agonist DEX and increasing concentrations of the RAR antagonists AGN (Figure 2G) were performed. Neither did AGN antagonize the DEX induced effect, nor did MP antagonize the a*t*RA induced effect revealing no interactions between the two signaling pathways. After both confirming receptor specificity of the agonists' effects and excluding receptor interactions, a test setup was designed to investigate the effects of the disruption of both pathways simultaneously (Figure 2H). For this hNPCs were treated with 0.06 µM DEX and 0.09 µM a*t*RA alone and combined. Moreover, co-treatments were performed with a*t*RA, DEX and 1 µM of each of the receptor antagonists AGN and MP alone and in combination. Figure 2G shows the effects of each condition compared to the solvent control. Single compound treatment with a*t*RA and DEX reduced proliferation to 64 % and 71 % while co-exposure of both agonists reduced proliferation to 28 % of the SC. Co-treatments of both agonists and either AGN or partially alleviated the agonists induced effect to 57 % and 58 %, mirroring single agonist treatment levels and treatment with both agonists and antagonists fully alleviated the agonist induced effect back to 86 % of the SC. Therefore, disruption of either RAR or GR signaling as well as disruption of both signaling pathways can be detected in this test system using a similar setup.

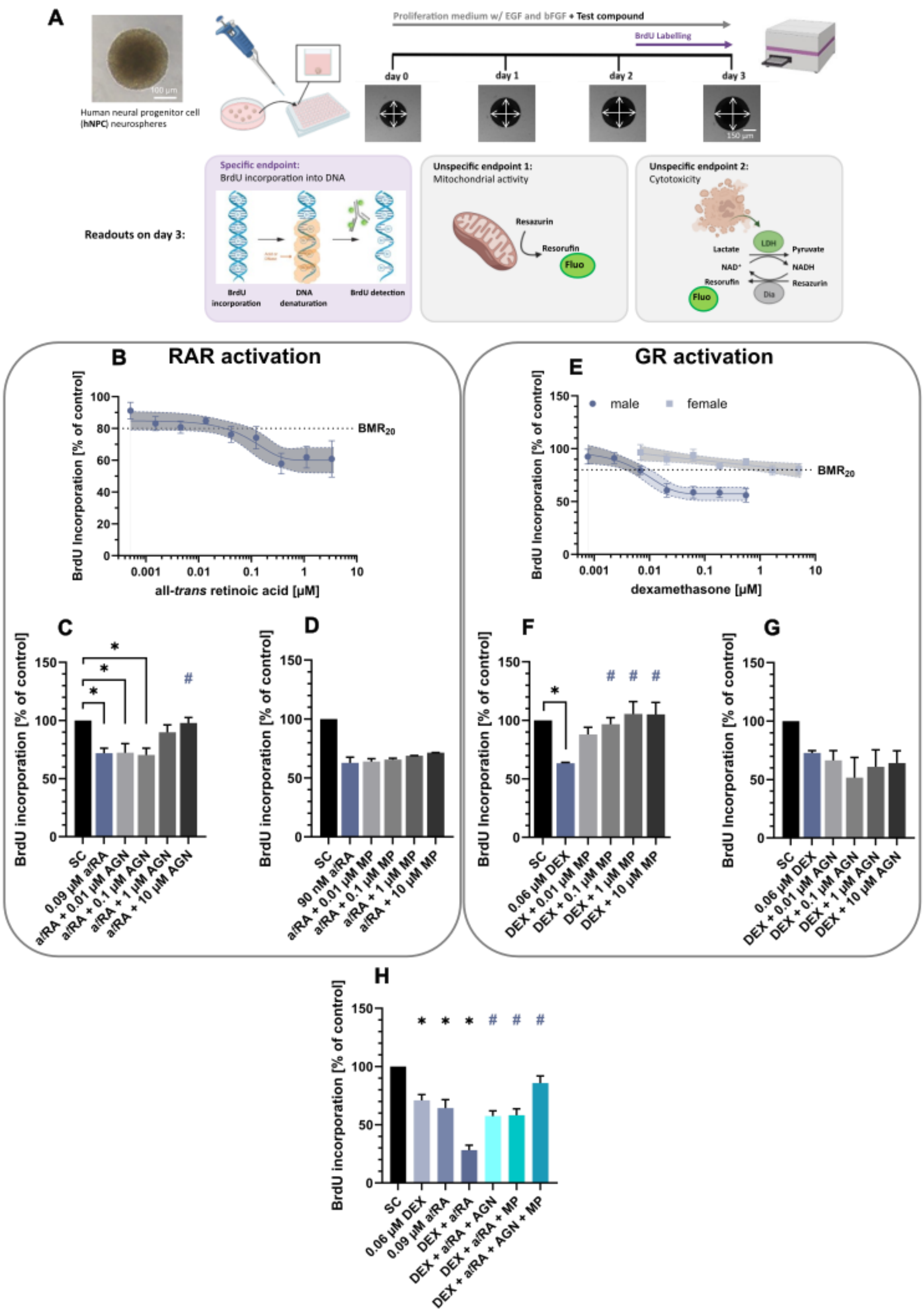


Figure 2: Characterization of RAR and GR dependent proliferation arrest. **A**. Exposure setup: Proliferating hNPC neurospheres were mechanically chopped for a diameter of 300 µm at the day of the experiments. hNPCs were exposed to the RAR or GR agonists *at*RA and DEX alone or in combination with the RAR or GR antagonists AGN and MP in presence of growth factors (EGF and FGF) for three days before hNPC proliferation was assessed by BrdU ELISA. In addition, mitochondrial activity and cytotoxicity were assessed by performing Alamar Blue and LDH release assays. **B.** Proliferating hNPCs were exposed to serial dilution of the RAR agonist all-*trans* retinoic acid (*at*RA) or solvent (0.1 % DMSO) for three days before proliferation was assessed by BrdU ELISA presented in % of the solvent control (SC). Proliferating hNPCs were exposed to a solvent (0.2 % DMSO), 0.09 µM *at*RA alone, or *at*RA in combination with increasing concentrations of **C.** the RAR antagonist AGN193109 (AGN, 0.01 – 10 µM) or **D.** the GR antagonist mifepristone (MP, 0.01 – 10 µM) for three days before proliferation was assessed by BrdU ELISA presented in % of SC. *Significant ($p<0.05$, two-tailed Student t-test) compared to the solvent control. #Significant ($p<0.05$, one-way ANOVA followed by post-hoc Dunnett's test) compared to 0.09 µM *at*RA. **E.** Proliferating hNPCs were exposed to serial dilution of the GR agonist dexamethasone (DEX) or solvent (0.1 % DMSO) for three days before proliferation was assessed by BrdU ELISA presented in % of the SC. Stratification of data by hNPC sex was performed Male proliferating hNPCs were exposed to a solvent (0.2 % DMSO), 0.06 µM DEX alone, or DEX in combination with increasing concentrations of **F.** the GR antagonist MP (0.01 – 10 µM) or **G.** the RAR antagonist AGN (0.01 – 10 µM)) for three days before proliferation was assessed by BrdU ELISA presented in % of SC. *Significant

($p<0.05$, two-tailed Student t-test) compared to the solvent control. #Significant ($p<0.05$, one-way ANOVA followed by post-hoc Dunnett's test) compared to 0.06 µM DEX. **H.** Male proliferating hNPCs were exposed to solvent (0.3 % DMSO), 0.09 µM a*t*RA, 0.06 µM DEX, 1 µM AGN, 1 µM MP alone or in combination for three days before proliferation was assessed by BrdU ELISA presented in % of SC. *Significant ($p<0.05$, two-tailed Student t-test) compared to the solvent control. #Significant ($p<0.05$, two-tailed Student t-test) compared to the combined 0.09 µM a*t*RA and 0.06 µM DEX treatment. All data (B-H) are expressed as mean ± SEM. Abbreviations: RAR, retinoic acid receptor; GR, glucocorticoid receptor; hNPC, human neural progenitor cells; a*t*RA, all-*trans* retinoic acid; DEX, dexamethasone; AGN, AGN193109; MP, mifepristone; EGF, epidermal growth factor; FGF, fibroblast growth factor; BrdU, bromodeoxyuridine; LDH, lactate dehydrogenase; DMSO, dimethyl sulfoxide.

Establishment of positive and negative controls for the NPC1_RAR_GR assay

A two-tiered approach was designed in order to identify ED-DNT compounds interfering with RAR or GR signaling (Figure 3A). In a tier 1 experiment, a non-endocrine proliferation assay is performed. Substances reducing proliferation are treated as putative RAR or GR agonists and in a tier 2 co-exposure experiment, the test compound is challenged with the receptor antagonists AGN and MP to test if the antagonists can rescue the compound induced endophenotype. Rescued proliferation corresponding to an increased BMC_{20} identifies the test compound as an RAR or GR agonist while no rescue reveals a different non-endocrine mode of action (MoA). On the other hand, substances not affecting proliferation in the tier 1 experiment are treated as putative RAR or GR antagonists and challenged with the hormone receptor (HR) agonists DEX and a*t*RA in the tier 2 co-exposure experiment to test if the test compound antagonizes the HR induced endophenotype. Increased proliferation by reaching BMC_{50} or by a significant increase compared to the lowest tested concentration identifies the test compound as an RAR or GR antagonist while no rescue reveals the compound to not disrupt RAR or GR signaling.

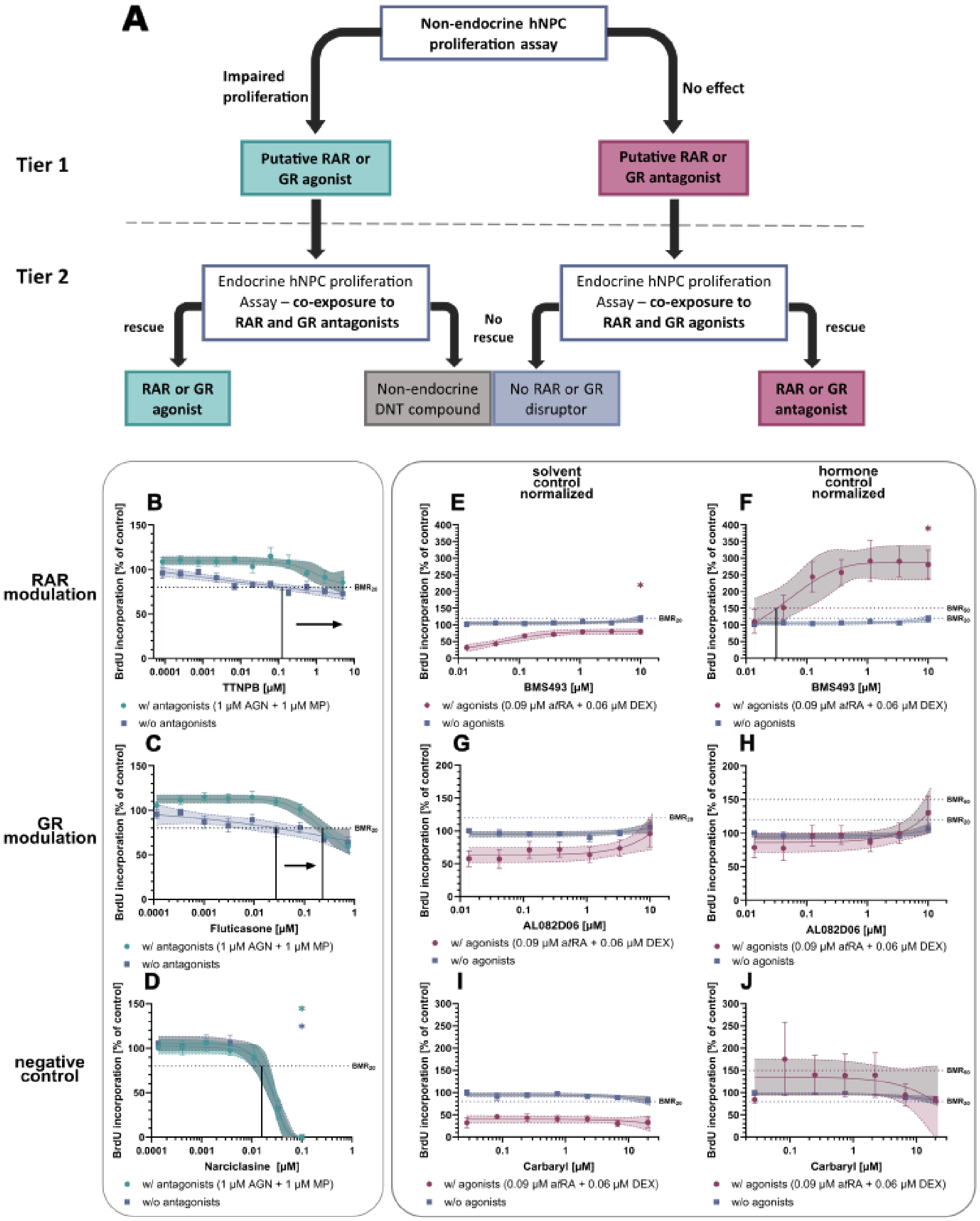


Figure 3: Two-tiered workflow for the NPC1_RAR_GR assay was established with positive controls for RAR and GR modulation as well as specific negative controls. A. Following a tier 1 non-endocrine hNPC proliferation assay a tier 2 endocrine hNPC proliferation assay is performed. Substances impairing proliferation in the tier 1 experiment are tested as putative RAR or GR agonists in presence of RAR and GR antagonists. Substances not affecting proliferation in the tier 1 experiment are tested as putative RAR or GR antagonists in presence of RAR and GR agonists. Proliferating hNPCs were exposed to serial dilution of **B.** the synthetic RAR agonist TTNPB **C.** the synthetic GR agonist Fluticasone or **D.** the negative control Narciclasine alone in a non-endocrine setup (blue-grey curve) or in presence of the synthetic RAR and GR antagonists AGN193109 (AGN, 1 µM) and mifepristone (MP, 1 µM) (cyan curve) for three days before proliferation was assessed by BrdU ELISA presented in % of the solvent control (SC) for the non-endocrine setup or in % of the antagonist control containing 1 µM AGN and 1 µM MP. Proliferating hNPCs were exposed to serial dilution of **E+F.** the synthetic RAR antagonist BMS493 **G+H.** the synthetic GR antagonist AL082D06 or **I+J.** the negative control Carbaryl alone in a non-endocrine setup (blue-grey curve) or in presence of the synthetic RAR and GR agonists all-*trans* retinoic acid (a*t*RA, 0.09 µM) and dexamethasone (DEX, 0.06 µM) (red curve) for three days before proliferation was assessed by BrdU ELISA presented in % of the SC for the non-endocrine setup and the co-exposure to the agonists (**E+G+I**) or in % of the SC for the non-endocrine setup or in % the agonist control containing 0.09 µM a*t*RA and 0.06 µM DEX (**F+H+J**). *Significant ($p<0.05$, one-way ANOVA followed by post-hoc

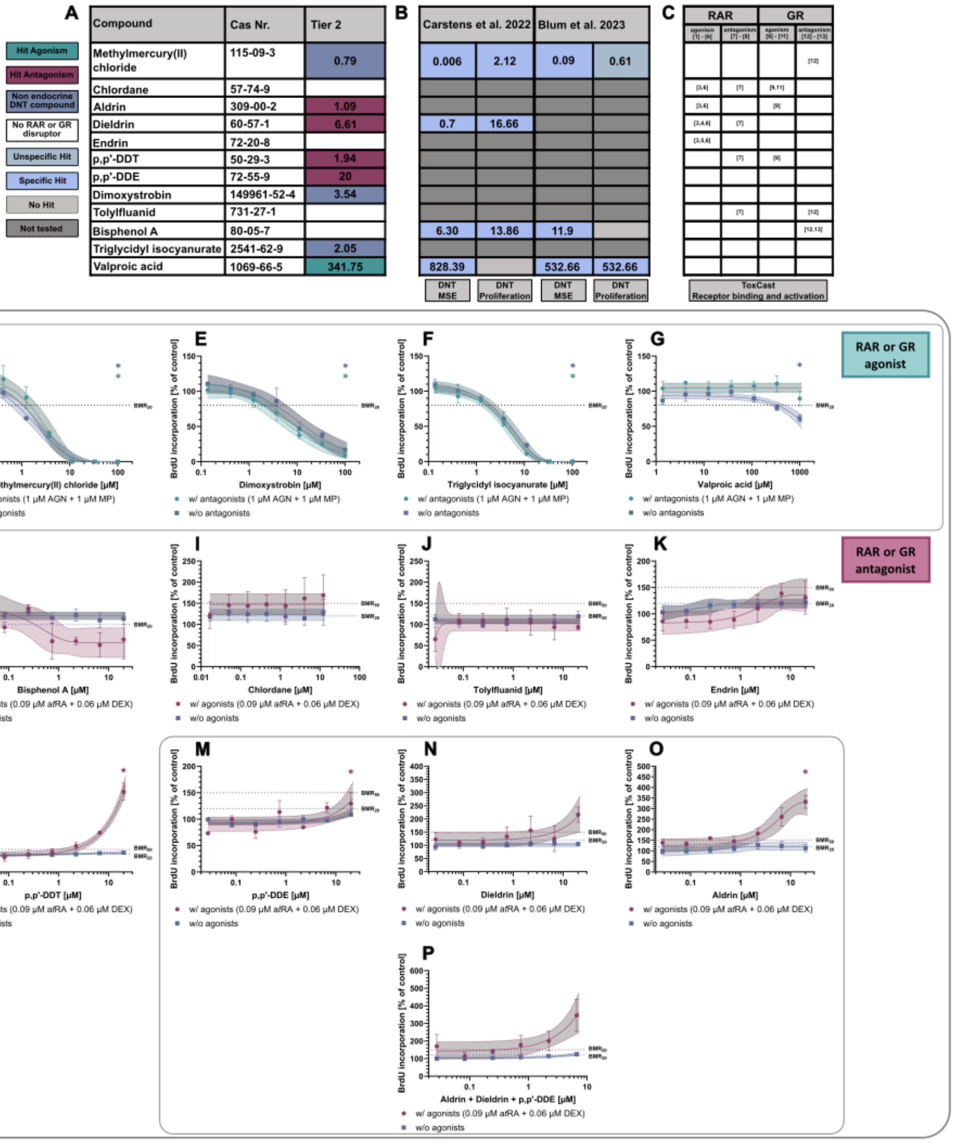
Dunnett's test). All data (B-J) are expressed as mean ± SEM. Abbreviations: RAR, retinoic acid receptor; GR, glucocorticoid receptor; hNPC, human neural progenitor cells; AGN, AGN193109; MP, mifepristone; BrdU, bromodeoxyuridine; SC, solvent control; a*t*RA, all-*trans* retinoic acid; DEX, dexamethasone).

In line with this two-tiered approach a set of positive and negative controls for each approach was established with the synthetic retinoid TTNPB (Figure 3B) and the synthetic glucocorticoid Fluticasone (Figure 3C) as positive controls for GR and RAR agonism respectively. In both cases the compound reduced proliferation to 70 % of the SC in the non-endocrine proliferation assay (grey-blue curve), whereas the co-exposure to the GR and RAR antagonists MP and AGN caused a shift of the curve and an increase of the BMC_{20} from 0.0008 µM to 0.2 µM for Fluticasone and from 0.035 µM to above the tested concentrations for TTNPB. In addition, Narciclasine, an alkaloid found in Amarrylidaceae, was established as the negative control for RAR and GR agonism. Narciclasine reduces hNPC proliferation in the tier 1 experiment and is not affected by co-exposure to MP and AGN with the BMC_{20} staying at 0.015 µM (Figure 3D). Two approaches were compared for receptor antagonism either normalizing both non-endocrine proliferation assay and co-exposure experiments to the receptor agonists to the SC (Figure 3 E+G+H) or normalizing the non-endocrine proliferation assay to the SC and the co-exposure experiment to the hormone control (HC) containing 0.09 µM a*t*RA and 0.06 µM DEX (Figure 3F+H+J).

For receptor antagonism the synthetic RAR antagonist BMS493 (Figure 3E+F) and the synthetic GR antagonist AL082D06 (AL08) (Figure 3G+H) were established. Both compounds did not affect proliferation in the tier 1 experiment. Co-exposure of BMS493 and the agonists caused a significant increase in proliferation from 32 % in lowest concentration to 79 % SC normalized (Figure 3E) as well as a significant increase compared to lowest concentration with a BMC_{50} of 0.032 µM HC normalized (Figure 3F). AL08 on the other hand increased proliferation from 58 % to 96 % of the SC (Figure 3G) and increased proliferation compared to the lowest concentration to 130 % of the HC at 10 µM (Figure 3H). In addition, the carbamate insecticide Carbaryl was established as the negative control for RAR and GR antagonism (Figure 3I+J). Carbaryl neither affected proliferation in the tier 1 experiment nor did it alleviate the agonist induced effect in the tier 2 experiment. Therefore, the establishment of controls for both mode and receptors confirmed the capacity of the NPC1_RAR_GR assay to identify RAR and GR agonists and antagonists and distinguish these from non-RAR and GR disruptors Moreover, for future assessment, co-exposure experiments are normalized to the HC for antagonism.

Compound screening and potential additional value of the NPC1_RAR_GR to the DNT-IVB

After the establishment of our test assay, a list of 12 putative EDCs disrupting RAR or GR signaling was compiled and tested (Figure 4A). The test compounds are comprised of putative EDCs which were identified as hits in at least 50 % of receptor binding and transactivation assays for RAR or GR agonism or antagonism included in the ToxCast data bank (Figure 4C). In addition, DDE was selected as a known EDC and metabolite of DDT and valproic acid, triglycidyl-isocyanurate, and dimoxystrobin were added to the list to assess whether these non-endocrine DNT compounds act as EDCs.



Compound	Cas Nr.	Tier 2	Carstens et al. 2022 DNT MSE	Carstens et al. 2022 DNT Proliferation	Blum et al. 2023 DNT MSE	Blum et al. 2023 DNT Proliferation	RAR agonism [1] - [6]	RAR antagonism [7] - [8]	GR agonism [9] - [11]	GR antagonism [12] - [13]
Methylmercury(II) chloride	115-09-3	0.79	0.006	2.12	0.09	0.61				[12]
Chlordane	57-74-9						[3,6]	[7]	[9,11]	
Aldrin	309-00-2	1.09					[3,6]		[9]	
Dieldrin	60-57-1	6.61	0.7	16.66			[3,4,6]	[7]		
Endrin	72-20-8						[3,5,6]			
p,p'-DDT	50-29-3	1.94						[7]	[9]	
p,p'-DDE	72-55-9	20								
Dimoxystrobin	149961-52-4	3.54								
Tolylfluanid	731-27-1							[7]		[12]
Bisphenol A	80-05-7		6.30	13.86	11.9					[12,13]
Triglycidyl isocyanurate	2541-62-9	2.05								
Valproic acid	1069-66-5	341.75	828.39		532.66	532.66				

Figure 4: Analysis of putative endocrine disrupting chemicals using the NPC1_RAR_GR assay revealed different environmental compounds to disrupt RAR and GR signaling. A. 12 different compounds were tested in the NPC1_RAR_GR assay with numbers corresponding to concentrations in µM for either reducing hNPC proliferation by 20 % for the non-DNT compounds and the substances identified as RAR or GR agonists (Hit agonism) or increasing proliferation significantly or by 50 % for the substances identified as RAR or GR antagonists (Hit antagonism). **B.** shows the results of compounds tested in the DNT NAM battery (Carstens et al. 2022) and the DNT IVB (Blum et al. 2023) with the concentrations corresponding to the most sensitive endpoint (MSE) identified in either battery as well as the concentration reducing proliferation by 20 %. **C.** shows RAR and GR binding and transactivation assays which revealed activity for the respective compound. The assay numbered [1] – [6] assess RAR agonism. [7] – [8] assess RAR antagonism. [9] – [11] assess GR agonism and [12] – [13] asses GR antagonism. Proliferating hNPCs were exposed to serial dilution of **D.** methylmercury(II) chloride, **E.** dimoxystrobin, **F.** triglycidyl isocyanurate, or **G.** valproic acid alone in a non-endocrine setup (blue-grey curve) or in presence of the synthetic RAR and GR antagonists AGN193109 (AGN, 1 µM) and mifepristone (MP, 1 µM) (cyan curve) for three days before proliferation was assessed by BrdU ELISA presented in % of the solvent control (SC) for the non-endocrine setup or in % of the antagonist control containing 1 µM AGN and 1 µM MP. Proliferating hNPCs were exposed to serial dilution of **H.** Bisphenol A, **I.** chlordane, **J.** tolylfluanid, **K.** endrin, **L.** p,p'-DDT, **M.** p,p'-DDE, **N.** dieldrin, **O.** aldrin, or **P.** a combination of p,p'-DDE, dieldrin, and aldrin alone in a non-endocrine setup (blue-grey curve) or in presence of the synthetic RAR and GR agonists all-*trans* retinoic acid (*at*RA, 0.09 µM) and dexamethasone (DEX, 0.06 µM) (red curve) for three days before proliferation was assessed by BrdU ELISA presented in % of the SC for the non-endocrine setup or in % the agonist control containing 0.09 µM *at*RA and 0.06 µM DEX. *Significant ($p<0.05$, one-way ANOVA followed by post-hoc Dunnett's test). All data (B-N) are expressed as mean ± SEM. [1] TOX21_RAR_LUC_Agonist; [2] NVS_NR_hRARa_Agonist; [3] ATG_DR5_RAR_CIS; [4] ATG_RARa_TRANS; [5] ATG_RARb_TRANS; [6] ATG_RARg_TRANS; [7] TOX21_RAR_LUC_Antagonist; [8] NVS_NR_hRAR_Antagonist; [9] TOX21_GR_BLA_Agonist_ratio; [10] ATG_GRE_CIS; [11] ATG_GR_TRANS; [12] TOX21_GR_BLA_Antagonist_ratio; [13] NVS_NR_hGR. Abbreviations:

RAR, retinoic acid receptor; GR, glucocorticoid receptor; hNPC, human neural progenitor cells; DNT, developmental neurotoxicity; MSE, most sensitive endpoint; AGN, AGN193109; mifepristone, MP; BrdU, bromodeoxyuridine; SC, solvent control; DDT, dichlorodiphenyltrichloroethane; DDE, dichlorodiphenyldichloroethylene; a*t*RA, all-*trans* retinoic acid; DEX, dexamethasone)

Among the test compounds, four substances, methylmercury, dimoxystrobin, triglycidyl-isocyanurate and valproic acid, reduced hNPC proliferation in the non-endocrine proliferation assay and were thus tested for RAR and GR agonism (Figure 4D-G). Methylmercury reduced proliferation concentration dependently with a BMC_{20} of 0.79 µM, an effect which was not alleviated by co-exposure to the receptor antagonists (Figure 4D). Moreover, methylmercury induced cytotoxicity in the tier 1 and tier 2 experiments at 4.8 µM and 9.2 µM respectively. Furthermore, dimoxystrobin and triglycidyl isocyanurate reduced proliferation to 38 % and 23 % of the SC in the tier 1 experiment respectively (Figure 4E+F). In both cases, the anti-proliferative effects were not antagonized in the tier 2 experiments. Therefore, methylmercury, dimoxystrobin and triglycidyl-isocyanurate were identified as non-endocrine DNT compounds. On the other hand, valproic acid was identified as an RAR or GR agonist. While valproic acid significantly reduced proliferation to 60 % at 1000 µM with a BMC_{20} of 342 µM (Figure 4G), co-exposure with the RAR and GR antagonists caused a shift of the curve with valproic acid not reducing proliferation and thus revealing that the valproic acid induced effect is RAR or GR mediated. The compounds Bisphenol A, Chlordane, Tolylfluanid, and Endrin neither affected proliferation in the tier 1 nor in the tier 2 experiments therefore identifying these compounds as no RAR or GR disruptors (Figure 4 H-K). On the other hand, we were able to identify the insecticide DDT and its metabolite DDE as RAR or GR antagonists (Figure 4L+M). Neither DDT nor DDE reduced proliferation in the tier 1 non-endocrine proliferation experiment. Co-exposure of DDT with the RAR and GR agonists caused a significant increase in proliferation to 532 % compared to the HC with a BMC_{50} of 1,94 µM (Figure 4L). On the other hand, DDE significantly increased proliferation to 129 % of the hormone control in the highest concentration (Figure 4M). Furthermore, the insecticide Dieldrin and its metabolite Aldrin did not impair proliferation in the tier 1 C-R experiment (Figure 4N+O). Co-exposure of the RAR and GR agonists with Dieldrin increased proliferation to 217 % of the HC with a BMC_{50} of 6.6 µM (Figure 4N) whereas co-exposure of the receptor agonists with Aldrin significantly increased proliferation to 332 % of the HC with a BMC_{50} of 1.09 µM (Figure 4O). While Dieldrin and Aldrin impaired RAR or GR signaling, the stereoisomer of Dieldrin, Endrin did not affect RAR or GR signaling, hinting at steric differences in the chemicals structure that allow or disallow binding and subsequently disruption of RAR or GR signaling. Therefore, by identifying agonists and antagonists of RAR or GR signaling we were able to show, that the NPC1_RAR_GR assay is capable of identifying environmental substances which disrupt RAR or GR signaling as a secondary, non-intended MoA. In addition, we compared the test set of this study with previous applications of the DNT-IVB (Blum et al. 2023) and the DNT NAM battery (Carstens et al. 2022) to identify potential additional value for identifying DNT compounds using the NPC1_RAR_GR assay (Figure 4B). While each compound tested in this study and at least one of the two previous studies was already identified as a DNT compound, information on eight compounds is still missing. Substances assessed for RAR or GR agonism were identified to reduce proliferation in this study and the DNT IVB at similar concentrations. Moreover, the concentrations necessary to induce any DNT effect in the previous studies were lower than the concentration causing ED-DNT in this study, with for example Dieldrin causing a DNT effect at 0.7 µM in the DNT NAM battery compared to the 6.61 µM which were necessary to antagonize RAR or GR signaling in this study (Figure 4B). Therefore, in order to assess the additional value given by identifying RAR and GR antagonism in the NPC1_RAR_GR assay regarding a purely DNT focused approach, the antagonists have to be tested in both the DNT-IVB and DNT NAM battery. However, comparing the results with the RAR and GR binding and transactivation assays from the ToxCast Data bank, we were able to identify DDE and valproic acid as RAR or GR disruptors thus elucidating the necessity to not only test for receptor activity but also assess different key processes such as KDNPs to adequately identify EDCs (Figure 4C). Furthermore, to provide a suitable data set to compare and discuss additional value aside from the identification of an endocrine mode of action will require the testing of a larger set of chemicals. In addition, a component-based mixture approach was attempted using a combination of Dieldrin, Aldrin, and DDE (Figure 4P). The mixture caused a small, not significant increase of proliferation in the tier 1 C-R experiment. Co-exposure of the RAR and GR agonists with the mixture increased proliferation to 346 % of the hormone control with a BMC_{50} of 0.296 µM of each compound. Therefore, in this mixture approach, a combined effect was observed decreasing the concentration necessary to antagonize the hormone induced effects, showing the possibility of a mixture-based approach in the NPC1_RAR_GR assay.

The NPC1_RAR_GR assay is more suited for hazard than for risk assessment purposes

In addition to the initial compound screening, an approach for risk assessment using a mixture of putative EDCs in concentrations comparable to prenatal blood concentrations which were measured within the Swedish SELMA birth cohort study (Svensson et al. 2021), was undertaken. Here both the mixture of seven environmental compounds and each compound individually were tested with the third lowest concentration corresponding to the concentrations measured in the study being 0.27 nM Bisphenol A, 0.003 nM 4,4-bisphenol F, 4.3 nM monoethyl phthalate (MEP), 4.8 nM monobenzyl phthalate (MBzP), 0.36 nM diphenylphosphate, 0.6 nM 3,5,6-trichloro-2-pyridinol (TCP) and 0.18 nM 3-phenoxybenzoic acid (3-PBA) (Figure 5). Neither of the seven individual compounds of the mixture (Figure 5A-G) nor the mixture (Figure 5H) affected proliferation in the tier 1 or tier 2 experiments. In order to assess if the individual compounds or the mixture have the potential to disrupt RAR or GR signaling, experiments with higher concentration would be necessary.

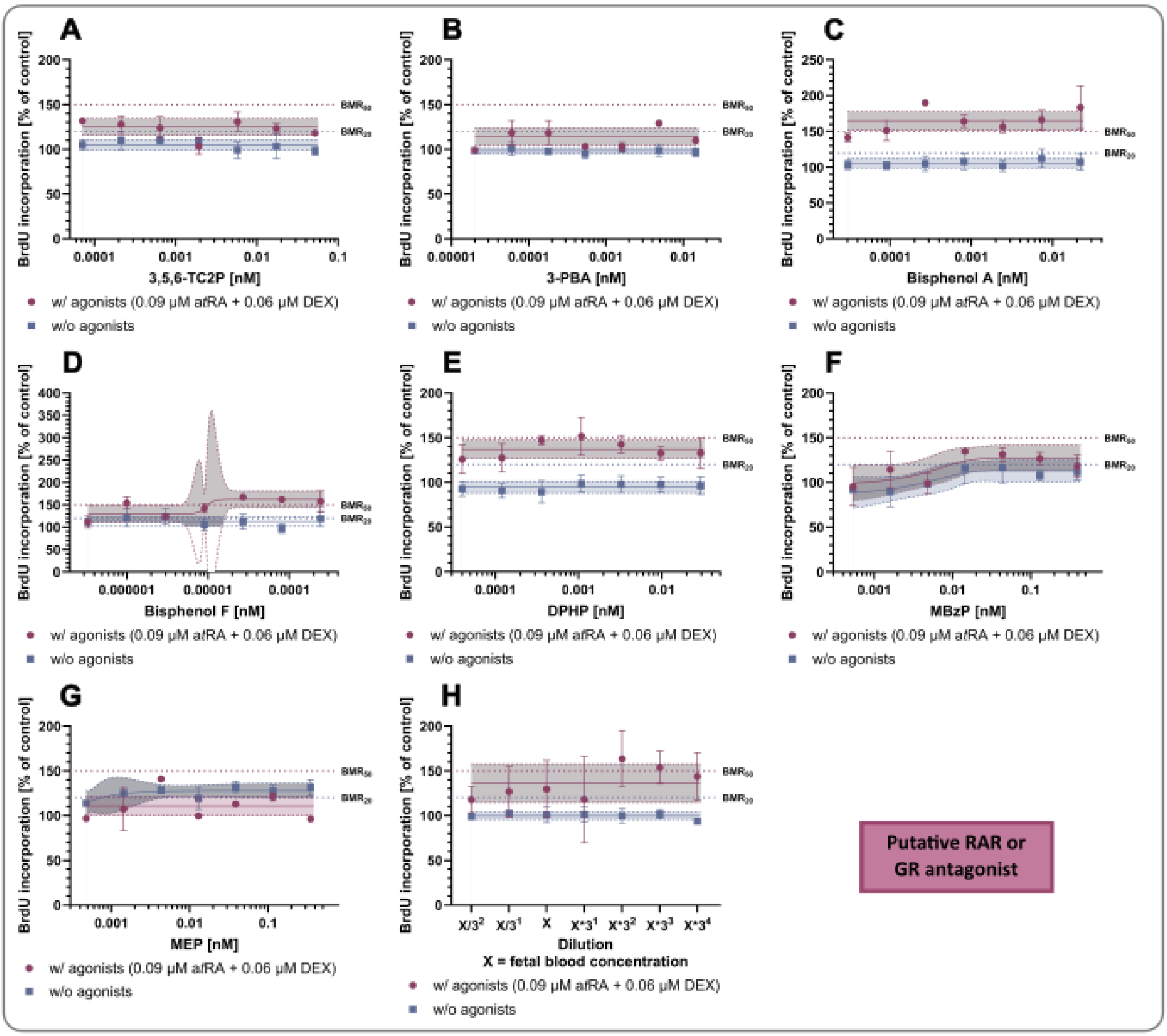


Figure 5: Mixture of putative endocrine disrupting chemicals identified in the SELMA birth cohort study was assessed using physiological concentrations in the NPC1_RAR_GR assay. Proliferating hNPCs were exposed to serial dilution of **A.** 3,5,6-trichloro-2-pyridinol (3,5,6-TC2P), **B.** 3-phenoxybenzoic acid (3-PBA), **C.** Bisphenol A, **D.** Bisphenol F, **E.** diphenylphosphate (DPHP), **F.** monobenzylphtalate (MBzP), **G.** monoethyl phthalate (MEP), or **H.** a combination of all seven compounds alone in a non-endocrine setup (blue-grey curve) or in presence of the synthetic RAR and GR agonists all-*trans* retinoic acid (a*t*RA, 0.09 µM) and dexamethasone (DEX, 0.06 µM) (red curve) for three days before proliferation was assessed by BrdU ELISA presented in % of the SC for the non-endocrine setup or in % the agonist control containing 0.09 µM a*t*RA and 0.06 µM DEX. All data (A-H) are expressed as mean ± SEM. Abbreviations: RAR, retinoic acid receptor; GR, glucocorticoid receptor; hNPC, human neural progenitor cells; DNT, developmental neurotoxicity; MSE, most sensitive endpoint; 3,5,6-TC2P, 3,5,6-trichloro-2-pyridinol; 3-PBA, 3-phenoxybenzoic acid; DPHP, diphenylphosphate; MBzP, monobenzyl phthalate; MEP, monoethyl phthalate; BrdU, bromodeoxyuridine; SC, solvent control; a*t*RA, all-*trans* retinoic acid; DEX, dexamethasone)

Discussion

Due to the emerging needs and efforts to increase chemical risk assessment by substituting animal studies with human alternatives, the necessity to understand the underlying processes of chemical MoA grows. Moreover, assessment and identification of EDCs is becoming a more and more important goal in chemical risk and hazard assessment. In the current Conceptual Framework for Testing and Assessment of Endocrine Disruptors the main focus in the *in vitro* studies lies on receptor binding and transactivation as well as enzyme activity for EATS modalities with for example the OECD TG 455 and TG 458 for estrogen and androgen receptor binding which investigate a defined MoA but often lacking cell activity and cell-cell interaction (OECD 2018a, OECD 2021, OECD 2023). Moreover, *in vivo* mechanistic studies such as the Uterotrophic and Hershberger assay provide only limited mechanistic information on disruption of estrogen and androgen signaling (OECD 2009, OECD 2018b). In addition, more complex *in vivo* guideline studies such as the OECD TG 426 and TG 443 cohort 2 provide only limited information on potential endocrine MoAs (OECD 2007, OECD 2018c). While the assessment of estrogenic and androgenic pathways is fairly advanced and applied in the US Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP), new concepts for screening of non-EATS modalities are emerging (Rotroff et al. 2013; Zgheib et al. 2021; Martyniuk et al. 2022).

In this study, we developed an alternative test method which is based on the DNT-IVB to provide additional value for the assessment of DNT, one of the more advanced fields for NAM and next generation risk assessment (NGRA) (Fritsche et al. 2018b; Sachana et al. 2019). The assay itself is based on the scientifically validated Neurosphere assay which has been well characterized over the past years (Nimtz et al. 2019; Masjosthusmann et al. 2020; Koch et al. 2022; Klose et al. 2022; Blum et al. 2023). Aside from the already abundant information of the importance of RAR signaling during very early brain development, especially neural tube formation and hindbrain patterning, this study elucidates the influence during later developmental stages (Wilson et al. 2003; Glover et al. 2006). Both the induction of cell cycle arrest and the differentiation processes show a RAR mediated shift into differentiating processes, which is in line with previous studies highlighting the necessity of retinoic acid and its interplay with FGF (Maden 2002; del Corral et al. 2003; Rhinn and Dollé 2012; Janesick et al. 2015). On the other hand, glucocorticoid signaling plays an important role during brain development and stress response (Miranda and Sousa 2018). Activation of GR signaling caused a downregulation of transcription hinting at different MoA of RAR and GR induced proliferation arrest (Sundberg et al. 2006; Moors et al. 2012). Both a*t*RA and DEX are substances known to disrupt brain development in rodents and in case of DEX, altered glucocorticoid signaling is even described to alter stress response in infants (Coluccia et al. 2008; Hossain et al. 2008; O'Connor et al. 2013). Moreover, due to their adverse effects on the developing brain and the general importance of RAR and GR signaling during brain development, atRA and DEX were included as reference compounds for alternative test methods indicating DNT potential thus further highlighting the necessity to identify compounds with similar MoAs (Aschner et al. 2017).

Current validation of NAMs is based on traditional animal studies, however a more fit for purpose process with a focus on human biology and mechanistic understanding has been proposed (Hartung et al. 2013; van der Zalm et al. 2022). While this is already important for NAMs the apparent species differences for endocrine signaling further highlight the importance of adapting a fit-for-purpose approach. Especially, since both sex and species differences in GR signaling were identified within the Neurosphere assay (Koch et al. in preparation). In order to understand and characterize the test system mechanistically, RNAseq and co-exposure experiments were performed. Furthermore, establishing a set of positive and negative controls is indispensable for developing new test methods. Using the synthetic RAR and GR agonists TTNPB and Fluticasone for RAR and GR agonism and the synthetic RAR and GR antagonists BMS493 and AL082D06 for RAR and GR antagonism, further confirmed receptor specificity of the endocrine MoA investigated in this assay and thus showed, that detection of RAR and GR disruptors is possible. Moreover, using Narciclasine as a negative control with a different MoA by inhibiting topoisomerase I activity and the FAK/JNK pathway (Qiu et al. 2022; Wang et al. 2023) and Carbaryl which was identified to disrupt estrogen and androgen receptor in vitro and adversely affect several reproductive endpoints in male rats (Fattahi et al. 2012; Tange et al. 2016). However, currently there is only sparse information on the effects of carbaryl on RAR or GR signaling with for example the ToxCast data base providing only negative hits for RAR and GR binding for carbaryl (Dix et al. 2007; Filer et al. 2014; Richard et al. 2016). Therefore, carbaryl was used as a negative control due to its endocrine properties that act on other endocrine mode of actions without disrupting RAR or GR signaling.

After establishment of the assay, an initial list of test compounds was compiled consisting of proposed DNT reference compounds and known human DNT compounds (Grandjean and Landrigan 2006, Grandjean and Landrigan 2014; Mundy et al. 2015; Aschner et al. 2017). In addition, substances that were identified to reduce proliferation in previous applications of the DNT-IVB and DNT NAM battery or in the currently ongoing DNT screening assay battery funded by the national toxicology program's (NTP) division of translational toxicology (DTT) were added to the compound list for a targeted assessment for RAR and GR agonism (Masjosthusmann et al. 2020; Carstens et al. 2022; Blum et al. 2023). Moreover, suspected DNT compounds were assessed for a risk assessment approach to further elucidate the applicability of the NPC1_RAR_GR assay (Svensson et al. 2021). One of the known human DNT compounds, methylmercury, was identified as a non-endocrine DNT compound reducing proliferation via a non- RAR or GR MoA. While methylmercury was described to inhibit GR signaling in zebrafish, identification of GR antagonism in this assay is difficult due to the inherent adverse effects on cell proliferation shown by methylmercury (Burke et al. 2006; Spulber et al. 2018). On the other hand, we identified DDT and its metabolite DDE as inhibitors of RAR or GR signaling which is in line with previous studies identifying DDT and DDE as GR antagonists in reporter gene assays (Wilson et al. 2016; Zhang et al. 2016). In addition, the insecticides Dieldrin and its metabolite Aldrin were identified to inhibit GR or RAR signaling in the NPR1_RAR_GR assay. While neither substance was tested in the DNT-IVB or NAM battery previously, both were described to activate RAR in transactivation assays thus showing their capacity to interfere in RAR signaling (Lemaire et al. 2005; Kamata et al. 2008). In addition to the four GR or RAR antagonists, valproic acid activates RAR or GR signaling in our test system. VPA has already been identified as an *in vitro* and *in vivo* DNT compound, causing behavioral changes in rats (Vorhees 1987; Schneider and Przewłocki 2005; Carstens et al. 2022; Blum et al. 2023). Moreover, VPA induces similar gene expression patterns as retinoic acid in zebrafish embryos and P19C5 stem cells (Li and Marikawa 2016; Samrani et al. 2023). Among the other tested chemicals, the non-endocrine DNT compounds were already identified in the previous screening projects or in the ongoing NTP-DDT DNT screening assay battery, while the other compounds did not affect hNPC proliferation or RAR or GR signaling. Therefore, while the assay did not identify novel DNT compounds within this data set, only a subset of the compounds from this study was evaluated in the previous applications of the DNT-IVB and the additional value in identifying DNT compounds needs to be further investigated by increasing the data set. However, by identifying several compounds as EDCs of RAR or GR signaling, we were able to show the capacity of the assay to identify putative ED-DNT compounds for pathways that play a pivotal role in brain development. Moreover, by identifying substances as receptor antagonists, which without the second-tier testing would have been identified as “no hit” in the proliferation assay we further show the potential implications of compounds not being identified as DNT substances because of their inherent absence of activity without a physiological receptor ligand present in the test system. Therefore, by substituting the hormonal activity of RAR and GR signaling, it is possible to circumvent the lack of physiological activity of this test system and thus also partially substitute the lack of complexity of *in vitro* systems have compared to their *in vivo* counterparts.

While the NPC1_RAR_GR assay is capable of detecting EDCs, the concentration needed to detect the endocrine MoA higher than those found in the environment where concentrations in human cord blood samples range from 1.9 nM to 13.84 nM for Dieldrin, 0.44 nM to 19.98 nM for Aldrin, 0.03 nM to 12.14 nM for DDT and 0.91 nM to 11.83 nM for DDE (Mustafa et al. 2010; Luo et al. 2016; Cabrera-Rodríguez et al. 2020; Junqué et al. 2020). This might be caused by these environmental compounds affecting RAR and GR only secondarily as an unintended MoA and thus a lower binding affinity to the receptors. Only valproic acid disrupts RAR or GR signaling within the therapeutic range of 50-100 µg/ml (Tseng et al. 2020). One of the potential MoAs for valproic acid mediated RAR disruption is by HDAC inhibition, and a subsequent increased accessibility of retinoic acid, which is present in the basic proliferation medium, to the RAR and thus increased activity of RAR signaling (Li and Marikawa 2016). In addition, the screening approach using seven potential EDCs with concentrations derived from the SELMA cohort study alone and as a mixture yielded only negative results (Svensson et al. 2021). This could either be due to the concentrations being too low to compete with the specific receptor agonists or no endocrine activity regarding RAR and GR signaling. Therefore, the assay might be more suited for chemical hazard assessment to identify the capability of test compounds to interfere with RAR or GR signaling. While a risk assessment approach might prove difficult using this assay, a hazard-based approach coincides with the current regulatory approach for endocrine disruptors, making this assay a potential candidate for regulatory use in the future (Andersson et al. 2018).

Subsequent tests for potential mixture assessment using three of the identified RAR or GR antagonists, Aldrin, Dieldrin, and p,p’-DDE were able to cause an enhanced proliferation at a lower concentration of the individual substances. This shows the potential of the NPC1_RAR_GR assay to be used for a component-based based mixture hazard assessment (Kumari and Kumar 2020). This further highlights the necessity to develop test systems capable of identifying endocrine disrupting mixtures which while not disrupting the endocrine signaling pathways alone might cause severe adverse effects in a mixture due to potential additive or synergistic natures of the individual EDCs (Caporale et al. 2022). In addition, with both Dieldrin and DDT and their respective metabolites eliciting ED, assessment of endocrine disruptors will need to be coupled with understanding the metabolism of test compounds and potentially performing combined treatments if the test system is incapable of metabolism.

Thus, in this study we not only expanded on the initial characterization for the potential to assess ED-DNT in the scientifically validated Neurosphere assay, we also developed a two-tiered approach to identify chemicals with the potential to disrupt RAR or GR signaling. Moreover, we provide mechanistic information as a validation of an endocrine mediated MoA and due to the inherent species differences in endocrine signaling which directly apply to GR signaling in our test system, we further underline the requirement to implement human based test methods for the characterization of ED and ED-DNT (Cediel-Ulloa et al. 2022; Koch et al. in prep). Furthermore, by providing endpoint specific controls, a prediction model and an initial test compound list, we demonstrate the readiness of this test system for inter-laboratory validation processes (Bal-Price et al. 2018; Crouzet et al. 2023). Moreover, the initial test compound set enables comparison for RAR and GR disruption with other cell-based systems to help both validate the generated data and subsequently increase the trust in these NAMs.

Moreover, by creating a comprehensive list of ED-DNT compounds, either for each receptor respectively or combined, *in silico* and potentially artificial intelligence (AI) based approaches could be further developed to aid in the emerging efforts of Integrated Approaches to Testing and Assessment (IATA) strategies in 21st century toxicity testing and potentially develop a test battery comprising of different assays identifying EDCs disrupting RAR and GR signaling (Devillers et al. 2015; Krewski et al. 2020; Aschner et al. 2022; Crofton et al. 2022; Stanojević et al. 2023). This however will require a bigger set of test chemicals and potentially a better understating of the exact MoA for the different chemicals in interfering with the different signaling pathways.

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3 Discussion

3.1 DNT NAMs in NGRA

Chemical risk assessment has been an essential part of ensuring human and environmental health throughout the development and production of new chemicals. During the 20th century this process mainly focused on animal studies and required large amounts of time and resources to receive appropriate information on potential adverse effects of chemicals (Krewski et al. 2009, Krewski et al. 2010; Andersen et al. 2010; Paparella et al. 2020). After the 3R concept was proposed by Russel and Burch during the 1960s a shift towards reducing the number of animals, refining the process of animal studies, and eventually replacing animal studies in toxicity testing began (Russel and Burch 1960). This resulted in the current paradigm shift in toxicology aiming to not only reduce the resource intensiveness of animal studies but also shift test method development to human-based models to better depict and thus predict toxicity of chemicals on human health (Sun et al. 2012; Krewski et al. 2020). This process using an exposure- and hypothesis-based concept integrating *in vitro, in silico,* and *in chemico* data is encompassed within NGRA (Dent et al. 2018). Moreover, the low throughput for testing of chemicals in classical *in vivo* toxicity testing coupled with the increasing amount of chemicals being developed for different uses necessitated the advancement of high-throughput approaches to cover the lack of information thereof (Sun et al. 2012; Krewski et al. 2020). However, alternative *in vitro* methods do not recapitulate the physiology of a living organism in its entirety. Therefore, a shift from assessing apical endpoints *in vivo* to a mechanistic approach focusing on endophenotypes of toxicity in alternative methods and combining multiple test methods to testing batteries can help substituting current *in vivo* guideline studies (Kleensang 2014).

Current regulatory guideline studies need to cover several different aspects of toxicology including acute and chronic toxicity, genotoxicity, carcinogenicity and developmental and reproductive toxicity (Kille 2017). In order to develop alternatives to the respective *in vivo* studies, the *in vitro* alternatives need to cover the different key events causing adversities (Sewell et al. 2024). By combining a set of NAMs targeting a specific adverse outcome (e.g., skin sensitization, eye damage and eye irritation), defined approaches have been developed to assess and interpret the data, use them for regulatory decision making, to ultimately faze out animal studies (Sewell et al. 2024). This approach has been successfully completed in the assessment of skin sensitization and serious eye damage and eye irritation (OECD 2023, 2024). However, to develop defined approaches assessing complex aspects of the human body such as organ development and crosstalk, more intricate test systems ranging from singular cell types to organoids containing different cell types and even microfluidic systems have been developed (Cakir et al. 2019; Benito-Kwiecinski and Lancaster 2020; Fritsche et al. 2021). In addition, certain animal models such as zebrafish or *Caenorhabditis elegans* are used more frequently due to their in-depth characterization and well described genomic profile allowing the assessment of behavioral aspects and the involvement of xenobiotic metabolism, which cannot be assessed using cell-based *in vitro* methods (Hunt 2017; Bauer et al. 2021). Furthermore, *in silico* models are gaining traction with increasing information to not only sustain and advance QSAR and read-across models but also utilize novel approaches such as artificial intelligence (AI)-based tools (Gini 2022; Tran et al. 2023; Schmeisser et al. 2023).

Approaches to replace DNT *in vivo* guideline studies have culminated in the development of the DNT-IVB (Crofton and Mundy 2021). Brain development is a highly intricate process, best modelled using highly complex models such as organoids and microfluidics. These models provide a recapitulation of the brain's cytoarchitecture, interplay between different organs and the involvement of barriers such as the BBB. However, this increased complexity is accompanied by reduced throughput and reproducibility, two parameters of utmost importance for *in vitro* test methods. On the other hand, the test methods applied in the DNT-IVB such as the Neurosphere Assay have higher complexity compared to 2-dimensional cell cultures and better recapitulate the physiology of the human brain while simultaneously keeping costs, time, and the variability within the system comparably lower than more complex organoid cultures (Wilkinson 2023). Moreover, the Neurosphere Assay models five different KNDPs in a multiplexed setup therefore decreasing the number of assays necessary to cover the key processes of brain development. By using a mixed neuron-glia cell culture, the interactions between the different cell types, required for the modelled KNDPs, are considered in contrast to test systems based on monocultures (manuscript 2.1).

3.2 Validation of NAMs

Alongside the development of novel test methods, their validation is equally as important to confirm the applicability, reproducibility, and predictivity for human health (Basketter et al. 2012; Leist et al. 2014). For NAMs, this process should focus less on the comparison with *in vivo* studies in elaborate ring trials but rather feature a mechanistic approach. This approach includes (i) the identification of the test method's applicability domain and underlying physiological pathways controlling the modelled KNDP, (ii) confirmation of the accurate representation of human biology, (iii) detection of DNT concern for chemicals reportedly interfering in the biological processes determining the modeled KNDP, and (iv) transfer into naïve laboratories without experience in performing the NAM but with the necessary technical know-how (Hartung et al. 2013; van der Zalm et al. 2022).

In general, classical validation frameworks follow similar criteria (Patterson et al. 2021). First, the test method needs to fulfill a defined scientific and regulatory need, and enable the derivation of a relationship of the investigated endpoint and the biological effect *in vivo.* The reproducibility of the assay must be ensured by providing standard operating procedures (SOP) and a detailed study plan for the validation process (OECD 2005). Moreover, a demonstration of the variability and intra- and interlaboratory reproducibility are required using reference chemicals in a comparison to their effects on the target species. Lastly, ensuring accessibility to the data is essential for the validation of a NAM (OECD 2005).

To reduce the workload and cost of an extensive interlaboratory validation, pre-validation studies are recommended. The pre-validation aims to (i) refine, optimize and standardize the SOPs to increase clarity and readiness for use in other laboratories (ii) obtain preliminary data on the transferability, reproducibility, and reliability of the test method and (iii) reduce and avoid costs in case of insufficient performance of the test method before initiating a large-scale multi-laboratory validation (OECD 2005). If the pre-validation shows acceptable test performance after optimization a main validation can be initiated. This process consists of one or more phases of interlaboratory testing including large scale testing of positive and negative controls and blind testing of reference chemicals. However, if pre-validation results indicate that the performance of a test method is not deemed acceptable for validation, changes in the method or a rejection altogether need to be considered (Fig. 5) (OECD 2005). In addition, current directions for the validation of NAMs move away from traditional, extensive ring trials to the assessment of intra- and inter-laboratory reproducibility in a smaller number of naïve laboratories (Hartung et al. 2004; van der Zalm et al. 2022). Here, the validation efforts focus primarily on performance assessment using a defined set of robust chemicals which target specific MoAs and an iterative proficiency and reproducibility assessment post-validation. This serves as a foundation to substitute large scale ring trials of NAMs whilte still ensuring the reliability and reproducibility and could further facilitate validation of NAMs simultaneously reducing cost and time (van der Zalm et al. 2022).

Since the current regulatory hazard and risk assessment is shifting from investigating apical endpoints in animals towards an IATA-based approach focusing on the assessment of endophenotypes of toxicities, the demand for fit-for-purpose NAMs is growing (Piersma et al. 2018; Parish et al. 2020). Moreover, a combination of test methods targeting different key events can be used in IATAs by combining information from different sources in WoE-based approaches to infer predictions on the potential hazard or risk of the test substance (OECD 2017b, OECD 2017a; Patterson et al. 2021). Furthermore, performing smaller, less extensive, interlaboratory validations offers the possibility to gain initial information on the potential reproducibility of the test method thus laying a foundation for further large-scale validation and simultaneously counteracting the reproducibility crisis in science (Baker 2016).

Before initiating the validation of a NAM, the method must first be scientifically sound and described in detail regarding its scientific rationale, reproducibility, and performance based on a set of reference chemicals and compared to information from the species of concern using existing toxicity data (OECD 2005). This comparison can be difficult especially in regards to DNT due to the low number of confirmed human positive DNT compounds (Grandjean and Landrigan 2014). A scientific validation can be used to increase confidence in the predictivity of the test method, assess the readiness, and identify uncertainties as a first step in preparing the test method for regulatory application (Bal-Price et al. 2018a). A scientific validation, as shown for the Neurosphere Assay in manuscript 2.1, should cover five steps for each endpoint independently. These steps include (i) the biological relevance of the test method endpoint (e.g., KNDP) for the modelled physiological *in vivo* process (i.e., brain development), the cellular (ii) and molecular (iii) characterization of the test system (e.g., cell type specific morphology and expression of cell type specific marker), (iv) adequate response to modulation of specific signaling pathway controlling KNDPs in vivo as well as (v) to DNT reference chemicals (manuscript 2.1).

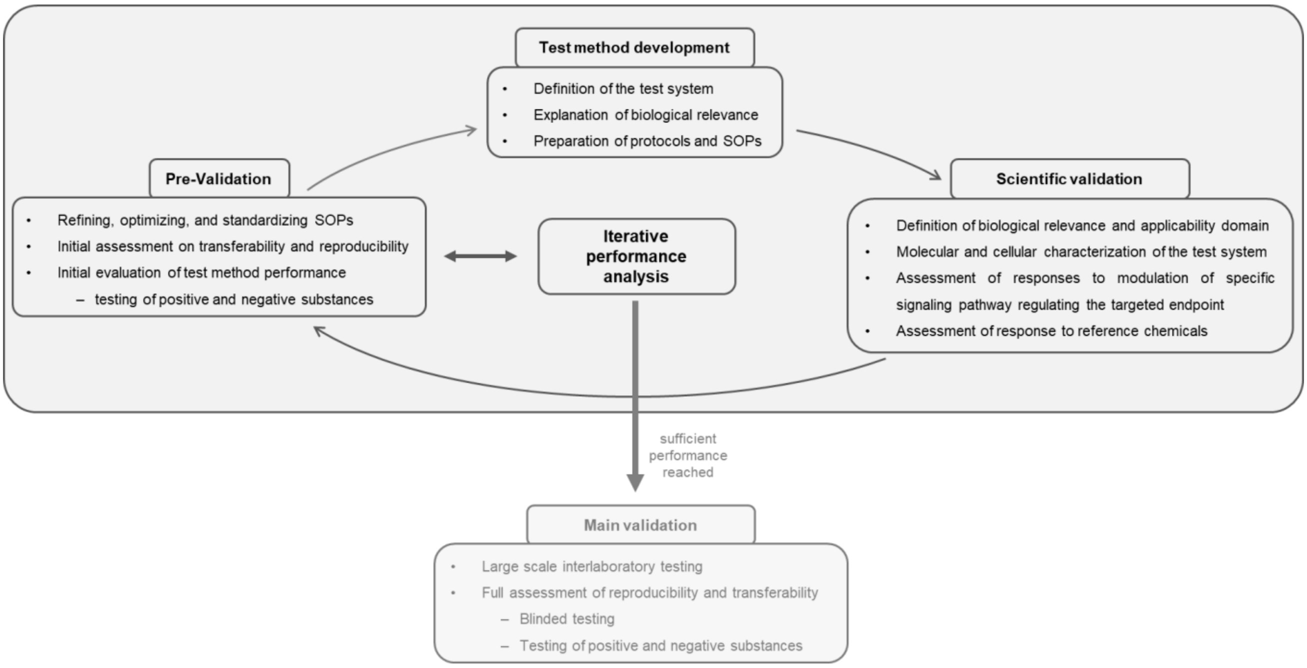


Figure 5: Process of test method validation and pre-validation (adapted from (OECD 2005). Abbreviations: SOP, Standard Operating Procedure.

The validity of the chosen approach for the scientific validation of the Neurosphere assay can be illustrated on the example of one central KNDP, oligodendrocyte differentiation. Oligodendrocytes are responsible for myelination and thus insulation of axons which serve to facilitate signal transduction in the brain. Oligodendrocytes differentiate from radial glia cells into oligodendrocyte precursor cells and ultimately mature into myelin producing oligodendrocytes (Bradl and Lassmann 2010). Functional and mature oligodendrocytes are pivotal for the physiological development of the brain whereas disrupted oligodendrogenesis is associated with neurodevelopmental and myelin-related disorders such as ADHD, multiple sclerosis, or perinatal white matters injuries (Stadelmann et al. 2019; Kuhn et al. 2019; Motavaf and Piao 2021). However, in part due to their high metabolic demand, oligodendrocytes are susceptible to outside stressors such as reactive oxygen species (ROS) and chemical insults i.e., the flame retardant TBBPA or the organophosphate flame retardants and pesticides tris(methylphenyl) phosphate (TMPP) or tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) (French et al. 2009; Klose et al. 2020, Klose et al. 2022; Spaas et al. 2021; Cohn et al. 2024). During differentiation and maturation, oligodendrocytes develop an increasingly complex morphology characterized by increased branching. Moreover, during differentiation, a number of different genes are expressed corresponding to the developmental stage of the oligodendrocyte, starting with *PDGFRA* (platelet-derived growth factor alpha) in immature, *O4* in maturing and *MBP* (myelin basic protein) in mature oligodendrocytes, which was confirmed for *PDGFRA* and *MBP* expression in manuscript 2.1 (Barateiro and Fernandes 2014; Marinelli et al. 2016).

Both neuronal and oligodendrocyte differentiation are processes highly regulated by notch signaling (Yoon and Gaiano 2005; Louvi and Artavanis-Tsakonas 2006; Pierfelice et al. 2011). Inhibition of notch signaling by treatment with the notch receptor antagonist DAPT induced neuronal differentiation and simultaneously reduced oligodendrocyte differentiation, confirming that neuronal and oligodendrocyte differentiation is under control of notch signaling in hNPCs (Koch et al. 2022). In addition, oligodendrocytes are especially susceptible to ROS with i.e., arsenic reducing oligodendrocyte differentiation by inducing ROS in hNPCs (Jomova et al. 2011; Masjosthusmann et al. 2019; Koch et al. 2022). In line with this, antioxidants such as vitamin C, which protect from ROS, induced oligodendrocyte differentiation and maturation in mouse and human derived NPCs (Dach et al. 2017; Guo et al. 2018; Koch et al. 2022). Lastly, to assess whether the test method can identify test substances targeting oligodendrogenesis thus inducing oligodendrocyte toxicity, deltamethrin and TBBPA were observed to reduce oligodendrocyte differentiation in human NPCs (Masjosthusmann et al. 2020; Koch et al. 2022; Klose et al. 2022). While deltamethrin exposure is associated with neurodevelopmental disorders such as ADHD and ASD, recent studies indicate both a depletion of the antioxidant system and dysregulation of lipid metabolism as potential MoAs for oligodendrocyte toxicity

(Pitzer et al. 2021; Wu et al. 2024). Similarly, TBBPA interferes with cholesterol biosynthesis, described as a potential MoA for oligodendrocyte toxicity (Klose et al. 2020). Due to the high metabolic rate and requirement of cholesterol and lipids for myelin synthesis, oligodendrocytes are dependent on lipids making oligodendrocytes more susceptible to alterations in lipid homeostasis (Montani 2021).

Brain development and its underlying KNDPs are not only regulated by more classically perceived signaling pathways as such as notch, FGF, or Wnt, but also by nuclear receptors and respective endocrine signaling pathways. This coupled with increasing concerns of potential effects of ED during development requires advancing the understanding of endocrine signaling during development and specifically brain development (OECD 2010).

3.3 Hormone receptors during brain development

Organ and tissue development is partially regulated and coordinated by hormones NRs (Oliveira et al. 2021). NRs regulate different processes ranging from organ development to lipid homeostasis (Jeong and Mangelsdorf 2009). During brain development, different processes such gliogenesis and neurogenesis are regulated by different hormones (Fig. 6) (e.g., RAR, RXR, PPARα, PPARγ) (Jeong and Mangelsdorf 2009; Gkikas et al. 2017). Due to the importance of endocrine signaling during brain development, exposure to EDCs, such as phthalates, bisphenols or PBDEs, correlates with neurodevelopmental outcomes such as ADHD or ASD (Lucaccioni et al. 2021; Özel and Rüegg 2023).

The impact of different endocrine signaling pathways on brain development varies greatly. Androgen and estrogen signaling play an important role in sexual differentiation during brain development (Wallen 2005; McCarthy 2008). Moreover, disruption of AR or ER signaling is associated with neurodevelopmental deficits such as ASD and with neurodegenerative diseases such as Alzheimer’s disease (Henningsson et al. 2009; Rosario et al. 2010; Lin et al. 2023). Regarding KNDPs, AR signaling was shown to induce NPC proliferation while simultaneously reducing neuronal differentiation in mouse embryos (La Rosa et al. 2021). Contrasting this, treatment of rat neural stem cells with the synthetic androgen nandronol was observed to reduce proliferation in presence of epidermal growth factor (EGF) and induce proliferation in absence of EGF (Brännvall et al. 2005). In a similar fashion, activation of ER signaling was shown to reduce rat neural stem cell proliferation in presence of EGF and induce proliferation in the absence of EGF (Brännvall et al. 2002). In addition, ER activation was observed to induce rat neural stem cell proliferation in the absence of FGF (Okada et al. 2008). Moreover, ER signaling was identified to induce oligodendrocyte differentiation in mouse embryonic stem cells or to induce differentiation into dopaminergic neurons in human neural stem cells (Kishi et al. 2005; Imamura et al. 2020). Although both AR and ER are implicated in several different KNDPs, they did not impact any endpoints modeled within the Neurosphere Assay. This could be caused by the relatively low expression of AR and ER in fetal brain samples and concomitantly in human NPCs which hints at limited involvement during this developmental window or between species in ER and AR signaling (manuscript 2.2).

On the other hand, the information on progesterone receptor (PR), prostaglandin E2 receptor (PGE2R), and VDR signaling during brain development are sparse. Vitamin D and prostaglandin E2 deficiency are associated with neurodevelopmental disorders including ASD (Wong et al. 2019; Siracusano et al. 2020; Wang et al. 2020). Moreover, VDR activation was shown to reduce proliferation in rat NPCs and mouse embryonic cells where it was accompanied by a simultaneous induction of cell differentiation and neurite outgrowth (Cui et al. 2007; Marini et al. 2010). Furthermore, VDR activation as a VDR-RXR heterodimer enhances oligodendrocyte differentiation in rat oligodendrocyte progenitor cell cultures (de la Fuente et al. 2015). PR signaling is highly involved in oligodendrogenesis and increased both proliferation in rat oligodendrocyte precursor cells and proliferation and differentiation of mouse oligodendrocyte progenitor cells (Labombarda et al. 2009; González-Orozco et al. 2020). PGE2R signaling on the other hand is primarily involved in neurite outgrowth, facilitating neuronal differentiation in mouse neuroblastoma-rat glioma hybrid cell cultures and neurite outgrowth in mouse NSC-34 cells (Kim et al. 2003; Nango et al. 2017). Although we observed alterations of the same endpoints for each of the receptors, the direction contrasts the data from rodent cells, identifying species differences in VDR, PGE2R, and PR signaling. Furthermore, the low number of effects after treatment with PGE2R, PR and VDR agonists could be due to the low expression of all three receptors in human NPCs (manuscript 2.2).

TH signaling contributes to different KNDPs and disruption of TH signaling is associated with neurodevelopmental deficits characterized by reduced IQ and deficits in speech and motor skills (Korevaar et al. 2016). THR activation was observed to induce neuronal differentiation in mouse embryonic stem cells and inhibit astrocyte differentiation (Chen et al. 2012, Chen 2015). Similarly, we observed an induction of neuronal differentiation after THR activation, however only in human- and not rat-based NPCs (manuscript 2.2). Moreover, TH signaling plays a pivotal role in oligodendrogenesis, promoting oligodendrocyte differentiation in rat neural stem cells whereas lack of TH reduced the number of mature oligodendrocytes in rat *in vivo* (Schoonover et al. 2004; Marziali et al. 2016). However, clear species differences were identified with TH signaling inducing oligodendrocyte differentiation in rat and mouse derived NPCs and oligodendrocyte maturation in hNPCs (Dach et al. 2017; Klose et al. 2020, manuscript 2.2).

Disruption of glucocorticoid signaling is associated with learning and memory deficits, lower IQ, anxiety disorders, and ADHD, which amongst others can be caused by maternal glucocorticoid treatment or elevated maternal stress levels (LeWinn et al. 2009; Moisiadis and Matthews 2014). GR-mediated proliferation arrest was reported not only in this work but also earlier in hNPCs, immortalized progenitor cells and rodent neural stem cells (Sundberg et al. 2006; Moors et al. 2012; Anacker et al. 2013; Alnoud et al. 2021, manuscript 2.2, manuscript 2.3). Moreover, the reduced proliferation was caused by cell cycle arrest which was observed in mouse NPCs and a downregulation of transcription-related genes identified after RNA sequencing in human NPCs in this work (Samarasinghe et al. 2011, manuscript 2.2, manuscript 2.3). Besides proliferation, GR signaling is involved in neurogenesis and activation of GR reduces neuronal differentiation in hNPCs (Nürnberg et al. 2018, manuscript 2.2). Furthermore, glucocorticoid signaling and concomitantly stress response differs between sexes with only male mice developing late-onset depression-like behavior after *in utero* exposure to dexamethasone (Elberling et al. 2023). In addition, male mice are more susceptible to glucocorticoid mediated stress response, highlighted by reduced long-term potentiation and altered recency memory (Velli et al. 2022). Concomitantly, we observed a reduction in proliferation and neuronal differentiation only in male hNPCs, while rNPC proliferation remained unaffected by GR activation identifying both sex and species differences in glucocorticoid signaling.

Perturbations of progenitor cell proliferation during brain development can cause severe pathologies such as micro- and megalocephaly with for example disruption of RA signaling being associated with the emergence of microcephaly and craniofacial malformations (Pirozzi et al. 2018; Petrelli et al. 2019; Gur et al. 2022). Furthermore, disrupted *at*RA signaling elicits teratogenic effects causing spina bifida, immune disorders, and severe malformations of different organs and structures such as the skeleton, eye, or heart during early development (Odermatt and Gumy 2008; Piersma et al. 2017; Nakajima 2019; Varticovski et al. 2022). Activation of RAR signaling caused a proliferation arrest by downregulating genes involved in cell cycle progression and is hypothesized to cause a switch from proliferation to differentiation in the brain (Janesick et al. 2015, manuscript 2.2, manuscript 2.3). In addition, inhibition of EGF signaling reduces proliferation in hNPCs and could be a MoA for RAR activation which represses EGFR expression in hNPCs and in keratinoid carcinoma cells (Zheng et al. 1992; Koch et al. 2022, manuscript 2.2). Adding to the effects on proliferation, RA is necessary for oligodendrogenesis with activation of RAR inducing the expression of inhibitors of oligodendrocyte differentiation in human neural stem cell derived oligodendrocyte precursor cells and reducing differentiation and proliferation of rat oligodendrocyte precursor cells (Laeng et al. 1994; Kim et al. 2017). Comparable results were observed in this work, indicating accelerated oligodendrocyte differentiation. The effects on oligodendrocyte differentiation however were suspected to be an increase in differentiation and maturation causing a clustering of oligodendrocytes within the sphere core, probably due to premature differentiation in the 3D neurospheres (manuscript 2.2).

Lipids, especially cholesterol, make up 73 % of myelin, and oligodendrocytes synthesize three times their weight in myelin per day. Therefore, lipid homeostasis remains essential for oligodendrocyte function (Norton and Poduslo 1973; Saher et al. 2005; Mathews and Appel 2016). Moreover, while excessive amounts of lipids can cause lipotoxicity, lack thereof can impair biosynthesis and subsequently myelination in oligodendrocytes (Zhang and Liu 2015; Berghoff et al. 2017). Oligodendrocyte disfunction caused by for example altered lipid metabolism is associated with neurodegenerative diseases such as multiple sclerosis and Huntington's disease during later life stages (López-Muguruza and Matute 2023; Ferrari Bardile et al. 2023). Both LXR and PPAR are essential signaling pathways regulating lipid homeostasis and energy metabolism in the body including the brain thus leaving these receptors as potential targets for ED-DNT (Theofilopoulos and Arenas 2015; Mouzat et al. 2019; Zhou et al. 2020). Concordant with this, LXR activation was shown to promote cholesterol efflux in primary neonatal rat oligodendrocytes (Nelissen et al. 2012). Furthermore, lack of LXR activity is associated with reduced oligodendrocyte differentiation and maturation in LXR knockout mice while LXR activation induces oligodendrocyte differentiation in rat radial glia cultures (Xu et al. 2014). Activation of PPARβ/δ and PPARγ was observed to induce oligodendrocyte differentiation and maturation in mouse primary glial cultures and rat primary oligodendrocyte progenitor cells (Saluja et al. 2001; Bernardo et al. 2017). Even though these effects contrast the observations in this work with LXR and PPAR activation reducing oligodendrocyte differentiation, altered lipid dysregulation could cause the reduced number of oligodendrocytes. This was further shown in manuscript 2.2 with both LXR and PPARα activation altering the expression of genes mediating cholesterol and fatty acid homeostasis. Besides their impact during oligodendrogenesis, PPAR and LXR signaling are involved in neuronal differentiation. For example, PPARβ activation induced neuronal differentiation in mouse embryonic stem cells while PPARα induced neuronal differentiation in human teratocarcinoma cells (Mei et al. 2016; Lin et al. 2018). Similarly, LXR activation induced neuronal differentiation in human iPSC-derived NPCs and rat pheochromocytoma cells (Schmidt et al. 1999; De La Fuente et al. 2024). In addition, LXR agonists promoted differentiation into dopaminergic neurons in human and mouse embryonic stem cells (Sacchetti et al. 2009). Concomitantly, LXR and PPARβ/δ activation induced neuronal differentiation in this work (manuscript 2.2). However, species comparison in manuscript 2.2 between rat and human NPCs revealed that LXR activation induced neuronal differentiation and that PPARβ/δ activation reduced oligodendrocyte differentiation only in hNPCs and not in time-matched rNPCs.

Lastly, RXR signaling is implicated in the signaling pathways of several other HRs due to the heterodimerization with LXR, PPAR, RAR, THR, and VDR (Evans and Mangelsdorf 2014). Most research regarding RXR focuses on its positive implications in remyelination and potential treatments of neurodegenerative diseases (Huang et al. 2011; Muñoz-Cabrera et al. 2019). However, RXR activation is involved in neurogenesis as well, promoting neuronal differentiation and neurite outgrowth in mouse embryonic stem cells (Mounier et al. 2015). During brain development, the main partners for heterodimerization in mouse neural stem and progenitor cells are LXR and RAR (Simandi et al. 2018). This could indicate, that the RXR induced effects are mediated by activation of LXR, RAR or potentially PPAR signaling, since activation of these pathways elicited similar responses (manuscript 2.2).

Studies on endocrine signaling pathways and the potential impact of EDCs on brain development are increasing. However, most of the information is based on animal models which might not adequately recapitulate human responses to endocrine signaling. These differences became apparent for eight pathways investigated in manuscript 2.2. These differences include a higher or selective sensitivity in hNPCs observed for GR-mediated proliferation arrest, LXR-, PPARγ-, RXR-, and THR-mediated induction of neuronal differentiation, and reduced oligodendrocyte differentiation after PPARβ/δ and VDR activation. Only THR-mediated induction of oligodendrocyte differentiation was specific for rNPCs. This further necessitates the development of human-based test methods to understand and investigate the impact of EDCs on the cellular and molecular processes of brain development and ultimately facilitate the testing and assessment of endocrine disruptors in a regulatory context.

	Human					Rodent (rat, mouse)				
Hormone receptor	**Proliferation**	**Migration**	**Neuronal differentiation**	**Neurite outgrowth**	**Oligodendrocyte differentiation**	**Proliferation**	**Migration**	**Neuronal differentiation**	**Neurite outgrowth**	**Oligodendrocyte differentiation**
AR						*In vitro*: ↑ (m) ↑↓ (r) [1,2]		*In vitro*: ↓ (m) [1]		
ER			*In vitro:* ↑ [3]			*in vitro*: ↑↓ (r) [4,5]				*In vitro:* ↑ (m) [6]
GR	*In vitro:* ↓ [7,8]		*In vitro:* ↓ [9]			*In vitro:* ↓ (m,r) [10,11,12]				
LXR			*In vitro:* ↑ [13,14]					*In vitro:* ↑ (m,r) [14,15]		*In vitro:* ↑ (r) *In vivo:* ↑ (m) [16]
PPARα			*In vitro:* ↑ [17]							
PPARβ/δ								*In vitro:* ↑ (m) [18]		*In vitro:* ↑ (m) [19
PPARγ										*In vitro:* ↑ (r) [20]
PR						*In vitro:* ↑ (m,r) [21,22]				*In vitro:* ↑ (m) [21]
PGE2R								*In vitro:* ↑ (m) [23]	*In vitro:* ↑ (m) [24]	
RAR					*In vitro:* ↓ [25]	*In vitro:* ↓ (r) [26]				*In vitro:* ↓ (r) [26]
RXR								*In vitro:* ↑ (m) [27]	*In vitro:* ↑ (m) [27]	
THR								*In vitro:* ↑ (m) [28]		*In vitro:* ↑ (m,r) *In vivo:* ↑ (r) [29,30,31,32]
VDR						*In vitro*: ↓ (m,r) [33,34]		*In vitro:* ↑ (m) [34]	*In vitro:* ↑ (m) [34]	*In vitro:* ↑ (r) [35]

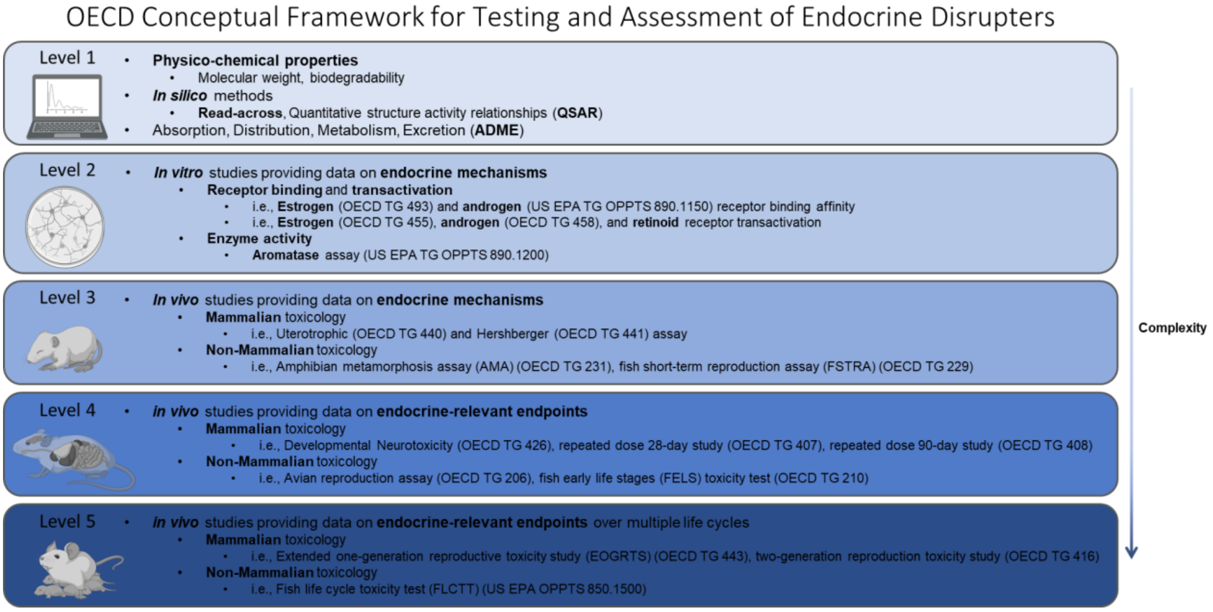
↑ : inducing effect **m: mouse**
↓ : reducing effect **r: rat**

Figure 6: Overview of the effects of hormone receptor signaling on KNDPs in human and rodents. Abbreviations: AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; PGE2R, prostaglandin E2 receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; THR, thyroid hormone receptor; VDR, vitamin D receptor; m, mouse; r, rat) [1] (La Rosa et al. 2021), [2] (Brännvall et al. 2005), [3] (Kishi et al. 2005), [4] (Brännvall et al. 2002), [5] (Okada et al. 2008), [6] (Imamura et al. 2020), [7] (Moors et al. 2012), [8] (Anacker et al. 2013), [9] (Nürnberg et al. 2018), [10] (Alnoud et al. 2021), [11] (Samarasinghe et al. 2011), [12] (Sundberg et al. 2006), [13] (De La Fuente et al. 2024), [14] (Sacchetti et al. 2009), [15] (Schmidt et al. 1999), [16] (Xu et al. 2014), [17] (Lin et al. 2018), [18] (Mei et al. 2016), [19] (Saluja et al. 2001), [20] (Bernardo et al. 2017), [21] (González-Orozco et al. 2020), [22] (Labombarda et al. 2009), [23] (Kim et al. 2003), [24] (Nango et al. 2017), [25] (Kim et al. 2017), [26] (Laeng et al. 1994), [27] (Mounier et al. 2015), [28] (Chen et al. 2012), [29] (Dach et al. 2017), [30] (Marziali et al. 2016), [31] (Klose et al. 2020), [32] (Schoonover et al. 2004), [33] (Cui et al. 2007), [34] (Marini et al. 2010), [35] (de la Fuente et al. 2015).

3.4 State of the art of ED and ED-DNT testing

Current testing and assessment of endocrine disrupters as defined in the OECD conceptual framework for testing and assessment of endocrine disrupters, is set up in a tiered testing strategy with a total of 5 levels increasing in complexity and biological relevance for humans (Fig. 7) (OECD 2018c). Level 1 investigates physico-chemical properties of the test compounds such as molecular weight and biodegradability to predict absorption, distribution, metabolism, and excretion of the test compound and derive toxic MoAs using *in silico* methods i.e., read across and QSAR. Level 2 assays cover *in vitro* assays providing data on specific endocrine mechanisms and pathways in mammalian and non-mammalian species. These assays mostly focus on certain key events within endocrine signaling pathways such as ER or AR binding and transactivation assays or enzyme activity assays such as the aromatase assay (Coady et al. 2017; OECD 2018c). Although the assays currently integrated into the OECD conceptual framework test primarily for EATS modalities, the US EPA's ToxCast program encompasses more than 700 high throughput assays that cover roughly 300 signaling pathways including endocrine signaling pathways. This lays a foundation for the initial assessment of endocrine signaling pathways which are currently not evaluated in the conceptual framework (Filer et al. 2014; Coady et al. 2017). Level 3 assays test for interference in selected endocrine pathways *in vivo*. These assays include the Uterotophic (OECD TG 440) and Hershberger (OECD TG 441) assay for mammalian toxicology and i.e., the amphibian metamorphosis assay (OECD TG 231), or the fish short-term reproduction assay (OECD TG 229) for non-mammalian toxicology (OECD 2009a, OECD 2009b, OECD 2012, OECD 2018c, OECD 2018d). However, these assays cover only specific steps and endpoints of endocrine signaling pathways such as the uterine weight in the Uterotrophic assay or changes in androgen-dependent tissues in the Hershberger assay (OECD 2009a, OECD 2018d). Therefore, since they do not assess the whole spectrum of potential EDC-mediated adverse effects, level 3 assays are more suited for hazard characterization (Coady et al. 2017). Level 4 studies further increase the complexity of the study design and increase the number of endpoints. Level 4 studies include the developmental neurotoxicity (OECD TG 426), repeated dose 28- and 90-day studies (OECD TG 407, OECD TG 408) or prenatal developmental toxicity study (OECD TG 414) (OECD 2007, OECD 2008, OECD 2018b, OECD 2018e, OECD 2018c). Nevertheless, level 4 studies do not consider the effect on multiple life-cycles (Coady et al. 2017). Lastly, the most complex level 5 studies span over multiple generations and evaluate long term exposure, early development, sexual differentiation, and active reproduction. Level 5 studies include the EOGRTS (OECD TG 443) and the two-generation reproduction toxicity study (OECD TG 416) (OECD 2001, OECD 2018a). However, it is difficult to identify an endocrine MoA using these studies. Although the different assays from each level enable the identification of perturbations caused by chemical insults, deriving a causal relationship from the endocrine MoA to the apical endpoint remains challenging (Coady et al. 2017; Andersson et al. 2018). In addition, even though the timing and duration of KNDPs varies between species, current investigation of endocrine signaling is primarily performed in rodents (Clancy et al. 2001; Workman et al. 2013). This in turn necessitates species comparison to investigate species differences in endocrine signaling covering aspects such as receptor binding, gene transcription, or cellular responses (Walker et al. 1999).

Developing AOPs to link the different mechanistic endpoints from *in vitro* and low tier *in vivo* studies to the apical endpoints observed in higher tiered *in vivo* studies will allow causally linking endocrine MoAs to adverse outcomes. This in turn will contribute to the substitution of *in vivo* studies with mechanistic *in vitro* alternatives. Moreover, integrating the information into IATAs, AOP networks, and ontologies will further support unraveling the mechanisms of toxicity and increase the confidence in NAMs (Ankley et al. 2010; Hardy et al. 2012; Knapen et al. 2018; Hessel et al. 2018; Bajard et al. 2023). Examples for such an IATAs that are implemented into regulatory frameworks are the defined approaches for skin sensitization and eye damage and eye irritation (OECD 2023, OECD 2024). In both cases comprehensive AOPs were used to develop NAMs based on the key events for the respective adverse outcomes and the data of the respective test methods is scored per assay. The scores are then combined and used to assign a category for the compound ranging from strong sensitizer/irritant over moderate sensitizer/irritant to no sensitizer/irritant (OECD 2023, OECD 2024). Furthermore, in the conceptual framework for testing and assessment of endocrine disruptors, ED-DNT is insufficiently covered and does not identify specific endocrine MoAs. Only the OECD TG for DNT (OECD TG 426) and EOGRTS cohort 2 (OECD TG 443) allow the investigation of DNT, however, without discriminating between endocrine and non-endocrine MoAs (OECD 2007, OECD 2018a).


OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters

Level 1
- **Physico-chemical properties**
  - Molecular weight, biodegradability
- ***In silico*** methods
  - **Read-across**, Quantitative structure activity relationships (**QSAR**)
- Absorption, Distribution, Metabolism, Excretion (**ADME**)

Level 2
- ***In vitro*** studies providing data on **endocrine mechanisms**
  - **Receptor binding** and **transactivation**
    - i.e., **Estrogen** (OECD TG 493) and **androgen** (US EPA TG OPPTS 890.1150) receptor binding affinity
    - i.e., **Estrogen** (OECD TG 455), **androgen** (OECD TG 458), and **retinoid** receptor transactivation
  - **Enzyme activity**
    - **Aromatase** assay (US EPA TG OPPTS 890.1200)

Level 3
- ***In vivo*** studies providing data on **endocrine mechanisms**
  - **Mammalian** toxicology
    - i.e., Uterotrophic (OECD TG 440) and Hershberger (OECD TG 441) assay
  - **Non-Mammalian** toxicology
    - i.e., Amphibian metamorphosis assay (AMA) (OECD TG 231), fish short-term reproduction assay (FSTRA) (OECD TG 229)

Level 4
- ***in vivo*** studies providing data on **endocrine-relevant endpoints**
  - **Mammalian** toxicology
    - i.e., Developmental Neurotoxicity (OECD TG 426), repeated dose 28-day study (OECD TG 407), repeated dose 90-day study (OECD TG 408)
  - **Non-Mammalian** toxicology
    - i.e., Avian reproduction assay (OECD TG 206), fish early life stages (FELS) toxicity test (OECD TG 210)

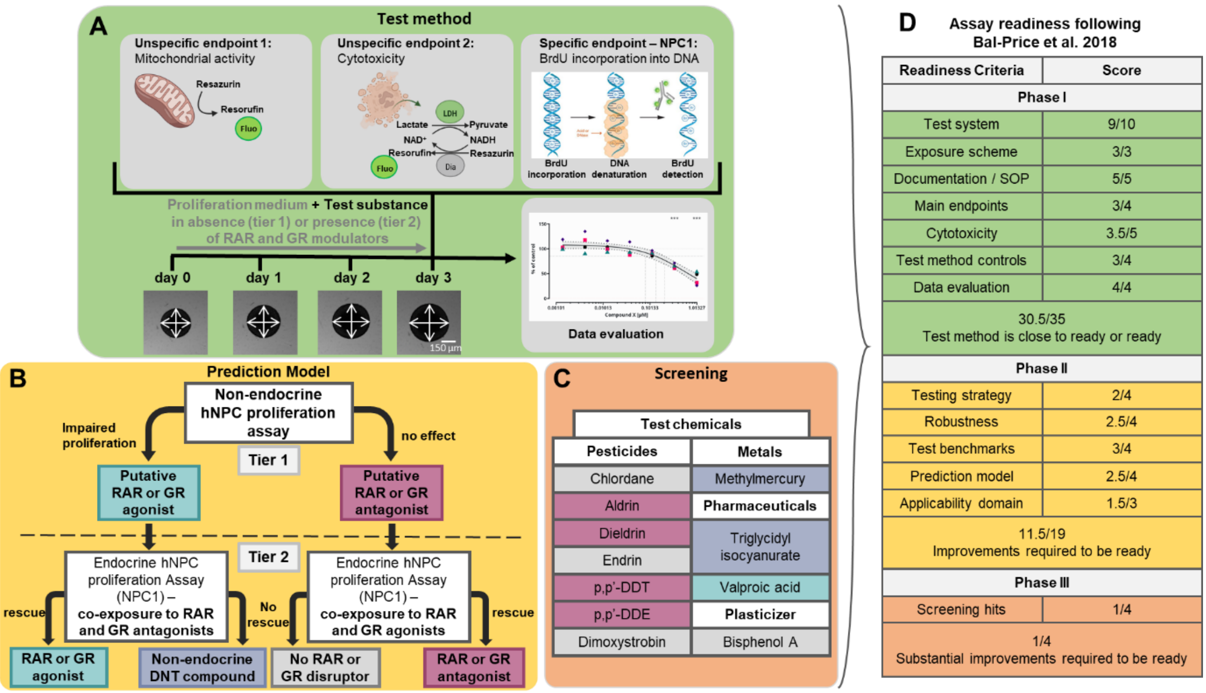
Level 5
- ***in vivo*** studies providing data on **endocrine-relevant endpoints** over multiple life cycles
  - **Mammalian** toxicology
    - i.e., Extended one-generation reproductive toxicity study (EOGRTS) (OECD TG 443), two-generation reproduction toxicity study (OECD TG 416)
  - **Non-Mammalian** toxicology
    - i.e., Fish life cycle toxicity test (FLCTT) (US EPA OPPTS 850.1500)

Figure 7: Overview of OECD conceptual framework for testing and assessment of endocrine disrupters. Testing and assessment of endocrine disruptors is divided into 5 tiers with increasing complexity from level 1 to level 5 (adapted from (OECD 2018c) Created with Biorender.com

However, current EDC risk assessment does not specifically identify ED-DNT. Therefore, within the ENDpoiNTs project the need for the development of ED-DNT NAMs targeting KNDPs is addressed (Cediel-Ulloa et al. 2022). Some of the endocrine signaling pathways which play important roles during brain development are RA and glucocorticoid signaling (Rhinn and Dollé 2012; Miranda and Sousa 2018). Disruption of each pathway is associated with neurodevelopmental disorders, yet currently only the retinoid receptor transactivation assay is included in the conceptual framework for testing and assessment of endocrine disruptors (OECD 2018c), while the assessment of GR disruption is missing. This creates a gap regarding the hazard assessment for RAR and GR disrupters.

Within this work, a novel assay adapted from the Neurosphere assay, the NPC1_RAR_GR assay was developed. Based on the initial characterization of hormone sensitive KNDPs, the assay was set up to detect RAR and GR dependent proliferation arrest (manuscript 2.3) (Fig. 8). The assay is developed in a two-tiered approach to identify substances disrupting proliferation and further discriminating between a non-endocrine and an RAR- or GR-specific MoA, thus covering DNT and ED-DNT effects on proliferating hNPCs. The tier 1 experiment enables the detection of non-endocrine DNT compounds and RAR and GR agonists, since the agonists' presence is sufficient to activate the receptors and induce a proliferation arrest in hNPCs. However, to confirm the endocrine MoA of RAR and GR agonists a tier 2 experiment is necessary. On the other hand, due to the inherent lack of RAR and GR agonists and thus receptor activity in the cultivation medium, a tier 2 experiment in the presence of the RAR and GR agonists is necessary to identify RAR and GR antagonists. The NPC1_RAR_GR assay therefore not only identifies ED-DNT compounds *in vitro,* it also covers a gap of the Neurosphere assay, which does not detect RAR and GR antagonists due to the inactivity of the receptor in the absence of physiological or synthetic agonists in the culture medium. This is highlighted by the confirmed synthetic (AGN193109, Mifepristone, BMS493, AL082D06) and here proposed environmental (Aldrin, Dieldrin, p,p'-DDT, p,p'-DDE) RAR and GR antagonists not affecting hNPC proliferation in absence of the RAR and GR agonists (manuscript 2.2, manuscript 2.3). Therefore, the NPC1_RAR_GR assay fills a potential gap in the DNT-IVB and the OECD conceptual framework for testing and assessment of endocrine disruptors. On the one hand, the DNT-IVB does not cover ED-DNT and thus might miss detecting RAR and GR antagonists which do not affect a KNDP in the current assays of the DNT-IVB. On the other hand, the OECD conceptual framework for testing and assessment of endocrine disruptors currently does not include methods to detect RAR and GR disruption on a cellular level *in vitro*, or *in vivo*.

The method was developed based on previously published recommendations for the development of DNT NAMs, that highlight 15 important features of a predictive DNT NAM including, amongst others, its biological relevance, clear endpoint measurement, endpoint-specific controls and reference chemicals, robust data analysis pipelines and a defined training and testing set of chemical (Crofton et al. 2010). The NPC1_RAR_GR assay was screened using synthetic agonists and antagonists as endpoint-specific controls and a list of test chemicals comprising (i) known human DNT positive compounds (methylmercury, valproic acid, p,p'-DDT, p,p'-DDE), (ii) substances suspected to disrupt RAR and GR signaling (chlordane, aldrin, dieldrin, endrin, tolylfluanid, bisphenol A) and (iii) chemicals that reduced hNPC proliferation in previous applications of the Neurosphere assay (dimoxystrobin, triglycidyl isocyanurate) (Lemaire et al. 2005; Kamata et al. 2008; Grandjean and Landrigan 2014; Wilson et al. 2016; Aschner et al. 2017; Zhang et al. 2019; Blum et al. 2023).The substances suspected to disrupt RAR and GR signaling were chosen based on experimental data on GR or RAR activity in receptor binding assays derived from the ToxCast data base (Kavlock et al. 2012). Within this list of test chemicals, the pesticides p,p'-DDT and Dieldrin as well as their respective metabolites p,p'-DDE and Aldrin were identified to inhibit RAR or GR signaling revealing a novel ED-DNT MoA for these compounds (Fig. 8). Both p,p'-DDT and Dieldrin are bio-accumulative and highly persistent organochlorine pesticides acting as teratogens, reproductive toxicants, and EDCs especially targeting estrogen and androgen signaling in mouse primary neuronal cultures and human adenocarcinoma cells *in vitro* and in different fish *in vivo* (Frigo et al. 2002; Briz et al. 2011; Sun et al. 2016; Martyniuk et al. 2020). Although Dieldrin was identified as a DNT compound in the DNT-IVB reducing neural network formation activity, it did not affect proliferation in the classical non-endocrine proliferation assay thus highlighting the potential gap of DNT-IVB in not identifying RAR and GR antagonists (Carstens et al. 2022).



Test chemicals	
Pesticides	**Metals**
Chlordane	Methylmercury
Aldrin	**Pharmaceuticals**
Dieldrin	Triglycidyl isocyanurate
Endrin	
p,p'-DDT	Valproic acid
p,p'-DDE	**Plasticizer**
Dimoxystrobin	Bisphenol A

Readiness Criteria	Score
Phase I	
Test system	9/10
Exposure scheme	3/3
Documentation / SOP	5/5
Main endpoints	3/4
Cytotoxicity	3.5/5
Test method controls	3/4
Data evaluation	4/4
30.5/35 Test method is close to ready or ready	
Phase II	
Testing strategy	2/4
Robustness	2.5/4
Test benchmarks	3/4
Prediction model	2.5/4
Applicability domain	1.5/3
11.5/19 Improvements required to be ready	
Phase III	
Screening hits	1/4
1/4 Substantial improvements required to be ready	

Figure 8: Overview of the NPC1_RAR_GR assay. A. Exposure setup of the NPC1_RAR_GR assay: Proliferating hNPC neurospheres were exposed to a test chemical in presence or absence of RAR and GR modulators for three days before hNPC proliferation was assessed by a BrdU ELISA. In addition, mitochondrial activity and cytotoxicity were assessed by performing Alamar Blue and LDH release assays to distinguish unspecific, cytotoxic effects from DNT effects. Benchmark concentrations (BMCs) were calculated for BrdU incorporation, cytotoxicity and mitochondrial activity **B.** Prediction model of the NPC1_RAR_GR assay: Following a tier 1 non-endocrine hNPC proliferation assay, a tier 2 endocrine hNPC proliferation assay is performed. Substances impairing proliferation in the tier 1 experiment are tested as putative RAR or GR agonists in presence of RAR and GR antagonists. Substances not affecting proliferation in the tier 1 experiment are tested as putative RAR or GR antagonists in

presence of RAR and GR agonists. **C.** List of chemicals screened in the NPC1_RAR_GR assay: A list of test chemicals was compiled and tested within the NPC1_RAR_GR assay revealing Aldrin, Dieldrin, p,p'-DDT, and p,p'-DDE as RAR or GR antagonists and valproic acid as RAR or GR agonists. Substances in gray did not affect hNPC proliferation, substances in blue were identified as non-endocrine DNT compounds reducing hNPC proliferation, substances in magenta were identified as RAR or GR antagonists, and substances in cyan were identified as RAR or GR agonists (adapted from manuscript 2.3) **D.** The readiness of the NPC1_RAR_GR assay was evaluated based on test readiness criteria published in (Bal-Price et al. 2018a). Abbreviations: BrdU, Bromodeoxyuridine; hNPC, human Neural progenitor cell; RAR, retinoic acid receptor; GR, glucocorticoid receptor; DNT, developmental neurotoxicity; SOP, Standard operating procedure. Created with Biorender.com

The NPC1_RAR_GR assay was evaluated for its readiness to proceed into pre-validation using test readiness criteria defined for NAMs in general and specifically targeting ED (Bal-Price et al. 2018a; Crouzet et al. 2023). This readiness evaluation focusses on the characterization of the test system, evaluation of the test method relevance for regulatory use, and underlying reliability and reproducibility (Bal-Price et al. 2018a; Crouzet et al. 2023). Using the test readiness criteria for the development of NAMs (Bal-Price et al. 2018a), the NPC1_RAR_GR assay was scored as ready or close to ready for validation, yet more chemicals need to be tested to yield more correctly identified EDCs using the assay (Fig. 8). A pre-validation of the NPC1_RAR_GR assay in cooperation with PEPPER, a French private-public platform for the pre-validation of test methods identifying endocrine disruptors, was initiated in order to assess its transferability into naïve laboratories prior to the main validation (Grignard et al. 2022; Gourmelon et al. 2024). This in combination with expanding the list of test chemicals will help build confidence in the suitability of test method for regulatory application in EDC hazard assessment (Parish et al. 2020; Patterson et al. 2021; Jacobs et al. 2024).

3.5 Application of NAMs in regulatory hazard and risk assessment

In order to use data from NAMs such as the DNT-IVB or the NPC1_RAR_GR assay for regulatory purposes, it is necessary to calculate points of departure (PoD) such as benchmark concentrations (BMC) for further evaluation. These PoDs are derived from concentration-response data and are interpreted using prediction models in a WoE-based approach by evaluating the outcomes of the different assays and integrating the results to classify compounds for the potential and potency to cause adverse effects (Hardy et al. 2017). This can be exemplified using the classifications for DNT compounds from the DNT-IVB. Compounds eliciting effects stronger than a predefined benchmark response (BMR) are classified as active, whereas compounds eliciting no effects or effects weaker than the BMR are defined as inactive. Moreover, effects accompanied by cytotoxicity at similar concentrations are defined as unspecific hits, since the MoA cannot be distinguished from effects caused by cytotoxicity (Masjosthusmann et al. 2020; Crofton and Mundy 2021; Blum et al. 2023). The data obtained from the different assays in the DNT IVB enables the clustering of the results to identify adverse effects on specific KNDPs. Using an IATA-based approach the test compounds is then either classified for a potential hazard or prioritized for further testing if applicable based on a WoE-approach (OECD 2017c).

To advance from hazard to risk characterization, additional considerations have to be made. These include the calculation of intracellular concentrations from the nominal concentration applied into the culture medium for the *in vitro* models which are influenced by for example evaporation, protein and plastic binding, cellular uptake, or lipophilicity and degradation of the test substance (Chang et al. 2022; Dimitrijevic et al. 2022). Moreover, to evaluate the margin of exposure between human exposure in the target tissue and in vitro PoDs, pharmacokinetic characteristics of the test compound need to be assessed, by investigating the absorption, distribution, metabolism, and excretion (ADME) characteristics. Different methods, including *in vitro* and *in silico* methods, investigating these characteristics can be assembled into physiologically based pharmacokinetic (PBPK) models which enable the estimation of the *in vivo* exposure and distribution of the test compound within target organs (Paini et al. 2019; Proença et al. 2021). These PBPK models use data from the physiology and anatomy of the investigated species, pharmacokinetics such as ADME and the physico-chemical properties of the test substance and estimate exposure in target tissues considering a specific route of administration and exposure scheme (Paini et al. 2019). The complexity of PBPK models depends on different parameters including the number of tissue compartments, transport processes, or metabolism (Bell et al. 2018). The data obtained from PBPK models can be used to reduce uncertainties due to default extrapolation factors in the calculation of health-based guidance values such as the acute reference dose (ARfD) or acceptable daily intake (ADI)). Moreover, the data can serve as a tool in quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) to estimate *in vivo* exposure concentrations in target tissues in combination with *in vitro* test method results (PoDs) and subsequently enables the comparison to human exposure data to assess the risk of a chemical (Bell et al. 2018; Paini et al. 2019; Niemann et al. 2023).

To investigate, whether the NPC1_RAR_GR assay can be utilized to detect EDCs in exposure relevant concentrations, first experiments were performed using compound concentrations ranging from 3 pM to 5 nM for Bisphenol A, 4,4-bisphenol F, monoethyl phthalate (MEP), monobenzyl phthalate (MBzP), diphenylphosphate, 3,5,6-trichloro-2-pyrinidol (TCP), and 3-phenoxybenzoic acid (3-PBA). The compounds chosen belong to different compound classes including phenols, plasticizers, and other short-lived compounds which had the highest contributions to a decreased IQ score observed within the Swedish SELMA birth cohort study. The fetal brain concentrations for the respective compounds of the mixture were calculated using PBPK modeling based on fetal urine sample measured within the SELMA study and were applied in the ratio of the compounds identified within the study (Bornehag et al. 2012; Tanner et al. 2020; Lupu et al. 2020).

While none of the seven compounds alone or as a mixture affected hNPC proliferation at exposure relevant concentrations, testing of higher concentrations is still missing which would allow to identify the general hazard potential of the compounds with regard to RAR or GR interference in proliferating hNPCs. Therefore, the lack of information on the comparison of *in vitro* test results at human exposure-relevant concentrations to higher concentrations in the micromolar range in manuscript 2.3 presents a comprehensive estimation of the DNT hazard potential of the tested chemicals. Combining the results from the NPC1_RAR_GR assay with methods from the OECD conceptual framework for testing and assessment of endocrine disruptors would enable the integration into a WoE-based approach to combine MoA-based information from lower-level assays with apical endpoints from higher level in vivo studies and hopefully eventually reduce the latter when more confidence has been gained into the predictivity of ED-DNT NAMs (OECD 2018c). The NPC1_RAR_GR could either serve as a follow-up assay within the DNT-IVB to test for ED-DNT or it could serve as a basis for a novel battery of test methods identifying EDCs in a similar approach as the DNT-IVB.

The field of ED encompasses a multitude of different signaling pathways, 14 of which were investigated within this work with a NAM being developed for two of these pathways in this work (RAR and GR) and additional ones in the ENDpoiNTs project. However, if these assays are to be used in an ED testing battery, preceding assays identifying hormone receptors as potential chemical targets could help to streamline and prioritize testing of relevant endocrine signaling pathways for the respective test compound. In a first tier, receptor binding and transactivation assays could serve as the basis for the prioritization of tier 2 assays depicting more complex, cellular key events associated with an adverse outcome in an IATA framework (OECD 2017c). Targeted tier 2 follow-up assays thus enable the identification of EDCs for their potential to disrupt specific hormone receptors identified in the tier 1 assay such as GR and RAR in the NPC1_RAR_GR assay on a more physiological level. Furthermore, developing a comprehensive list of reference chemicals to be tested in NAMs for ED-DNT assessment is of utmost importance to evaluate their applicability in EDC risk assessment and will serve as a foundation for the validation and development of the ED-DNT test battery. The development of a list of reference compounds for DNT can be used as a guideline utilizing peer-reviewed data focusing on compounds disrupting KNDPs linked to adverse outcomes. Here, to increase confidence in the data, results preferably observed in different test systems *in vitro* and *in vivo* were used (Aschner et al. 2017). In regards to ED, compiling compounds disrupting both a multitude of different hormone receptors and singular, defined receptors would enable the assessment of assays detecting a broader range of hormone receptors while also evaluating the specificity of the test method to identify disruptors of specific hormone receptors. In a similar approach to the DNT reference compounds, using data from different test systems and different species would increase confidence and evaluate the influence of species differences. Furthermore, this chemical list could in turn be used as a basis for *in silico* QSAR and read-across approaches to identify regions of concern in the chemical structure that drive the ED-potential of a test compound thus following current directions of toxicity and neurotoxicity assessment (Krewski et al. 2020; Crofton et al. 2022). Moreover, with the recent advances in AI, a reliable set of ED-DNT test chemicals could serve as a basis for leveraging AI and machine learning tools (ML) to advance the identification of EDCs disrupting human brain development (Aschner et al. 2022; Lin and Chou 2022).

4 Conclusion

This thesis highlights the potential of developing *in vitro* test methods for ED-DNT with high confidence in the scientific rationale by investigating the involvement of hormones and their receptors in KNDPs modelled in already established human-based DNT *in vitro* test methods. Here, test method development can build upon the previous knowledge on and confidence in the human-relevant representation of key processes of neurodevelopment *in vitro,* using a test system which has been successfully evaluated for its performance in identifying chemicals of DNT concern in multiple previous studies. Moreover, comprehensive investigations of species specificities regarding the involvement of hormones and their receptors in KNDPs, in this thesis and the current literature, question the predictivity of the rodent-based *in vivo* studies applied in EDC risk assessment for the human population. Moreover, broadening the study of hormone-sensitive KNDPs and the subsequent establishment of ED-DNT NAMs beyond EATS modalities, to include the plethora of hormone receptors expressed in the developing brain, will help reducing uncertainties in the current EDC risk assessment process by covering EDC MoAs to a larger extent. ED-DNT NAMs developed in this work can either complement the current DNT-IVB, which is insufficient in covering endocrine MoAs in its entirety, or be the starting point for the development of a defined approach including a testing battery of human-based *in vitro* and *in silico* ED-DNT NAMs.

5 Abstract

The human brain develops in the complex, spatiotemporal interplay of a plethora of intricate processes. These processes are highly susceptible to chemical insults which cause developmental neurotoxicity (DNT). The underlying processes of brain development are partially reliant on endocrine signaling pathways, whose disruption by endocrine disrupting chemicals (EDCs) is associated with adverse neurodevelopmental outcomes such as attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD). However, current EDC risk assessment is performed in animal studies and focuses on only few signaling pathways being estrogenic, androgenic, thyroidogenic, and steroidogenic (EATS) modalities thus creating a data gap regarding the effects of putative EDCs on other endocrine signaling pathways, such the retinoic acid receptor (RAR) or glucocorticoid receptor (GR) signaling. Moreover, inherent species differences with regards to brain development and hormonal involvement complicate the extrapolation from animal data to humans, and the resource and time-intensiveness of *in vivo* studies prevents the testing a high number of chemicals. This thesis focused on closing the scientific data gap regarding hormone-sensitive key neurodevelopmental processes (KNDPs) and developing New Approach Methodologies (NAMs) identifying EDCs causing endocrine disruption (ED)-mediated DNT. Here, this thesis applied the human Neurosphere Assay, a multiplexed, human-based NAM which models five different KNDPs and has been integrated into a DNT-*in vitro* battery (IVB) for DNT hazard assessment and chemical screening and prioritization. In manuscript 2.1 the Neurosphere Assay was scientifically validated to confirm the biological relevance of the test system and to evaluate its adequate responsiveness to physiological signaling pathway modulators and DNT compounds to increase confidence in its predictivity to detect DNT chemicals. The second manuscript (2.2) describes the efforts to close the scientific data gap on ED-DNT by investigating the effects of 14 endocrine signaling pathways on the KNDP modeled in the Neurosphere Assay in a comparative approach using rat and human-derived neural progenitor cells (NPCs). Modulation of 12 of the 14 signaling pathways affected at least one KNDP in human NPCs, with species differences being observed for nine of the pathways. Based on the newly identified hormone sensitive KNDPs, the NPC1_RAR_GR assay was developed to detect RAR- and GR-mediated proliferation arrest of human NPCs. Positive and negative controls were established and a set of putative EDCs was tested for their potential to disrupt RAR or GR signaling (manuscript 2.3). This thesis contributed to the scientific validation of the Neurosphere Assay and the advancing efforts to improve EDC risk assessment by the identification of KNDPs susceptible to EDC exposure and the development of a novel test methods capable of identifying chemicals causing ED-DNT by RAR and GR disruption.

6 Zusammenfassung

Das menschliche Gehirn entwickelt sich in einem komplexem räumlich-zeitlichen Zusammenspiel verschiedener Prozesse. Diese Prozesse sind anfällig für chemische Einflüsse, welche die Entstehung von Entwicklungsneurotoxizität (DNT) begünstigen. Die der Gehirnentwicklung zugrunde liegenden Prozesse sind teilweise von endokrinen Signalwegen abhängig. Eine Störung dieser Signalwege durch endokrine Disruptoren (EDCs) ist mit adverser neurologischer Entwicklung, wie Aufmerksamkeitsdefizit-Hyperaktivitätsstörung (ADHS) und Autismus-Spektrum-Störung (ASD) assoziiert. Die derzeitige Risikobewertung von EDCs erfolgt in Tierstudien und beschränkt sich auf wenige Signalwege, vornehmlich östrogene, androgene, thyroidale und steroidale (EATS) Modalitäten, wodurch eine Datenlücke in Bezug auf den Einfluss anderer endokriner Signalwege, wie dem Retinsäure-Rezeptor (RAR) und dem Glukokortikoid-Rezeptor (GR) Signalweg, entsteht. Zudem erschweren Speziesunterschiede eine Extrapolation der Daten von dem Tier auf den Menschen und der hohe Ressourcen- und Zeitaufwand der Tierstudien verhindert die Testung großer Mengen an Chemikalien. Hierfür wurde in dieser Arbeit der Fokus auf das Schließen der wissenschaftlichen Datenlücke im Feld der endokrinen Disruption (ED)- vermittelten DNT und auf die Entwicklung neuer alternativer Testmethoden (NAMs) zu Identifizierung von EDCs gelegt. Eine dieser Testmethoden basiert auf dem Neurosphärenassay, der es erlaubt fünf Schlüsselprozesse der Gehirnentwicklung (KNDP) zu modellieren. Dieser ist Teil der DNT-*in vitro* Batterie (DNT-IVB), einer Zusammenstellung von Testmethoden für die DNT Gefahrenbeurteilung und Testung von Chemikalien. In Manuskript 2.1 wurde hierzu der Neurosphärenassay wissenschaftlich validiert, um das Testsystem zu charakterisieren und die Reaktion auf spezifische Signalwege und definierte DNT-verursachen Substanzen zu bewerten und so die das Vertrauen in die Testmethoden zu erhöhen. Manuskript 2.2 beschreibt Versuche, um die wissenschaftliche Datenlücke in Bezug auf ED-DNT zu schließen. Hier wurde der Einfluss von 14 endokrinen Signalwegen auf KNDPs, die in dem Neurosphärenassay sowohl in neuralen Vorläuferzellen (NPC) der Ratte und des Menschen modelliert werden, untersucht. Veränderung der Aktivität von 12 der 14 Signalwege beeinflusste mindestens einen KNDP, wobei in neun dieser Signalwege Speziesunterschiede beobachtet wurden. Basierend auf diesen Beobachtungen wurde der NPC1_RAR_GR Assay entwickelt, um RAR- und GR-vermittelte Proliferationsstopps in humanen NPCs nachzuweisen (Manuskript 2.3). Es wurden Positiv- und Negativkontrollen etabliert und erste Substanzen getestet. Diese Arbeit leistete einen Beitrag zu der wissenschaftlichen Validierung des Neuosphärenassays und zur Entwicklung der Risikobewertung von EDCs durch die Identifizierung von ED-sensitiven KNDPs und der Entwicklung einer neuen Testmethode zu Identifizierung von ED-DNT

Abbreviations

3-PBA	3-phenoxybenzoic acid
ADHD	attention-deficit hyperactivity disorder
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, and excretion
AhR	aryl hydrocarbon receptor
AI	artificial intelligence
AOP	adverse Outcome Pathway
AR	androgen receptor
ARfD	acute reference dose
ASD	autism spectrum disorder
*at*RA	all-*trans* retinoic acid
BBB	blood-brain-barrier
BMC	benchmark concentration
BMR	benchmark response
CNN	convolutional neural network
CNS	central nervous system
DES	Diethylstilbestrol
DNA	deoxyribonucleic acid
DNT	developmental neurotoxicity
DNT-IVB	developmental neurotoxicity *in vitro* testing battery
EATS	estrogenic, androgenic, thyroidogenic, and steroidogenic
ED	endocrine disruption
EDC	endocrine disrupting chemical
ED-DNT	endocrine disruption-mediated developmental neurotoxicity
EGF	epidermal growth factor

EOGRTS	extended one generation toxicity study
EPA	Environmental Protection Agency
ER	estrogen receptor
FABP	fatty acid binding proteins
FASD	fetal alcohol spectrum disorder
FGF	fibroblast growth factor
GR	glucocorticoid receptor
GW	gestational week
hNPC	human-derived neural progenitor cell
IATA	Integrated approaches for testing and assessment
IUF	Leibniz – Research Institute for Environmental Medicine
KNDP	key neurodevelopmental process
LXR	liver X receptor
MBP	myelin basic protein
MBzP	monobenzyl phthalate
MEP	monoethyl phthalate
ML	machine learning
MoA	mode of Action
MSE	most sensitive endpoint
NGRA	next generation risk assessment
NPC	neural progenitor cell
NR	nuclear receptor
PBPK	physiologically-based pharmacokinetic
PCB	polychlorinated biphenyl
PDGFRA	platelet-derived growth factor alpha
PGE2R	prostaglandin E2 receptor

PoD	point of departure
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
QIVIVE	quantitative *in vitro* to *in vivo* extrapolation
QSAR	quantitative structure-activity relationship
RA	retinoic acid
RAR	retinoic acid receptor
rNPC	rat-derived neural progenitor cell
ROS	reactive oxygen species
RXR	retinoid X receptor
SHH	Sonic hedgehog
SOP	standard operating procedure
T3	Triiodothyronine
T4	Thyroxine
TBBPA	tetrabromobisphenol A
TCP	3,5,6-trichloro-2-pyrinidol
TDCIPP	tris(1,3-dichloro-2-propyl) phosphate
TG	test guideline
TH	thyroid hormone
THR	thyroid hormone receptor
TMPP	tris(methylphenyl) phosphate
UKN	University of Konstanz
US	United States
VDR	vitamin D receptor
WoE	weight of evidence

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Eidesstattliche Erklärung/Declaration

Hiermit versichere ich an Eides statt, dass die vorliegende Dissertation „Entwicklung von Testmethoden zur Identifizierung von endokriner Disruption-vermittelter Entwicklungsneurotoxizität“ von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis and der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder einer ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

I declare that I have developed and written the enclosed thesis ‘Test method development for the identification of endocrine disruption-mediated developmental neurotoxicity’ completely by myself, and have not used sources or means without declaration in the text. Any thoughts from others or literal quotations are clearly marked. The thesis was prepared in compliance with the principles of ‘Good Scientific Practice at the Heinrich-Heine-University Düsseldorf’. The thesis was not used in the same or in a similar version to achieve an academic grading elsewhere.

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