Mechanistic studies on the developmental neurotoxicity of polybrominated diphenyl ethers (PBDEs) in human and murine 3D *in vitro* models

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1. Introduction

1.1 Brain development

Brain development involves multiple complex processes including proliferation of neural progenitor cells (NPCs) and their maturation to neuronal and glial precursor cells. Precursor cells then differentiate into neurons or astrocytes and oligodendrocytes, the major cell types of the brain, while they migrate to their final position. After neurons reach their final position they form synapses and build neuronal networks. Insufficiently connected neurons undergo apoptosis. Oligodendrocytes and astrocytes are formed after neuronal differentiation and oligodendrocytes then insulate and myelinate the axons of neurons (Figure 1; and reviewed in Stiles and Jernigan 2010).

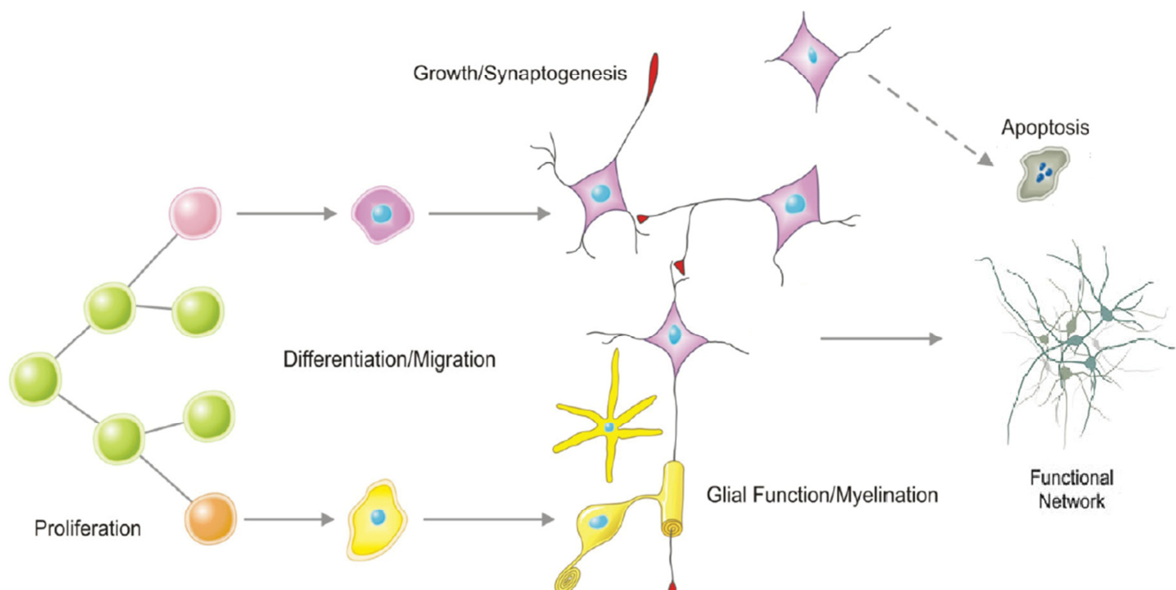

Figure 1: Scheme of cellular processes during brain development. Neural progenitor cells (green) proliferate and then migrate and differentiate into neurons (purple) and glial cells, oligodendrocytes and astroycytes, (yellow). Neurons form synapses (red) and functional networks while insufficient connected neurons undergo apoptosis (grey). Oligodendrocytes insulate and myelinate axons of neurons (with courtesy from William Mundy, US. EPA and John Havel, SRA International Inc).

Individual processes of brain development are highly complex and last from weeks up to years and persist until the puberty (Figure 2). It is mandatory that those processes take place in a concerted order and timing. Therefore, the developing brain is especially vulnerable towards chemicals disturbing specific processes over the whole time (Andersen 2003). However, the fetal brain is especially susceptible since the blood brain barrier (BBB) is not fully developed allowing also hydrophilic and ionic substances to enter the brain (Claudio et al. 2000; Pardridge 2006; Slikker 1994). Understanding the time course of the

development of brain structures and their function is crucial to understand the impact of various risk factors on brain development (Andersen 2003).

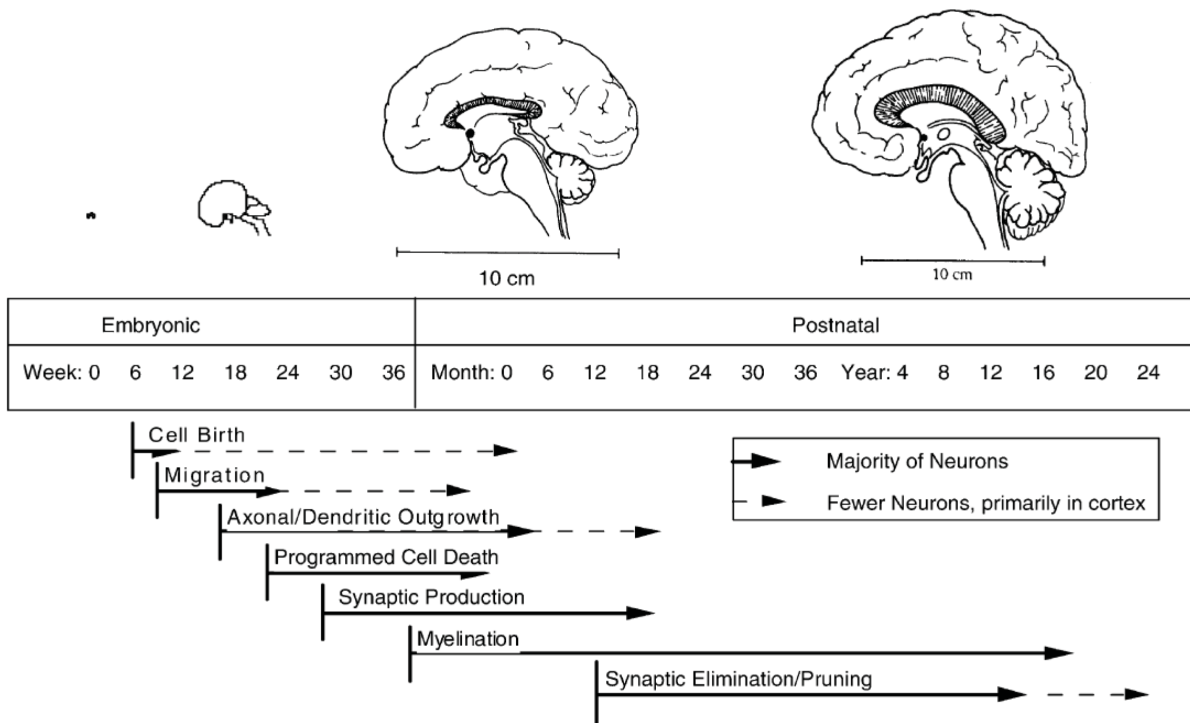

Figure 2: Stages of human brain development. Processes of human brain development (proliferation, migration, differentiation, apoptosis, synaptogenesis and myelination) are shown time-dependently (Andersen 2003).

1.1.1 Species-specific brain development.

A review by Rice and Barone (2000) compares rodent and human brain development. The gross regional development of the brain is comparable between humans and rodents and maturation of the nervous system in both species starts in the hindbrain and proceeds to the forebrain. Although the main processes of brain development like proliferation, migration, differentiation and apoptosis are conserved between humans (Figure 2) and rats (Figure 3), those processes occur in rodents in days or weeks while lasting in humans for months or years. Moreover, rodent neural development happens more postnatally than human neurodevelopment. Although the processes occur in both species within the same order, the overlap of the different processes during brain development is different between rodents and humans.

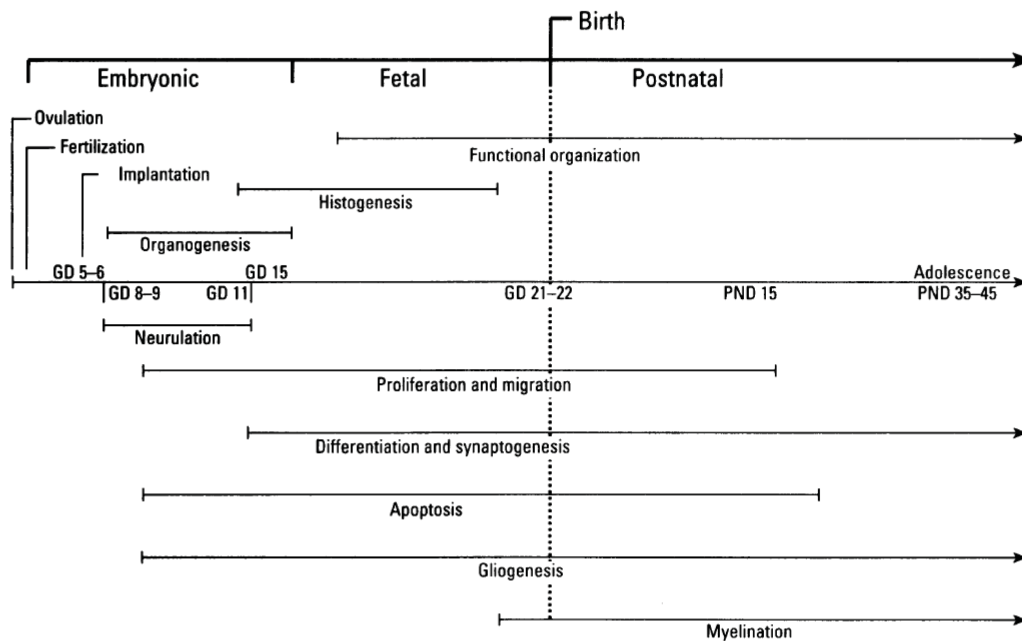

Figure 3: Timeline of rat brain development. Processes of rat brain development (proliferation, migration, differentiation, apoptosis, synaptogenesis and myelination) occur within days and most processes occur postnatal and last until adulthood (Rice and Barone 2000).

1.1.2 Role of thyroid hormones in brain development.

Thyroid hormones (THs), L-thyroxine (T4) and the more active form L-triiodothyronine (T3) are essential for proper brain development, since they are involved in basic processes like proliferation, migration, differentiation, myelination and synaptogenesis (reviewed in Anderson (2001); Bernal and Nunez (1995) and Bernal (2005)). Children who develop under severe thyroid hormone deficiency suffer postnatally from symptoms like severe mental retardation, spasticity, deaf-mutism and hearing loss, also known as endemic cretinism (Boyages and Halpern 1993; Cao et al. 1994; DeLong et al. 1985). Less severe TH deficiency can cause delayed mental and motor function and lower IQs (Haddow et al. 1999; Pop et al. 2003).

THs exert their effects by binding to thyroid hormone receptors (TRs). These are members of the nuclear receptor superfamily and contain a ligand and a DNA binding domain. Two human genes, THRA and THRB, and two murine genes, Thra and Thrb, encode TRα and TRβ isoforms (TRα1, TRα2, Δα1, Δα2; TRβ1, TRβ2, TRβ3, Δβ3) of which only four (TRα1, TRβ1, TRβ2 and TRβ3) contain both binding domains and are functional (Brent 2012; Harvey and Williams 2002), while TRβ3 was only found to be present in rodents. TRα1 is the major fetal isoform, the most abundant isoform in the brain and has a predominant role during brain development. TRβ1 is also present in many brain regions and mediates actions in the brain and the auditory system (Jones et al. 2003). TRs are present in all three major cell types of the brain, neurons, oligodendrocytes and astrocytes (Carlson et al. 1994, 1996;

Carre et al. 1998; Lebel et al. 1993; Puymirat et al. 1992; Strait et al. 1991). However, there is evidence that TRα is involved in earlier functions of brain development than TRβ (Jones et al. 2003). This observation is supported by the fact that TRα is present in both, oligodendrocyte progenitor cells and differentiated oligodendrocytes, and is involved in mediating the starting of oligodendrocyte differentiation while TRβ is only present in differentiated oligodendrocytes (Billon et al. 2002; Carre et al. 1998).

The classical way of TH action includes the transport of THs, T4 and T3, into the cell by transporters and binding of T3 to TRs that regulate as heterodimers with retinoic X receptor (RXR) the transcription of target genes (Figure 4). Furthermore, T3 and T4 are metabolized by deiodinases 2 and 3 (D2 and D3). TRs can also interact upon T3 binding with the p85 subunit of PI3 kinase (PI3K) and activate downstream signaling cascades like nitric oxide (NO) synthesis. T4 can directly influence the actin polymerization state and can activate mitogen-activated protein (MAP) kinase pathways upon integrin aVb3 binding (Horn and Heuer 2010).

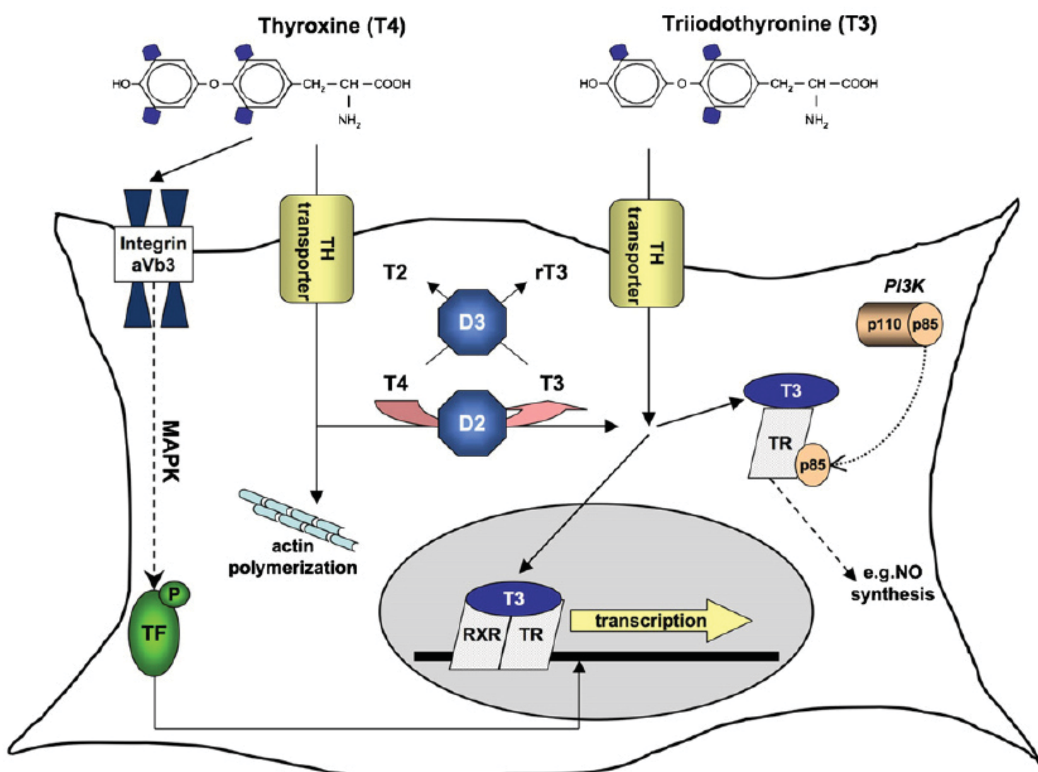

Figure 4: Classical and non-classical modes of thyroid hormone actions on target cell. Classical TH modes of actions include thyroid hormone transport into the cell, TR-mediated transcription in the nucleus and TH metabolism by deiodinases 2 and 3 (D2 and D3). Furthermore TH can activate downstream signaling cascades, influence the actin polymerization state and activate MAP kinase pathways (non-classical pathways) (Horn and Heuer 2010).

Although it is known that TH influences basic processes of brain development and considerable progress in the understanding of TH signaling pathways was achieved, the underlying molecular mechanisms of THs' effects on different processes of brain development remain elusive.

1.2 Developmental Neurotoxicity (DNT)

1.2.1 Developmental neurotoxicity testing

Substances, which after prenatal or postnatal exposure affect the developing nervous system by leading to pathological changes in the developing brain, are defined as developmentally neurotoxic (Slikker 1994). Effects on the developing brain result in neurological deficits in children like lower IQs, learning disability and mental retardation causing high costs for the whole society due to medication and individual support (Bellanger et al. 2013; Landrigan et al. 2002). The Mt. Sinai Children's Environmental Health Care center investigated that a reduction of the mean society intelligence quotient of 5 points leads to an increase in the number of mentally retarded people of 57%. Of 214 known neurotoxic compounds for humans in total, 12 are known to be developmentally neurotoxic: Lead, methylmercury, polychlorinated biphenyls (PCB), arsenic, toluene, ethanol, manganese, fluoride, chlorpyrifos, dichloro-diphenyltrichloroethane, tetrachloroethylene and polybrominated diphenyl ethers (PBDE) (Grandjean and Landrigan 2014). The last six compounds were classified between 2006 and 2014 (Grandjean and Landrigan 2014). The extensive data gap on the rest of the chemical universe is attributed to the fact that regulatory agency's demand for DNT testing is limited to neurotoxic or endocrine disrupting chemicals in Europe, while in the USA additionally all pesticides have to be tested.

Developmental neurotoxicity testing according to the current guidelines of OECD (Testing Guideline 426) and US EPA (OPPTS 870.6300) is performed in animal experiments (OECD 2007; USEPA 1998). Those animal studies are very time-consuming, cost-intensive and questionable from an ethical point of view: testing of one substance implies the use of 140 dams and 1000 pubs (Crofton et al. 2012; Lein et al. 2005). Within the last two decades only 100 chemicals were tested according to OECD guideline 426 (Makris et al. 2009). Moreover, species differences are a major problem in toxicology and lead up to 40% false classification of compounds (Hartung 2009; Leist and Hartung 2013). Due to the high costs and ethical concerns of animal models and their possibly low predictivity for humans a lot of effort has been put into the development of new approaches for DNT testing (Coecke et al. 2007; Lein

et al. 2005, 2007), which imply refinement, reduction and replacement (3Rs) of animal experiments. The 3R principle was developed by Russel and Burch (Russel et al. 1959).

1.2.2 Paradigm shift in toxicology

In 2007 the US National Research Council (NRC) released a report 'Toxicity Testing in the 21st Century: A Vision and a strategy' (NRC 2007). They recommended the development of new testing methods and models for human risk and hazard assessment which improve the throughput by combining novel technologies like e.g. *in silico* methods with *in vitro* based cell models or model organisms (*c. elegans*, zebrafish and *drosophila*) instead of reliance on expensive, time-consuming and ethically questionable animal models. The new testing paradigm is focused on mechanistic evaluations of critical toxicity pathways instead of apical endpoints assessment in *in vivo* animal studies. The novel technologies to screen for critical toxicity pathways comprise toxicogenomics, bioinformatics, high-throughput screening (HTS) assays and systems biology.

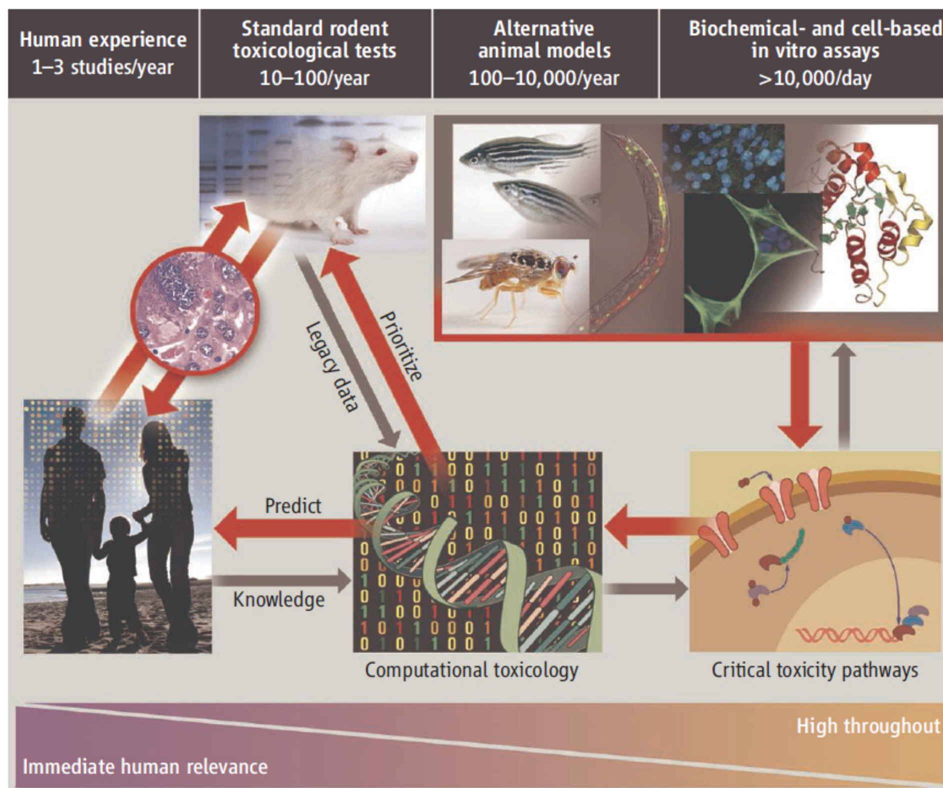

Figure 5: Paradigm shift in toxicology. Within Tox21 a paradigm shift in toxicology is proposed away from animal models towards human-based *in vitro* models. By a combination of *in vitro* systems and novel technologies the compound throughput as well as the hazard prediction will be improved. Furthermore this approach allows to overcome species-differences and reduces animal numbers according to the 3R principle. *In vitro* data in combination with computational toxicology will enable the prioritization of chemicals which need to be tested in rodents and improve human risk assessment (Collins et al. 2008).

Obtained data is analyzed using *in silico* methods like (quantitative) structure relationships ((Q)SAR) and read across for substance priorization for further animal experiments and to elucidate the underlying mode of action (MOA). Furthermore, the comparability of the human and rodent situation is addressed by comparing human *in vitro* data with legacy *in vivo* data from rodents (Collins et al. 2008; Crofton et al. 2012; Gibb 2008 and NRC 2007). Figure 5 visualizes this new toxicity testing approach.
In this context the US EPA started the ToxCast21 program, in which molecular and pathway perturbations caused by environmental chemicals are evaluated by utilizing new techniques like computational chemistry and HTS in combination with *in vitro* models. The aim is to test chemicals with methods, which allow hazard identification to human health and further prioritization for *in vivo* testing (Dix et al. 2007; Judson et al. 2010).

One possibility for implementing molecular mechanisms obtained from the above described strategy into risk assessment is the 'adverse outcome pathway' (AOP) concept (Ankley et al. 2010). An AOP describes the toxicity of a compound starting from the molecular initiating event (MIE) and ending with the adverse outcome (AO) for an individual or the population by linking causally related key events (KE) on different stages of biological organization (OECD 2013) (Figure 6).

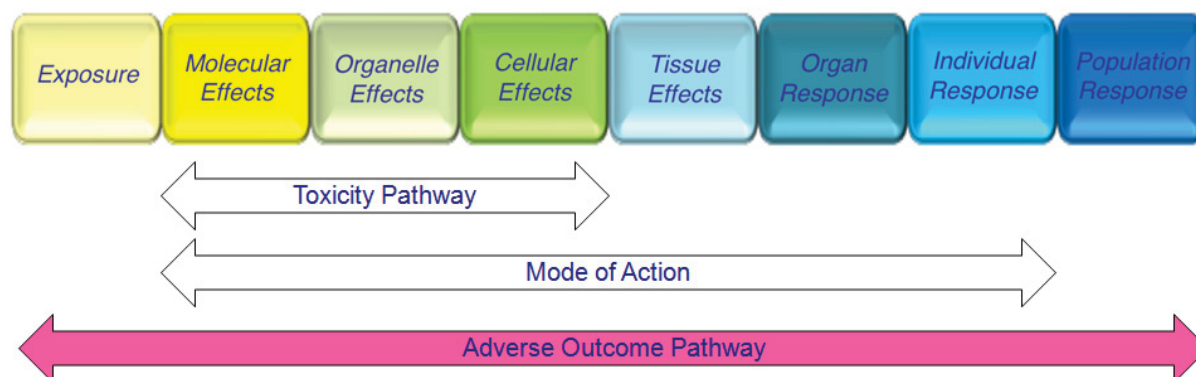

Figure 6: Schematic illustration of the AOP concept. A molecular effect after chemical exposure leads to responses on the different biological organization stages and in the end to an adverse outcome on the individual or the population (modified after Maurice Whelan, Joint Research Center).

The aim of the AOP concept is to achieve a better and more predictive hazard characterization as well as a better classification of the chemicals humans are exposed to.
AOPs deliver valuable additions to current testing strategies. First, MIEs shared by different compounds can be used in read across approaches for substance categorization, and second, KEs can be used for the development of biomarkers (Andersen et al. 2012; OECD 2013).

In order to obtain physiologically relevant data, based on a KE defined within an AOP, the applied *in vitro* system has to reflect the human *in vivo* situation as precisely as possible. Furthermore, a comparison of human and rodents within the AOP allows identification of species differences in toxicologically relevant pathways. Species-specific based elucidation of involved toxicity pathways should contribute to reduce uncertainty in hazard assessment (Bal-Price et al. 2015a).

Development of AOPs for DNT is extremely challenging since many complex processes are involved in brain development, which last over a long time period (see Chapter 1.1). This implies that timing and duration of exposure determines the effects of chemicals on brain development. Although a large number of cellular and molecular processes, which are important for brain development, are known, there are only few examples of well-documented pathways that include causally linked MIEs and KEs that result in an observed AO (Bal-Price et al. 2015b).

1.2.3 Primary neural progenitor cells as *in vitro* models for studying DNT

To date, the mouse embryonic stem cell test (EST) is the only *in vitro* test based on mammalian cells successfully validated as an alternative for *in vivo* DNT testing (Coecke et al. 2007; Genschow et al. 2002). Other rodent based *in vitro* models, which are considered as models for testing DNT are organotypic cultures (embryonic brain or spinal cord tissue), rotating brain cell cultures and primary dissociated cultures. The detailed advantages and disadvantages of each system are summarized in Coecke et al. (2007). The advantage of primary cells isolated from rodent brain tissue and cultured *in vitro* is the maintenance of the signaling functions and responses towards xenobiotics (Burke et al. 2006; Foti et al. 2013; Go et al. 2012; He et al. 2010; Simpson et al. 2011). However, the main limitation of all those models is their rodent origin in regard to species differences towards humans. Furthermore, human immortalized cells, tumor cell lines or transformed cells, which differentiate into neuronal cells, were used to test developmental neurotoxicity (Abdulla et al. 1995; Hong et al. 2003; Påhlman et al. 1990; Scholz et al. 2011; Stern et al. 2014). Those cells are of human origin, but the main concern about those immortalized cells is that they are not comparable to “normal” cells and results are therefore difficult to interpret (Coecke et al. 2007). Primary stem or progenitor cells, which can be generated from human tissue, become more prominent in the field of DNT testing (Baumann et al. 2014; Fritsche 2014; Hayess et al. 2013).

The ‘Neurosphere Assay’ (Figure 7) is a three-dimensional (3D) primary *in vitro* cell culture model based on normal neural progenitor cells (NPCs), which mimics basic processes of fetal brain development like proliferation, differentiation, migration and apoptosis (Baumann

et al. 2014; Breier et al. 2010; Moors et al. 2009). Those NPCs are cultured as 3D cell aggregates, so called ‘neurospheres’. NPCs can be generated from full brain homogenates of human or rodent fetuses and therefore allow the comparison of substance effects between species in very similar cell systems. Recently, it has been brought to attention that 3D cultures maintain physiological conditions better than 2D cell systems (Cukierman et al. 2001; Yamada and Cukierman 2007) supporting usage of NPCs growing in a 3D fashion (Alépée 2014).

The ‘Neurosphere Assay’ is described in Baumann et al. (2014) in detail. In the presence of growth factors like epidermal growth factor (EGF) and fibroblast growth factor (FGF) (proliferating conditions) NPCs proliferate in suspension and form 3D neurospheres (Buc-Caron 1995; Chalmers-Redman et al. 1997; Reynolds et al. 1992; Svendsen et al. 1995). NPCs maintain their proliferative character over months in culture and can be passaged mechanically with a tissue chopper (Svendsen et al. 1997).

In absence of growth factors and in presence of an extracellular matrix consisting of poly-D-lysine (PDL) and laminin (differentiating conditions) NPCs radially migrate out of the neurosphere core and differentiate into the three major cell types of the brain neurons (βIII-tubulin$^+$), oligodendrocytes (O4$^+$) and astrocytes (GFAP$^+$) (Brannen and Sugaya 2000; Lobo et al. 2003; Piper et al. 2001; Reubinoff et al. 2001). The ratio of neurons and glia cells in the migration area seems to reflect the human physiological *in vivo* situation (Baumann et al. 2014). Furthermore NPCs can undergo caspase-dependent or -independent apoptosis (Moors et al. 2009).

The ‘Neurosphere Assay’ allows studying effects of substances on basic processes of fetal brain development. Effects on proliferation are assessed by measuring the neurosphere diameter increase or by measuring bromodeoxyuridine (BrdU) incorporation into the DNA. Migration is determined by measuring the distance between the neurosphere core and the furthest migrated cells. Differentiation is assessed after fixation of the cells by performing immunocytochemical stainings for above named markers and a counterstaining with Hoechst to visualize cell nuclei. Then the percentage of positive cells per nuclei is determined.

To distinguish effects on proliferation, migration or differentiation from general cytotoxicity an AlamarBlue-Assay (CellTiter Blue, Promega) is performed, which measures viability by detecting mitochondrial activity.

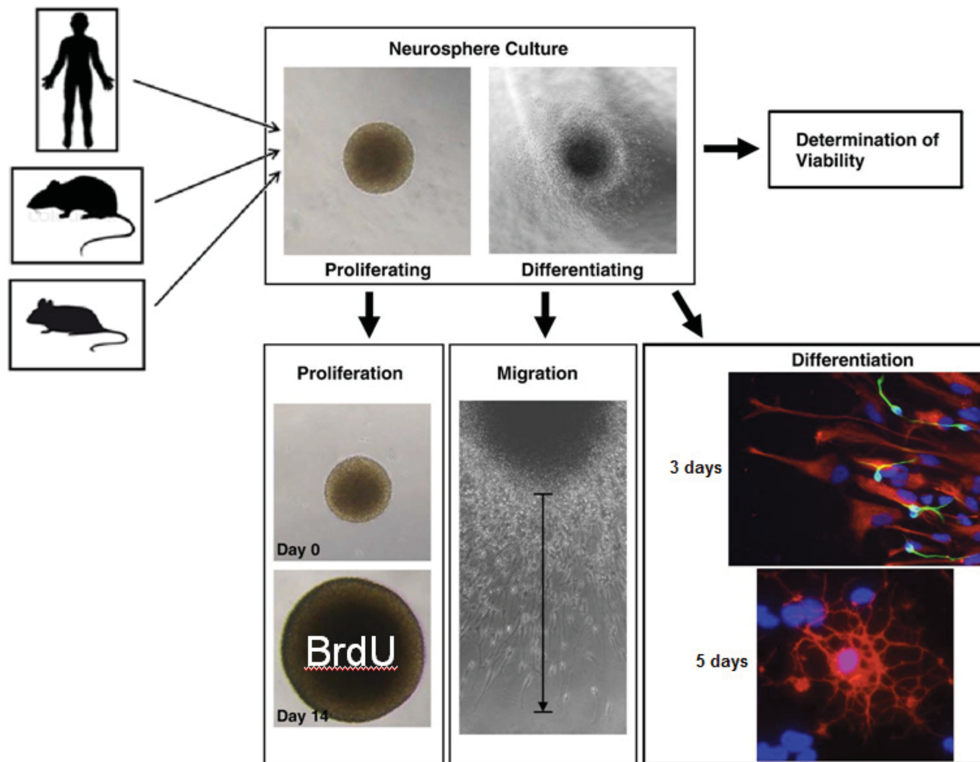

Figure 7: The 'Neurosphere Assay'. (modified from Breier et al. (2010)). Neural progenitor cells (NPCs) are cultured as floating neurospheres. They mimic basic processes of brain development like proliferation, migration and differentiation into the three main brain cell types: neurons, oligodendrocytes and astrocytes. In presence of growth factors NPCs proliferate and proliferation is assessed by measuring the diameter increase or BrdU incorporation. In absence of growth factors neurospheres settle down on PDL/laminin coated surfaces and cells start to radially migrate out and differentiate. Migration is determined by measuring the distance between the neurosphere core and the furthest migrated cells. Neurons, oligodendrocytes and astrocytes can be visualized by immunocytochemical staining with βIII-tubulin, O4 or GFAP antibodies and a counterstaining with Hoechst for nuclei.

Species differences can be studied in very similar cell culture models by comparing compound effects on human NPCs (GW 16-19) with those on PND1-derived rodent NPCs reflecting similar developing stages (www.translatingtime.net; Workman et al. 2013). In a parallelogram approach rodent and human *in vitro* data generated with equivalent cell systems (e.g. the 'Neurosphere Assay') can be compared with regards to species-specific susceptibilities as well as species-specific involved toxicity pathways. Furthermore, rodent *in vitro* data can be compared to rodent *in vivo* data from the published literature to elucidate *in vitro/in vivo* similarities and differences. This approach allows the extrapolation of rodent *in vivo* and human *in vitro* effects to the human *in vivo* situation (Figure 8).

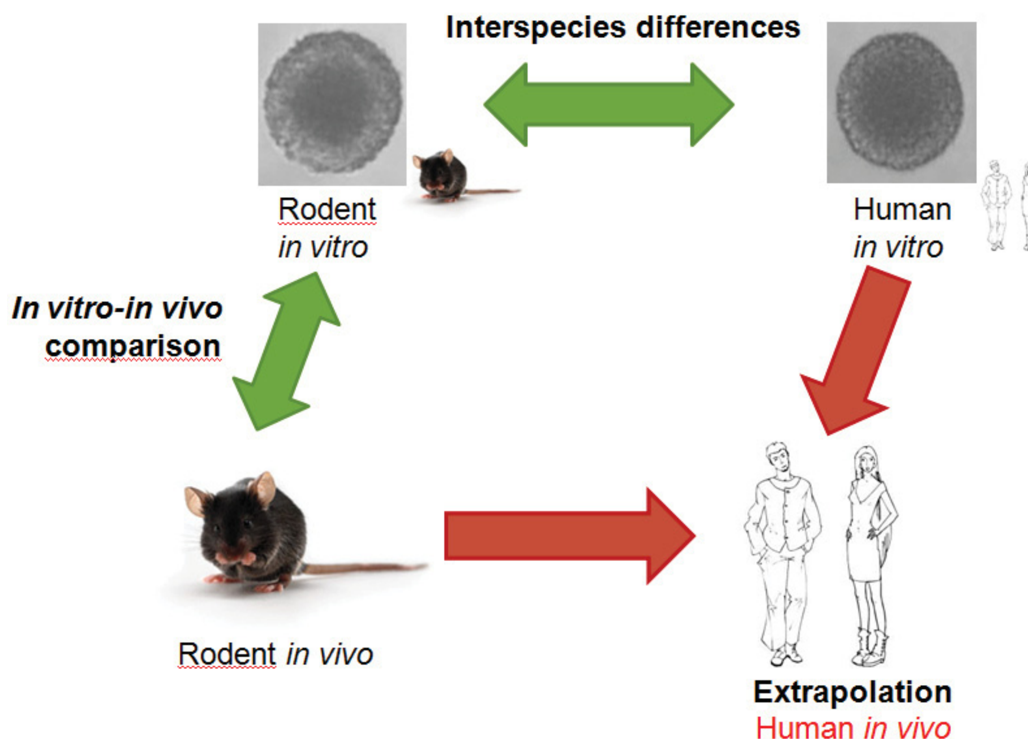

Figure 8: Parallelogram approach for extrapolation to the human *in vivo* situation. Rodent and human *in vitro* data generated in an equivalent cell system is compared to determine species differences in susceptibility as well as in involved toxicity pathways. Rodent *in vitro* data is compared to rodent *in vivo* data to elucidate *in vitro/in vivo* differences or similarities. This approach allows the extrapolation to the human *in vivo* situation.

A training set of nine chemicals, six DNT positive compounds and three DNT negative compounds, was tested by using the 'Neurosphere Assay' (Baumann et al. 2015). The 'Neurosphere Assay' was able to classify most of the chemicals correctly compared to literature data on internal exposure and it was observed that rodent and human NPCs strongly differed in susceptibility towards chemicals as well as their most sensitive endpoints. However, the known DNT compound chlorpyrifos was not detected as developmental neurotoxic by the 'Neurosphere Assay'. The reason might be that the 'Neurosphere Assay' only mimics early fetal brain development but not embryonic or later fetal brain development (Figure 9) and chlorpyrifos might act on earlier phases of brain development (embryonic) or on later fetal neurodevelopmental endpoints such as axon and dendrite formation or synaptogenesis. Although the 'Neurosphere Assay' is not able to cover all stages of brain development by itself it is an appropriate candidate to be included into a proposed DNT strategy consisting of several cell systems covering different stages and processes of brain development (Figure 9; Baumann et al. 2015).

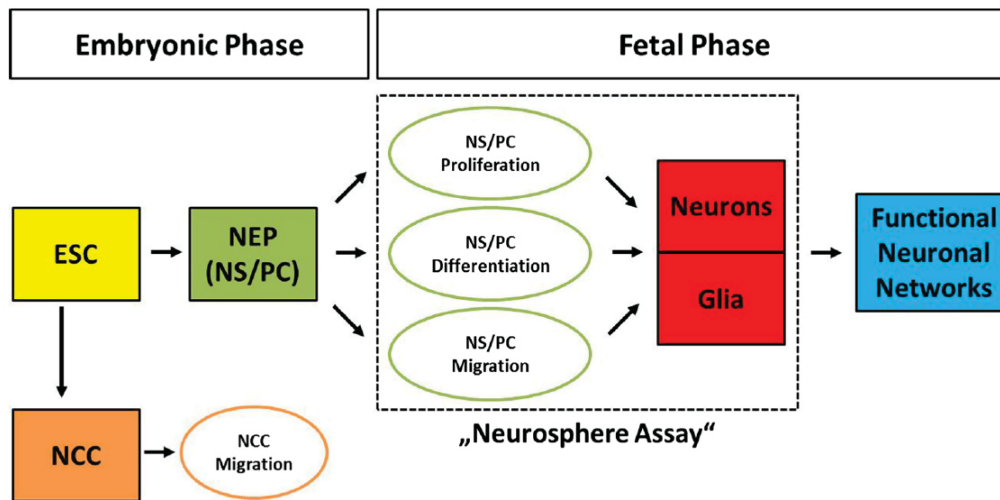

Figure 9: Testing strategy for *in vitro* DNT testing. The endpoints evaluated within the 'Neurosphere Assay' cover early fetal brain development. For assessment of all brain development stages a combination of different cell systems is needed. Embryonic brain development can be evaluated with embryonic stem cells (ESCs), neural crest cells (NCCs) and neuroepithelial precursors (NEPs) and later fetal brain by measuring neuronal activity on multielectrode arrays (MEAs) (Baumann et al. 2015).

1.3 Polybrominated diphenyl ethers (PBDEs)

Of the 214 known neurotoxic compounds for humans only 12 are known be developmentally neurotoxic (Grandjean and Landrigan 2014). Those include polybrominated diphenyl ethers (PBDEs), which were lately classified as human DNT compounds (Grandjean and Landrigan 2014). PBDEs are brominated flame retardants and were widely used in a lot of consumer products like electronics, plastics and textiles (Alaee et al. 2003).

1.3.1 Chemical properties of PBDEs

The chemical structure of PBDEs consists of two benzene rings bound by an oxygen atom (diphenyl ether) and between 1 to 10 (mono to deca) bromine atoms bound to the benzene rings (Figure 10). Their molecular formula is $C_{12}H_{(10-(m+n))}Br_{(m+n)}O$ with m+n = 1-10. PBDEs are numbered from BDE-1 to BDE-209 depending on their degree of bromination and bromine positions. In case of fire the relatively weak bromine bond is cleaved and bromine radicals scavenge oxygen radicals.

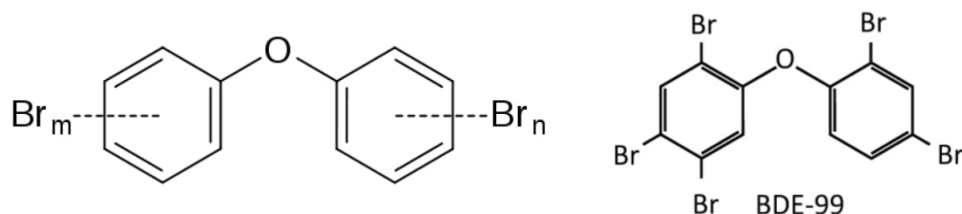

Figure 10: General structure of PBDEs and of the penta-brominated BDE-99 used within this thesis. PBDEs consist of two benzene rings bound by an oxygen atom (diphenyl ether) and contain between 1 and 10 bromine atoms.

1.3.2 PBDEs in the environment and human exposure

PBDEs are additive flames retardants, which are not chemically bound to the polymer matrix. Therefore, they leak out of the matrix and accumulate in the environment where they are found in water, air, sediments, soil, house dust, food, animal and human tissues (Darnerud et al. 2001; de Wit 2002; Law et al. 2006). Although they were mostly banned from the markets, they remain present in the environment (Law et al. 2014; Ma et al. 2013; Shaw and Kannan 2009; Yogui and Sericano 2009). Main exposure sources are house dust and the food chain. Among food, fish contains the highest PBDE concentration followed by meat and fatty products like butter, fat and oils (reviewed by Frederiksen et al. 2009). Cord blood and breast milk, which present high levels of PBDEs, are an additional exposure source for fetuses and babies (reviewed by Frederiksen et al. 2009 and Sjödin et al. 2003). Toddlers have the highest PBDE body burdens due to exposure by house dust and breast milk intake compared to babies, older children and adults (Fischer et al. 2006; Rose et al. 2010; Toms et al. 2009). PBDE exposure is one to two orders of magnitude higher in the USA than in Europe or Asia (reviewed by Frederiksen et al. 2009). Five congeners, tetra-, penta- and hexa-BDE congeners (PBDE-47, -99, -100, -153, and -154), predominate in human tissues and usually account for more than 90% of the total PBDE body burden in most individuals who are not occupationally exposed (McDonald 2005).

1.3.3 Developmental neurotoxicity of PBDEs

The current DNT testing guidelines, OECD (Testing Guideline 426) and US EPA (OPPTS 870.6300), demand for testing in rats (OECD 2007; USEPA 1998). Rodent studies revealed that prenatal and/or postnatal exposure to different PBDEs can cause long-lasting behavioral abnormalities, impair learning and memory and cause hyperactivity (e.g. Branchi et al. 2002; Dufault et al. 2005; Gee et al. 2008; Kodavanti et al. 2010; Suvorov et al. 2009; Viberg et al. 2003, 2004). Epidemiological studies found that high PBDE exposure during pre- and postnatal brain development influences motor function, behavior, attention and

cognition in children at school age and correlated high PBDE exposure with hyperactivity and lower IQs (Chao et al. 2007; Eskenazi et al. 2013; Roze et al. 2009; Shy et al. 2011).
Although several studies have shown the DNT potential of PBDEs, the mechanisms of PBDE-induced DNT remain elusive (reviewed by Costa et al. (2014)). It was observed that PBDE treated animals show the same behavioral abnormalities than animals suffering from hypothyroidism during brain development (Negishi et al. 2005). Costa et al. (2014) therefore discussed two MOAs for PBDE-induced DNT: first, interference of PBDEs with THs/TH signaling and second non TH-related effects on brain cells (reactive oxygen species (ROS) formation, DNA damage, apoptosis, interference with Ca^{2+}-signaling and neurotransmitter systems) .
Many studies reported decreased T4 and increased thyroid-stimulating hormone (TSH) levels in rodents after developmental exposure to different PBDEs (reviewed by Costa et al. (2014)) and also for humans PBDE exposure was correlated with changes in TH and TSH levels. However, human data were inconsistent: decreased, increased or unchanged T4 levels and increased or decreased TSH blood levels were reported (Chevrier et al. 2011; Herbstman et al. 2008; Stapleton et al. 2011; Zota et al. 2011). Moreover, it was shown that hydroxylated but not parent PBDEs can interfere with TH transport by binding to the TH transport protein transthyretin (TTR; Hamers et al. 2006; Meerts et al. 2000). Additionally, hydroxylated, but not parent PBDEs, can directly bind to TRs (Hamers et al. 2006; Kojima et al. 2009; Li et al. 2010; Ren et al. 2013; Suvorov et al. 2011). Lower brominated PBDEs are TR agonists while higher brominated congeners are TR antagonists (Ren et al. 2013). Furthermore, PBDEs deregulate the expression of genes encoding TR isoforms (Blanco et al. 2011; Lema et al. 2008) and interfere with T(H,R)-mediated transcription (Blanco et al. 2011; Ibhazehiebo et al. 2011; Lema et al. 2008). However, here the results were contradictory dependent on the studied congener, the cell system and the used method.

1.4 Aim of this thesis

DNT studies are routinely performed in rats, which are cost and time intensive, ethically questionable and often not predictive for humans. Stakeholders agreed on the need to move away from those animal models towards *in vitro* models, which also allow the identification of toxicity pathways and consider species differences. The ‘Neurosphere Assay’ is a promising tool for *in vitro* DNT evaluation since it mimics several basic processes of brain development and allows studying cultures generated from different species. PBDEs were classified to be developmental neurotoxic in humans and rodents. However, the mechanisms how PBDEs induce DNT are poorly understood. Since THs are involved in many processes of brain development and PBDEs alter TH levels in rodents and humans, also interaction with TH signaling is discussed as a mechanism for PBDE-induced DNT. Therefore, the overall aim of this thesis was to investigate whether TH disruption is involved in PBDE effects on basic processes of fetal brain development. Human and murine NPCs were used to compare the molecular mechanisms of PBDE-induced DNT between species. BDE-99 was chosen since it is one of the most abundant congeners found in human tissue. The following questions were addressed:

1. Establishment of the mouse neurosphere culture.
2. Investigation of the involvement of THs in basic processes of human and murine brain development and characterization of neurospheres as appropriate models to study TH signaling.
3. Elucidation of the influence of BDE-99 on basic processes of brain development in human and murine neurospheres and comparison of the sensitivity towards BDE-99 between species.
4. Establishment of a test assay to identify human- and mouse-specific TH disruptors and application of this assay to assess TH-disruption potential of BDE-99 on human and mouse neurospheres.

2. Manuscripts

The publications, which emerged from this thesis, are attached below.

The first publication ‘Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations’ (manuscript 2.1) is a book chapter comparing basic fetal processes of brain development (proliferation, migration and differentiation) between human and rodent (rat, mouse) neural progenitor cells (NPCs). Within this publication for the first time the murine NPC system was characterized by employing data generated within this thesis. The publication explains how those processes of brain development are analyzed within the ‘Neurosphere Assay’ for developmental neurotoxicity (DNT). Furthermore, the relevance of this cell model for modeling *in vivo* brain development is demonstrated and the strengths and limitations of the ‘Neurosphere Assay’ as a DNT testing model versus existing approaches are critically discussed.

The second publication ‘BDE-99 impairs human and mouse oligodendrogenesis by species-specific modes of action’ (manuscript 2.2; original work) describes BDE-99 effects on oligodendrocyte formation and maturation of human and murine NPCs and emphasizes species differences. This publication is based on the newly established murine NPC system characterized in manuscript 2.1. The focus lies on the investigation whether BDE-99 affects oligodendrogenesis by TH disruption. Susceptibility towards BDE-99-impaired oligodendrogenesis as well as its mode of action differed between both species.

2.1 Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations

Jenny Baumann*, **Katharina Dach*,** Marta Barenys, Susanne Giersiefer, Janette Goniwiecha, Pamela J. Lein, and Ellen Fritsche

* shared first-authorship

Mechanisms and Predictive Modeling with *In vitro* and *In vivo* Approaches
(book chapter) (*in press*, corrected author’s proof was sent back in August)

Die Gehirnentwicklung ist durch ein Zusammenspiel von komplexen Prozessen, die in einer geregelten zeitlichen Abfolge geschehen, gekennzeichnet. Diese Prozesse werden in frühe (embryonale) und späte (fetale) Prozesse unterteilt.
Während der embryonalen Organentwicklung werden durch Differenzierung von embryonalen Stammzellen zu neuroepithelialen Vorläuferzellen, die die neurale Stamm-/Progenitorpopulation bilden, die Neuralplatte und das Neuralrohr gebildet. Wir verwenden neurale Stamm-/Progenitorzellen vom Menschen und vom Nager, die als Neurosphären kultiviert werden, um Prozesse der fetalen Gehirnentwicklung (Proliferation, Migration und Differenzierung) zwischen den Spezies zu vergleichen und um adverse Effekte von Chemikalien auf diese Prozesse zu untersuchen. Wir beschreiben, wie diese neuroentwicklungsrelevanten Prozesse im „Neurosphären Assay“ für die *in vitro* Entwicklungsneurotoxizitätstestung untersucht werden. Dabei betonen wir endpunkt-spezifische Kontrollen für die einzelnen Prozesse und zeigen durch deren Vergleich mit der bekannten *in vivo* Situation, dass *in vitro* Ergebnisse die *in vivo* Situation widerspiegeln. Außerdem beschreiben wir drei Methoden, um transgene humane Neurosphären für biomolekulare Grundlagenforschung zu generieren. Die Daten werden kritisch diskutiert, indem die Stärken und Grenzen des „Neurosphären Assays“ für die Beurteilung des Gefährdungspotentials von Chemikalien für die Gehirnentwicklung hervorgehoben werden.

Metadata of the chapter that will be visualized online

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Abstract	Brain development is an integration of developmental processes that take place in a time-dependent manner. These processes can be divided into early (embryonic) and late (fetal) neurodevelopmental events. During embryonic organogenesis, the neural plate and neural tube are formed by embryonic stem cell differentiation into neuroepithelial precursors, which represent the neural stem/progenitor cell population of the developing brain. We use neural stem/progenitor cells of humans and rodents growing as neurospheres to investigate fetal neurodevelopmental events (proliferation, migration, and differentiation) across species and to assess adverse effects of chemicals on these processes. We describe how these specific neurodevelopmental processes are analyzed within the "Neurosphere Assay" for developmental neurotoxicity testing in vitro. Thereby, we emphasize endpoint-specific controls for these processes and relate those to the in vivo situation to demonstrate how in vitro outcomes reflect the actual in vivo situation for developmental neurotoxicity. In addition, we describe three methods for creating transgenic human neurospheres for basic biomolecular research. These data are critically discussed by pointing out strengths and limitations of the "Neurosphere Assay" for developmental neurotoxicity hazard assessment.

Keywords (separated by '-')	Developmental neurotoxicity - Neural progenitor cell - Brain development - Species differences - Human - Mouse - Rat - In vitro

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Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations

Jenny Baumann*, Katharina Dach*, Marta Barenys, Susanne Giersiefer, Janette Goniwiecha, Pamela J. Lein, and Ellen Fritsche

Abstract

Brain development is an integration of developmental processes that take place in a time-dependent manner. These processes can be divided into early (embryonic) and late (fetal) neurodevelopmental events. During embryonic organogenesis, the neural plate and neural tube are formed by embryonic stem cell differentiation into neuroepithelial precursors, which represent the neural stem/progenitor cell population of the developing brain. We use neural stem/progenitor cells of humans and rodents growing as neurospheres to investigate fetal neurodevelopmental events (proliferation, migration, and differentiation) across species and to assess adverse effects of chemicals on these processes. We describe how these specific neurodevelopmental processes are analyzed within the "Neurosphere Assay" for developmental neurotoxicity testing in vitro. Thereby, we emphasize endpoint-specific controls for these processes and relate those to the in vivo situation to demonstrate how in vitro outcomes reflect the actual in vivo situation for developmental neurotoxicity. In addition, we describe three methods for creating transgenic human neurospheres for basic biomolecular research. These data are critically discussed by pointing out strengths and limitations of the "Neurosphere Assay" for developmental neurotoxicity hazard assessment.

Keywords: Developmental neurotoxicity, Neural progenitor cell, Brain development, Species differences, Human, Mouse, Rat, In vitro

1 Introduction

Brain development is an integration of developmental processes orchestrated in a time-dependent manner (reviewed in refs. [1, 2]). These processes can be divided into early (embryonic) and late (fetal) neurodevelopmental events. During embryonic organogenesis, the neural plate and neural tube are formed by embryonic stem cell (ESC) differentiation into neuroepithelial precursors (NEP), which represent the neural stem/progenitor cell (NS/PC) population of the developing brain. During the same period of time, neural crest cells (NCC) also develop, which are precursor cells that give rise to peripheral sensory neurons and glia in addition to other peripheral cell types (reviewed in ref. [3]).

*Author contributed equally with all other contributors.

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In the fetal phase of development, significant organ growth takes place: NEP form radial glia; both types of NS/PC proliferate, radial glia develop scaffolds, and cells migrate along those scaffolds and differentiate into young neurons and glia cells (reviewed in ref. [4]). Later maturational stages include elongation of axons and dendrites, specification of neurotransmitters and receptors, formation and pruning of synapses, programmed cell death to eliminate surplus cells, formation of neuronal networks, myelination, and formation of the blood–brain barrier (reviewed in refs. [5], and [6]). These key neurodevelopmental stages are summarized in Fig. 1. A multitude of cell types (i.e., ESC, NEP, NS/PC, different neuronal and glial subtypes, endothelial cells) at distinct maturation stages in a brain region-specific manner are involved in these neurodevelopmental processes. These provide numerous targets for compounds acting as developmental neurotoxicants through a variety of different modes of action (MoA; reviewed in ref. [7]).

Toxicological testing for regulation of chemicals is currently undergoing a paradigm shift from apical endpoint evaluation in whole animals towards a mechanism-based assessment of compounds' toxicity as determined using multidisciplinary approaches [8]. In vitro hazard assessment using cell cultures is one of the main pillars of these approaches. Caution is warranted when choosing appropriate cells for chemical testing, because depending on cell

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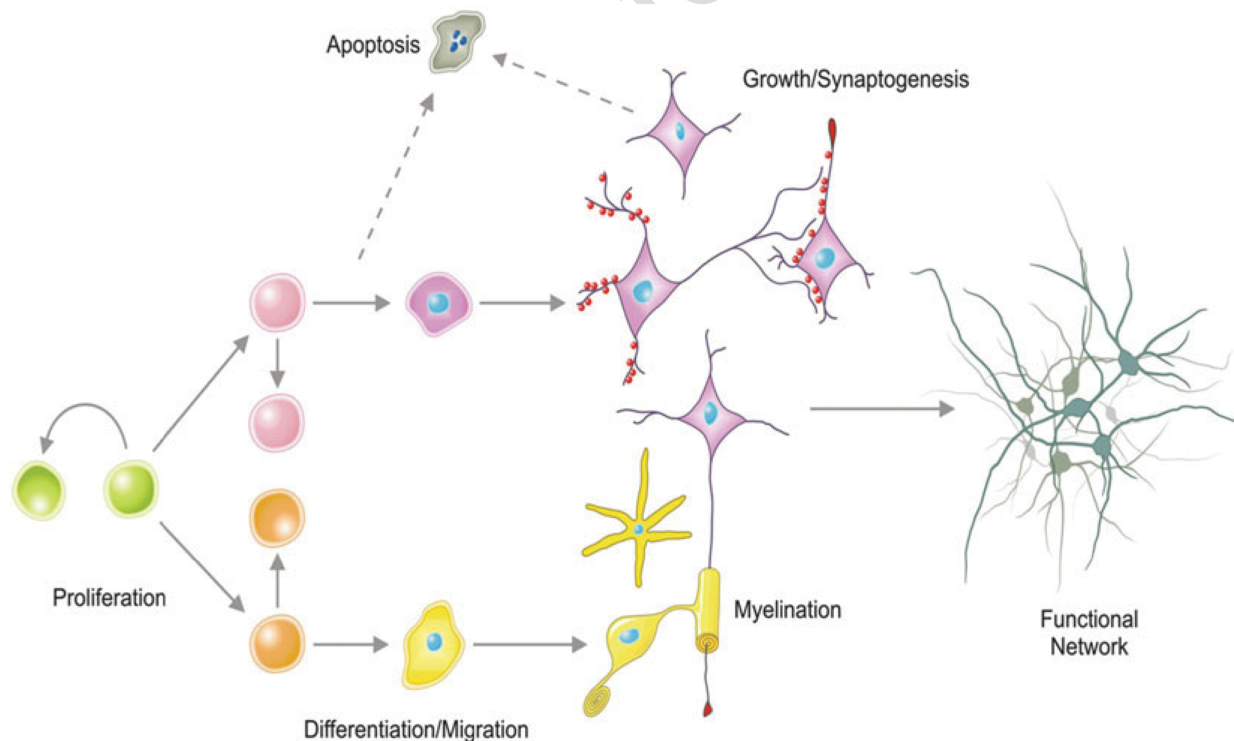

Fig. 1 Basic processes of brain development necessary for proper organ function. Neural Progenitor Cells (*green*) proliferate to provide an excess amount of cells, which then migrate and differentiate into neurons (*purple*) and glia (*yellow*). These form synapses (*red*) and excess cells undergo apoptosis (*grey*). When these processes happen in the appropriate and coordinated way, functional neuronal networks form (*olive*). With courtesy from William Mundy, U.S. Environmental Protection Agency and John Havel, SRA International, Inc

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type and culture conditions, results from in vitro testing can differ tremendously for the same compounds. Using the examples of in vitro testing for developmental neurotoxicity (DNT) endpoints, species [9–11] and cell type (tumor versus primary cell; [12]) might influence testing outcome. Moreover, recent advances in tissue engineering have clearly indicated that cells cultured in a conventional two-dimensional (2D) fashion can differ in their cell physiology from their counterparts growing in 3D ([13], reviewed in refs. [14, 15]). This observation, which was initially based on data from fibroblasts, also seems to hold true for neural cells [16].

Current international guidelines for DNT testing (OECD Testing Guideline 426, U.S. EPA OPPTS 870.6300) are very resource-intensive when it comes to the number of animals used and the time and costs required ([17, 18], reviewed in refs. [19], and [20]). In concert with the knowledge on species-specificities of cell and organ responses [21, 22], there is international consensus on the need for an alternative strategy for DNT testing with regulatory acceptance ([23, 24], reviewed in refs. [25, 26]). The "Neurosphere Assay" presented herein is regarded as one tool for such an alternative testing strategy because it mimics specific neurodevelopmental processes (key events)—NS/PC proliferation, migration, and differentiation into neural effector cells (neurons, astrocytes, and oligodendrocytes)—in vitro (Fig. 1). In the following sections, we describe how these individual processes are assessed within the "Neurosphere Assay" for in vitro DNT testing. In our discussion, we emphasize endpoint-specific controls for these key events and relate those to the in vivo situation.

2 The Neurosphere Assay: How to Evaluate Different Processes of Brain Development In Vitro

2.1 Proliferation

The disturbance of proliferation during brain development leads to substantial alterations of brain morphology including a reduction in the size, weight, and volume of the whole brain [27] or individual brain structures [28]. Assessing disturbance of proliferation as an endpoint for DNT is thus of high importance.

We use human second trimester NS/PC growing as 3D cell aggregates called neurospheres (Lonza, Verviers, Belgium). As part of the "Neurosphere Assay" we established a proliferation assay to evaluate the ability of NS/PC to divide and generate new cells by using two different methods for the assessment of proliferation: (1) Bromodeoxy-Uridine (BrdU) incorporation and (2) increase in the diameter of the neurosphere. BrdU-incorporation into the DNA of dividing cells is a direct measure of proliferation. The BrdU Assay is feasible for short exposure times and is a very sensitive method, but results obtained using BrdU incorporation as an endpoint strongly depend on neurosphere size, and inaccurate performance of the assay might lead to false positive results. The alternative method is

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simpler and assesses proliferation indirectly by measuring the increase in the diameter of floating neurospheres over time. However, this method is less sensitive and only robust changes in proliferation are detected. For this reason, exposure times of at least 1 week are recommended for this assay (for detailed information on these two methods *see* ref. [29]).

When performing DNT testing in an in vitro model it is essential to guarantee that the basic biology of the tested endpoint is functional and the endpoints can be modulated in vitro by factors known to modulate them in vivo. Therefore, the use of endpoint specific controls for quality assurance is necessary. The selected endpoint specific control for proliferation is growth factor (GF; epidermal growth factor (EGF) and fibroblast growth factor (FGF-2)) withdrawal. NS/PC proliferation is tested both in proliferation medium (B27) including GF and in GF-free medium (B27 w/o EGF and FGF-2). For both methods, GF withdrawal causes at least 60–70 % reduction in proliferation relative to controls (Fig. 2). NS/PC proliferation is well known to be dependent on EGF and FGF [30–33]. EGF-receptor (EGF-R)-deficient mice develop smaller brains with smaller germinal zones, yet on E17, proliferation in these proliferative areas was not reduced. This might be due to inhibition of proliferation earlier during development and/or

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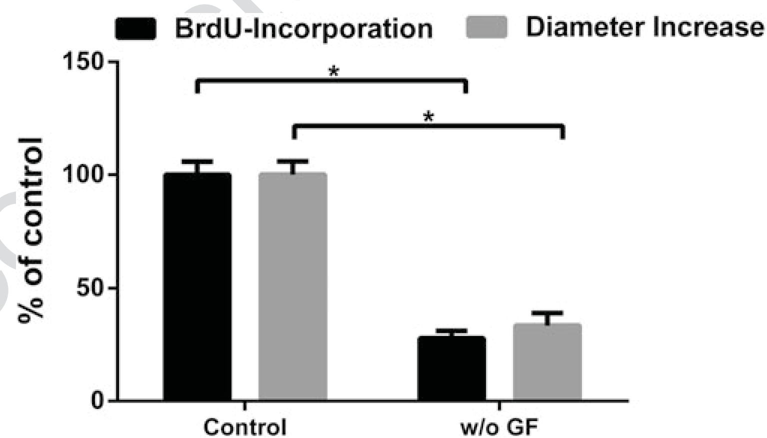

Fig. 2 Quality control of the endpoint proliferation in human NS/PC. Proliferation was assessed by performing a BrdU Cell Proliferation ELISA (Roche, *black bars*) or measuring the diameter increase (*grey bars*). Therefore, six neurospheres were plated in a 96-Well plate (one sphere per well) in normal proliferation medium with (control) or without growth factors (w/o GF) for 72 h. For assessment of BrdU incorporation, 16 h prior to the end of the experiment BrdU was added to the spheres. The assay was performed according to the manufacturer's instructions. For the assessment of the diameter increase, the diameter was measured on day 0 and day 3. Data (% of control) is shown as mean of 3–4 independent experiments $\pm$ SEM. *p-value $\leq$ 0.05 (students *t*-test) was considered as significant compared to control (adapted from Baumann et al. 2015 [112])

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compensatory effects of FGF [34]. Yet, EGF-R-deficient animals die within the first 14 days postnatally.

It is essential that an endpoint specific control is specifically acting on the neurodevelopmental process of interest rather than influencing the general viability of the neurospheres. An option for assessing viability is to perform an Alamar Blue Assay, which monitors mitochondrial reductase activity. However, for the endpoint of proliferation, the information obtained by this assay does not accurately reflect cell viability, as sphere diameter and mitochondrial activity are directly correlates because of different cell numbers in different sized spheres [35]. For this reason, e.g., the lactate dehydrogenase (LDH) assay, which measures cellular membrane integrity, is the preferred method for monitoring cytotoxic effects of compounds in proliferating spheres.

One benefit of the neurosphere model is the opportunity to compare neurodevelopmental processes of different species in parallel in vitro, which facilitates extrapolation between species and enables a direct in vivo–in vitro correlation for rodents. For proliferation, the BrdU and the neurosphere size assay indicated that human, mouse, and rat NS/PC proliferate at different rates in culture, and thus present different BrdU incorporation levels after 3 days (Fig. 3a) or different diameter increases after 7 days (Fig. 3b) of proliferation.

How does NS/PC proliferation respond to DNT compounds in vitro and in vivo? For the majority of DNT compounds (reviewed in refs. [36], and [37]) there is no mechanistic data available for their MoA in humans; therefore, the rodent model is of great value. Comparative human–rodent in vitro studies are therefore extremely useful in assessing whether the proposed mechanism of action

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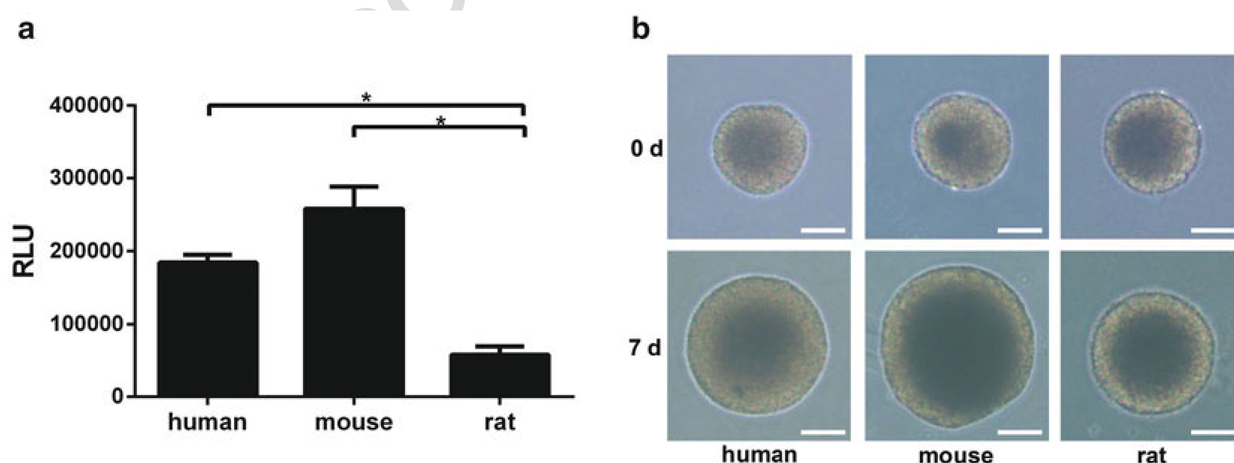

Fig. 3 Species comparison of the endpoint proliferation. (**a**) Proliferation was assessed by performing a BrdU Cell Proliferation ELISA (Roche). Control data (RLU) of 3–5 independent experiments is shown as mean ± SEM. **p*-value $\leq$ 0.05 (students *t*-test) was considered as significant between the species. (**b**) Phase-contrast images of human, mouse, and rat neurospheres taken after 0 days (*0 d*) or 7 days (*7 d*) of proliferation, scale bar = 100 μm (adapted from Baumann et al. 2015 [112])

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based on rodent studies is of relevance to humans. Currently, the best-studied DNT compound is methyl mercury (MeHg). Two independent labs demonstrated that MeHg reduced proliferation of rat primary neural cells in vitro and in the hippocampi of mice [38] or rats [39] in vivo. Furthermore, experimental data from low-dose exposure humans studies demonstrated a decreased mitotic index in lymphocytes, which reflects an inhibition of cell-cycle progression and/or a loss of proliferative capacity [40]. MeHg was also shown to inhibit NS/PC proliferation in the nM range in human umbilical cord blood-derived neural stem cells [41]. Besides MeHg, the antiepileptic drug valproic acid (VPA) is also a well-known developmental toxicant causing autism spectrum disorders in prenatally exposed infants (reviewed in ref. [42]). The mechanisms underlying VPA-induced DNT are not well understood because VPA acts on a multitude of cellular targets (reviewed in ref. [43]). Go and coworkers found that after an injection of VPA in rats at E12, postnatal brain weight was significantly increased. Moreover, NS/PC isolated from rat brains either exposed prenatally to VPA or treated with VPA in vitro displayed increased proliferation in vitro [44]. The opposite effects of VPA on proliferation of brain cells in vivo were seen when VPA was administered postnatally, and this inhibition of proliferation was recapitulated in vitro [45]. These data demonstrate that for some compounds, effects on rodent NS/PC proliferation in vivo and in vitro correlate well, and thus, ex vivo NS/PC seem to preserve their in vivo molecular program. Therefore, it seems likely that primary human NS/PC cultures, such as the above-described primary human neurospheres, will generate data relevant to the human DNT compound hazard on this endpoint. In the context of an alternative DNT testing battery it has to be pointed out that the “Neurosphere Assay” described here is based on fetal (GW 16-20) cells, which will most accurately reflect fetal NS/PC proliferation. This is important to note, because adverse compound effects on NS/PC proliferation might be dependent on the timing of insult [44, 45]. How similar hESC- or human induced pluripotent stem cell (hiPSC)-derived NS/PC are to primary hNS/PC and their in vivo counterparts and—most importantly—which window of development they reflect, needs to be urgently tested to correctly determine which phase of neurodevelopment they represent in a DNT testing strategy.

2.2 Migration

Throughout and then after the division of proliferating NS/PC, radial glia as well as postmitotic differentiating cells migrate towards their final destinations in the brain. Several human developmental brain disorders have been associated with disruptions of the migration process, including heterotopia and lissencephaly [46]. Migration defects of specific types of interneurons have also been associated with schizophrenia and epilepsy ([47, 48],

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reviewed in ref. [49]). Therefore, an in vitro DNT testing battery needs to include a model capable of detecting compound effects on migration (Fig. 1).

The "Neurosphere Assay" includes two simple methods to evaluate the ability of NS/PC to migrate simultaneously, mimicking their ability to move from proliferating niches to their final positions in the developing brain. First, migration speed can be assessed by measuring the distance cells migrate over time. A second gauge of successful migration is the evaluation of absolute numbers of cells that migrate out of the neurosphere during this period. To quantify both endpoints, neurospheres are plated onto a poly-D-lysine/laminin coated surface of a well filled with differentiation medium (for more details on the migration protocol *see* ref. [29]). After adhering to the laminin extracellular matrix, cells spontaneously start to migrate out of the neurosphere ([35], Supplementary Material 2). At the desired evaluation time point (e.g., 24 h), phase-contrast pictures of migration areas are taken. Migration distance is evaluated by measuring four radii of the migration area of each neurosphere in perpendicular angles from the edge of the neurosphere to the furthest migrated cells (Fig. 4 left). To count the number of migrated cells, neurospheres are fixed with 4 % paraformaldehyde and cell nuclei are stained with Hoechst dye.

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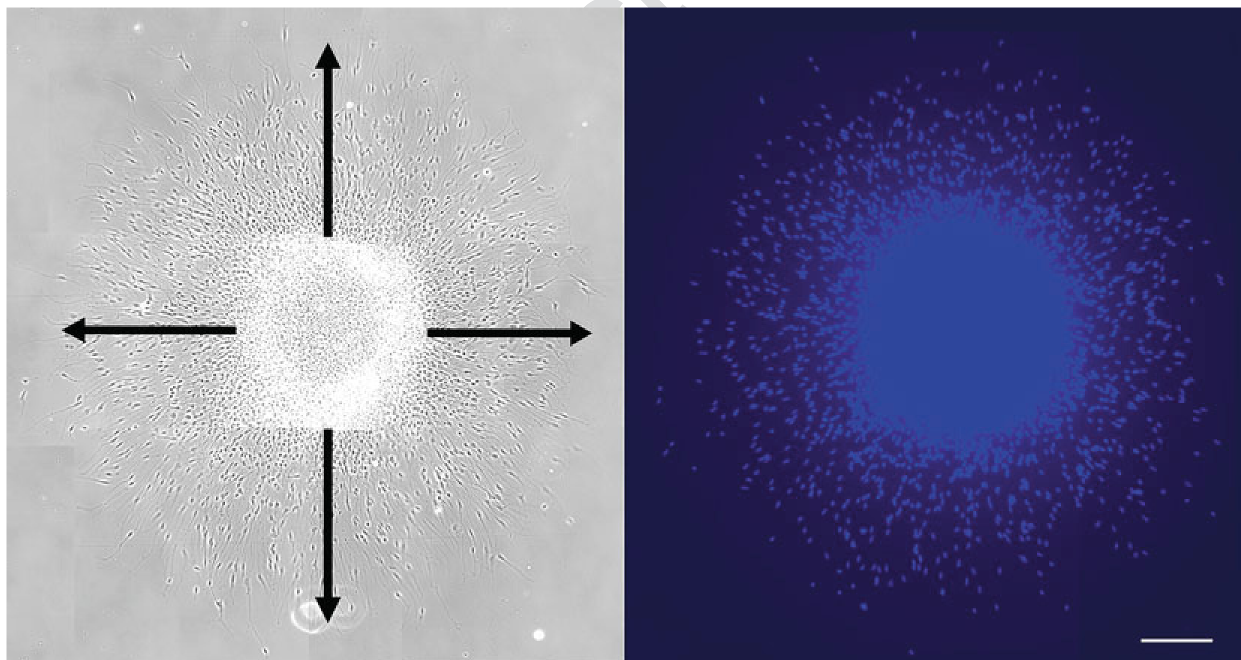

Fig. 4 Measuring migration within the "Neurosphere Assay." (*Left*) Phase-contrast image of the whole migration area of a human neurosphere after 24 h of culture on a PDL/laminin coated surface of a well filled with differentiation medium. *Arrows* exemplify the assessment of the migration distance by measuring four perpendicular radii from the neurosphere edge to the end of the migration area. (*Right*) Fluorescent picture of the whole migration area of the same human neurosphere, where migrated nuclei stained with Hoechst can be counted, scale bar = 100 μm

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The total number of Hoechst-positive cell nuclei is then counted in fluorescence pictures of the whole migrating area (Fig. 4 right).

These two straightforward ways of evaluating migration allow the comparison of the migration behavior of control cells to compound-exposed cells after a certain exposure time. A close monitoring of the control migration by time-lapse pictures has shown that cells migrating out of the neurosphere exhibit classical migration features required for migration dynamics. Migrating cells present a very active growth cone protrusion moving away from the cell body. This growth cone explores the local microenvironment, and later the cell nucleus is translocated toward the new position (nucleokinesis). In the "Neurosphere Assay" most cells migrate in a radial trajectory using a scaffold built by $GFAP^+$ cells; however, different trajectories and speed rates can also be observed ([35], Supplementary Material 2).

Several signaling pathways drive these migration dynamics and one of those is mediated by activation of src kinases [50]. For this reason, the src kinase inhibitor PP2 is used as the endpoint specific control for migration to assess general assay performance (Fig. 5a). PP2 does not completely inhibit migration because several other pathways contribute to the migration process, but the distance is decreased to around 60% of control values [50]. It is important to note that because src kinases participate in the regulation of many signaling pathways, one of which is cellular survival [51], a prolonged exposure (72 h) to this compound also impacts cell viability (Fig. 5b). As there is cross talk among several pathways involved in migration and the cell cycle [52], compounds affecting signaling pathways involved in migration are also thought to eventually

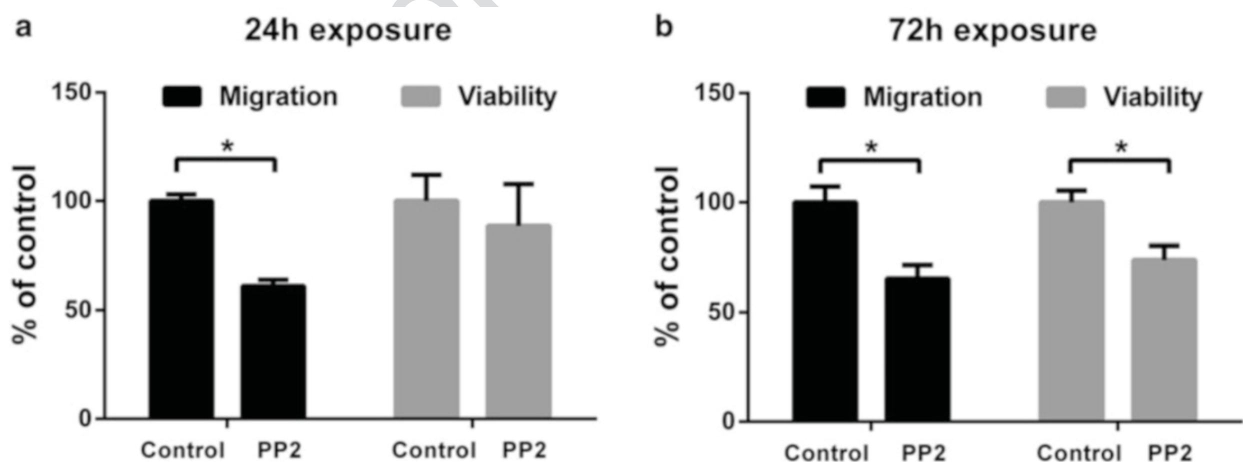

Fig. 5 Quality control of the endpoint migration in human NS/PC. Human neurospheres were cultured for 24 or 72 h on a PDL/laminin coated surface of a well filled with differentiation medium (control) or differentiation medium with 10 μM PP2. Migration distance was measured in phase-contrast images of five neurospheres per assay. Viability of the same spheres was measured by Alamar Blue Assay. *Bars* represent the mean of four independent experiments ± SEM in % of control. *p-value ≤ 0.05 (students t-test) was considered as significant versus control (adapted from Baumann et al. 2015 [112])

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influence cell viability. Therefore, it is important to measure cell viability as early as migration effects are observed when testing the effects of unknown compounds. If migration is reduced as a result of general cytotoxicity, both migration and viability will be reduced, while in the case of specific migration pathway interference, migration will be altered first without showing signs of cytotoxicity. In our experience, this timing effect in vitro is true for all studied endpoints, yet migration seems to be very sensitive. In addition to detecting decreases in migration distance, the assay can identify increased migration rates relative to untreated controls [53]. In these cases, general viability pathways are commonly not blocked, and therefore cytotoxic effects are generally not an issue.

Evaluating migration after 24 h is also a convenient time point when comparing the effects of compounds on migration of NS/PC of different species, because neurospheres from humans, rats, and mice exhibit similar migration dynamics in culture during the first 24 h (Fig. 6), with a migration distance covering between 400 and 500 μm during this period (around 20 μm/h). In contrast, during the second 24 h (24–48 h of the migration assay) the migration rates between species differ with 17.2, 9.6, and 1.9 μm/h for human, mouse, and rat, respectively. Within this time frame, the migration rate of rodent cultures is significantly slower than the migration speed of the human culture. During the next 24 h (48–72 h of the migration assay) human migration pace further decreases to 7.6 μm/h, while rodent speeds stay about the same (Fig. 6). It is noteworthy that migration speed during the first 24 h in all species is in agreement with real-time observed granule cell

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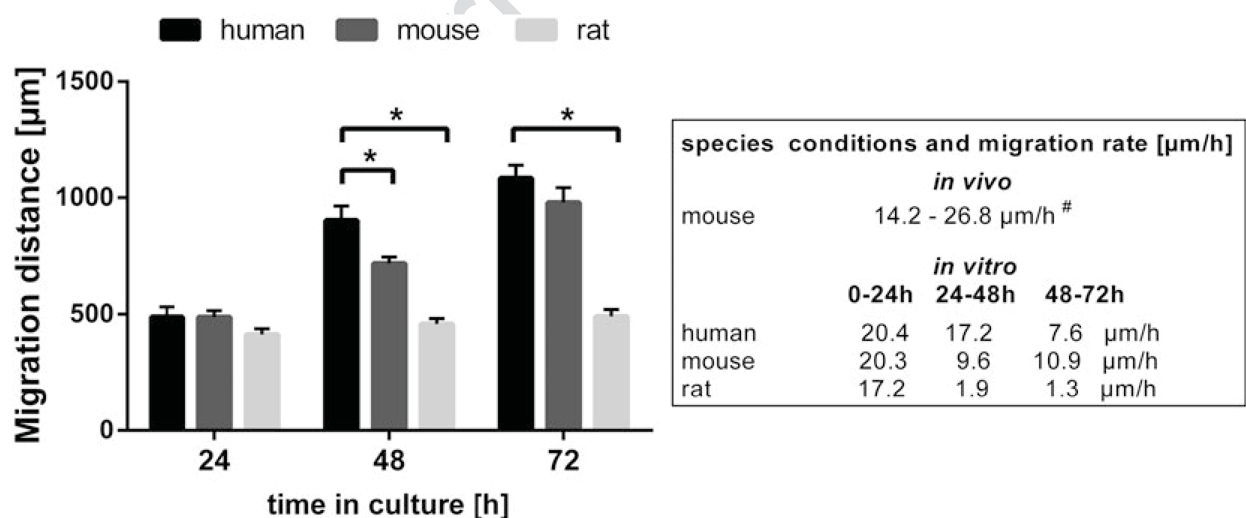

Fig. 6 Species comparison on NS/PC migration distance over time. Human, mouse, and rat neurospheres were cultured for 72 h in differentiation medium on PDL/laminin coated wells. Phase-contrast images were taken every 24 h and migration distance was measured in five neurospheres per assay. *Bars* represent the mean of four independent experiments ± SEM. Statistical analysis was performed by multiple *t*-test with Holm–Sidak multiple comparison with the GraphPrism 6 program, and significant threshold was established at $p < 0.05$.* was considered statistically significant different versus human value of the same time-point# [54]

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migration rates in vivo in mice, which range between 14.2 and 26.8 μm/h [54]. This in vitro–in vivo similarity of migration dynamics strongly underlines the physiological relevance of the neurosphere migration assay.

Most importantly, this migration assay not only accurately reflects the in vivo migration process but also detects migration alterations caused by substances known to disturb human neural migration in vivo, like MeHg [54]. Humans developmentally exposed to MeHg suffer from mental retardation and cerebral palsy [55–57]. Their brains are hypoplasic and present many ectopic neurons, indicating disrupted migration among other alterations (reviewed in refs. [58], and [59]). Disturbances of neural migration after developmental exposure to MeHg have also been observed in animal models in vivo and in vitro [60, 61]. In agreement with the literature, the "Neurosphere Assay" detects a significant reduction in NS/PC migration at in vivo relevant concentrations of MeHg, which are not cytotoxic (LOAEC = 0.5 μM; [35]). In essence, the neurosphere migration assay offers the possibility to study and compare effects of chemicals on NS/PC migration and to elucidate the mechanisms behind migration alterations of compounds in human and rodent cultures.

2.3 Differentiation

During the migration period and also afterwards, NS/PC differentiate into neural effector cells to correctly form the different brain regions. NS/PC differentiate into either neurons or glia cells among which the most abundant cell types are astroglial and oligodendroglial cells. The differentiation of NS/PC into these three cell types within the "Neurosphere Assay" will be the subject of the following section. In general, NS/PC differentiation is initiated by growth factor withdrawal and offering a specific extracellular matrix. Therefore, we routinely plate our neurospheres on poly-D-lysine/laminin coated surfaces in medium without growth factors and culture them for 3 (neurons and astrocytes) or 5 days (oligodendrocytes). After this culture period, we fix the neurospheres and perform an immunocytochemical staining for specific epitopes of the different cell types (neurons: ßIII tubulin; astrocytes: GFAP, oligodendrocytes: O4) which are then evaluated by fluorescence microscopy. For mouse NS/PC, the differentiation medium is supplemented with 1% FCS to prevent apoptosis. For a detailed method description, please *see* Baumann et al. [29].

2.3.1 Neurons

The differentiation of NS/PC to neurons during brain development is essential for the appropriate cellular composition of the different brain regions and functionality of neuronal networks. Disturbing neuronal differentiation during normal brain development, therefore, results in structural changes of the brain, which can manifest as behavioral disorders (reviewed in ref. [62]).

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Thus, the investigation of NS/PC differentiation is another essential endpoint to be evaluated for alternative DNT testing. 331 332

We assess the ability of NS/PC to differentiate into neurons within the "Neurosphere Assay" by evaluating the percentage of cells in the migration area that differentiated into neurons. A manual counting of neurons strongly depends on high quality immunocytochemical staining for a clear identification of neurons in the migration area. 333 334 335 336 337 338

To control general assay performance for neuronal differentiation, neurospheres are differentiated in the presence of EGF (20 ng/ml) as an endpoint specific control to reliably inhibit NS/PC differentiation to neurons [63]. For a valid endpoint specific control, EGF in the culture medium should reduce the number of neurons to around 20 % of the control value (Fig. 7). EGF does not reduce viability when administered during differentiation, showing that our endpoint specific control inhibits neuronal differentiation without causing cytotoxicity. 339 340 341 342 343 344 345 346 347

In order to compare neuronal differentiation across species we compared the percentage of neurons in the migration area after 3 days of differentiation under standard differentiation culture conditions in humans, rat, and mouse cultures. Quantitative evaluation of the three species revealed statistically significant differences in the amount of differentiated ßIII tubulin + cells. In human neurospheres approximately 10 % of the cells in the migration area were ßIII-tubulin+, whereas in mouse and rat neurospheres, the 348 349 350 351 352 353 354 355

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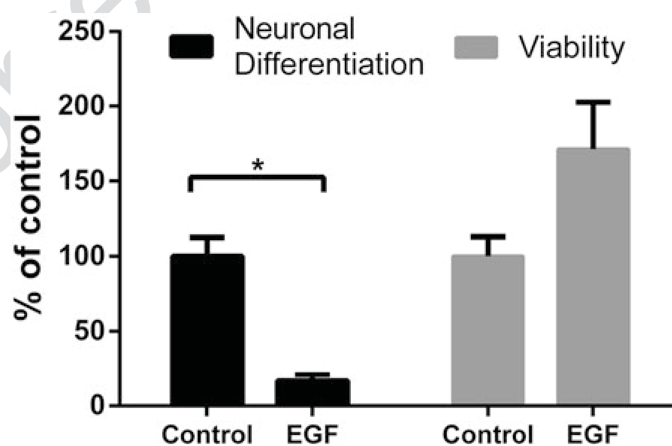

Fig. 7 Quality control of the endpoint neuronal differentiation in human NS/PC. (**a**) Neuronal differentiation was assessed by immunocytochemical staining of migrated human NS/PC after 3 days of differentiation for the neuronal marker ßIII tubulin and Hoechst for nuclear counterstaining. Therefore, five neurospheres were plated in one well of an 8-chamber slide in normal differentiation medium (control) or differentiation medium with 20 ng/mL EGF for 72 h. (**b**) For the assessment of NS/PC viability an Alamar Blue Assay was performed according to the manufacturer's instructions after 3 days of differentiation. Data (% of control) is shown as mean of four independent experiments ± SEM. *p-value ≤ 0.05 (students t-test) was considered as significant compared to control (adapted from Baumann et al. 2015 [112])

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percentage of ßIII-tubulin + cells was approximately 5 and 15 %, respectively (Fig. 8).

When testing chemicals for their DNT potential, how does the endpoint neuronal differentiation measured in in vitro reflect in vivo data? Kim and coworkers (2009) showed that Bisphenol A (BPA), an endocrine disruptor that potentially disrupts neuronal development (reviewed in ref. [64]), promoted neuronal differentiation in the murine immortalized C17.2 progenitor cell line and in primary embryonic hippocampal neurons. These in vitro data demonstrating accelerated neurogenesis were reflected by an enhanced dentate gyrus formation in mice on PND1 after

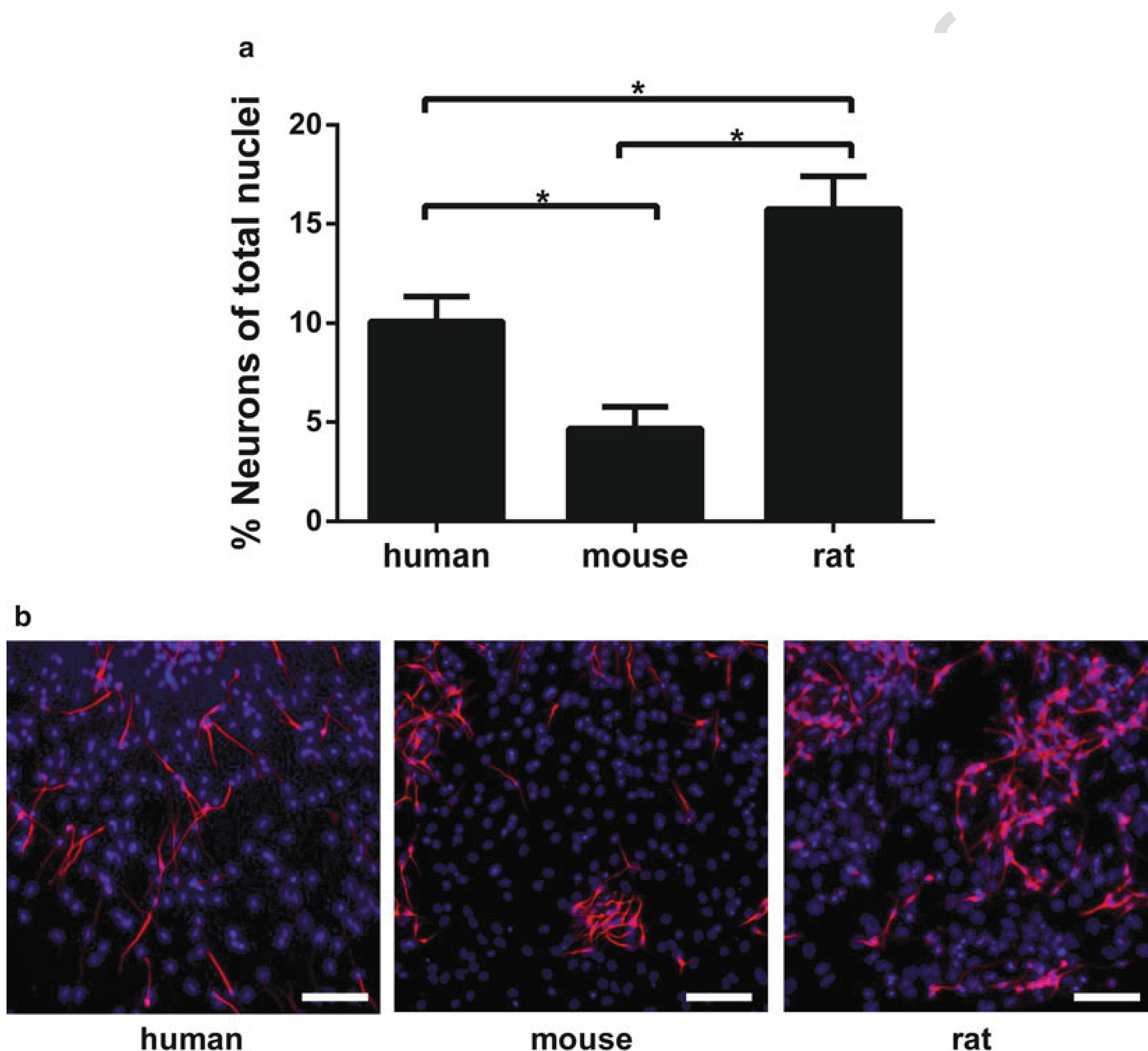

Fig. 8 Species comparison of the endpoint neuronal differentiation. (**a**) Neuronal differentiation was assessed by determining the percentage of neurons in the migration area after immunocytochemical staining for ßIII tubulin and cell nuclei. Control data (% Neurons of total nuclei) of four independent experiments is shown as mean ± SEM. *p-value $\leq$ 0.05 (students t-test) was considered as significantly different between the species. (**b**) Fluorescent images of human, mouse, and rat neurospheres taken after 3 days of differentiation (*blue*: cell nuclei; *red*: ßIII tubulin positive cells), scale bar = 100 μm (adapted from Baumann et al. 2015 [112])

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administering BPA during pregnancy between E14.5 and 18.5 [65]. Hence, in this study BPA affects murine brain development by altering neuronal differentiation both in vivo and in vitro. Another study from Go and coworkers [44] demonstrated that VPA induced neuronal differentiation of NS/PC generated from E14 rat cortex after exposure in vitro and in vivo after a single injection of rat dams with VPA at E12. Studies like these clearly show that an affected neuronal differentiation in vitro in primary cells correlates with corresponding in vivo data, illustrating that the endpoint of neuronal differentiation is a suitable and important endpoint for in vitro DNT testing using NS/PC. Similarly as discussed in the proliferation part of this chapter, we expect each species to be representative of its respective molecular profile, and thus, human primary cells as described above resemble human physiology. And again, application of hESC/hiPSC methods for assessing the endpoint neuronal differentiation, especially have to be characterized with regard to developmental time points.

2.3.2 Astrocytes

Differentiation of NS/PC to astroglial cells during brain development is essential for processes like neuronal migration, neurite guidance, and the formation of functioning synapses (reviewed in ref. [66]). Disturbing astroglial cells or neuronal–astroglial interactions during critical periods of brain development (e.g., during neuronal migration) can induce substantial deficits in the function of the central nervous system (reviewed in ref. [67]). Therefore, the assessment of NS/PC differentiation to astrocytes is thought to serve as an important functional endpoint during DNT testing.

We assess the ability of hNS/PC to differentiate into astrocytes by evaluating quantitative and qualitative changes in astrocytes differentiation and maturation using immunocytochemical staining of migrated cells for GFAP. Bone morphogenetic protein (BMP)-7-dependent astroglial differentiation serves as an endpoint specific control [68]. Figure 9 clearly demonstrates that human NS/PC differentiated in the presence of BMP-7 generated more astrocytes with a higher GFAP content and a more mature phenotype with shorter processes and a more stellate-like phenotype.

Comparing the morphology of astrocytes differentiated for 3 days from human, rat, and mouse NS/PC reveals similar astroglial morphologies in mouse and rat NS/PC with stellate astrocytes, whereas human NS/PC mainly show elongated astrocytes with more radial glia-like structures (Fig. 10). This might indicate that astrocytes differentiated from rodent NS/PC are already more mature after 3 days of differentiation when compared to human NS/PC, which again indicates that human and rodent NS/PC differ in their rate of differentiation and maturation [69, 70].

The endpoint of astroglial differentiation for DNT testing has been investigated in vivo and in vitro earlier. Burry and coworkers

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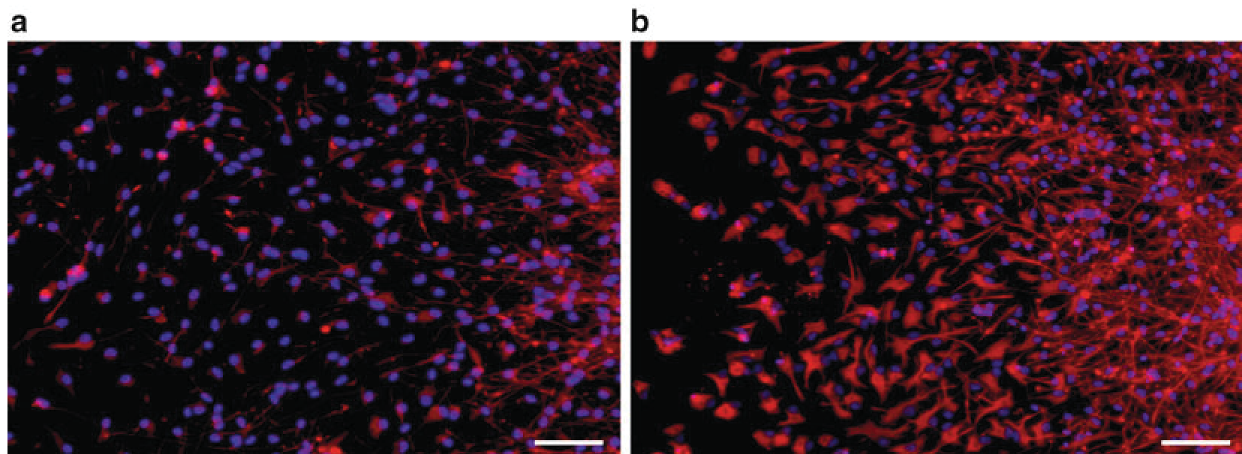

Fig. 9 Quality control of the endpoint astrocyte differentiation in human NS/PC. Astrocyte differentiation was assessed by immunocytochemical staining of migrated human NS/PC after 7 days of differentiation for the astrocyte marker GFAP and Hoechst for nuclear counterstaining. Therefore, five neurospheres were plated in one well of an 8-chamber slide coated with PDL/laminin in normal differentiation medium (**a**) or differentiation medium with 100 ng/mL BMP-7 (**b**) for 7 days (*blue*: cell nuclei; *red*: GFAP positive cells), scale bar = 100 μm

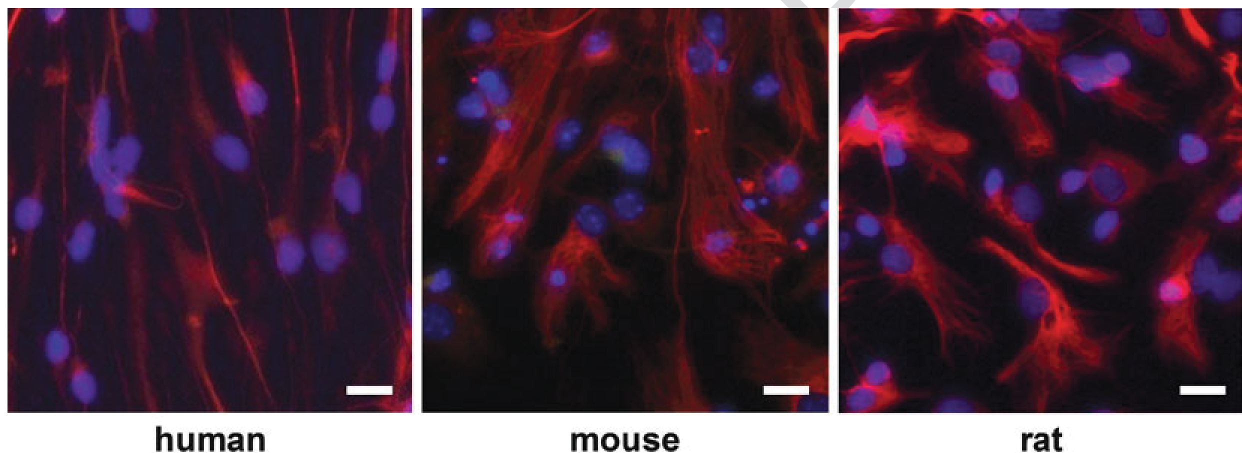

Fig. 10 Species comparison of the endpoint astrocyte differentiation. Astrocyte differentiation was evaluated by immunocytochemical staining of the migration area after 3 days of differentiation for the astrocyte marker GFAP (*red*) and cell nuclei (*blue*), scale bar = 20 μm

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observed that postnatal exposure of rats to toluene, one of the known human DNT compounds (reviewed in ref. [37]), impaired astrocyte development in the developing brain, which was measured by reduced brain GFAP content in rats treated with toluene daily between PND4 and 10. This toluene-induced GFAP reduction could be the result of reduced astrocyte maturation, diminished astrocyte number or a combination of both. In vitro analyses in the same study revealed that proliferation of GD 21 rat cortical astrocytes was reduced after toluene treatment for 24 h in a concentration-dependent manner in the absence of cytotoxicity [71]. Similarly, ethanol, which is also a confirmed human developmental neurotoxicant (reviewed in ref. [36]), delays the appearance

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of GFAP during brain development and decreases its expression after prenatal exposure of rats. Radial glial cultures of rats that were prenatally exposed to ethanol showed a similar reduction in GFAP content [72], and an in vitro treatment of rat NS/PC with ethanol resulted in a reduced number of astrocytes after differentiation [73]. Ethanol causes fetal alcohol syndrome (FAS), and there is human evidence that ethanol impairs astrogliogenesis, as evidenced by aberrant neural and glial tissue and other abnormalities in neural and glial migration in postmortem brain of FAS children (reviewed in ref. [74]). These studies demonstrate that results obtained for the endpoint astrogliogenesis both in vivo and in vitro correlate well, which makes it a valuable endpoint for evaluating the developmentally neurotoxic potential of chemicals.

2.3.3 Oligodendrocytes

Oligodendrogenesis during brain development is necessary for proper brain function as oligodendrocytes form and maintain myelin sheaths around axons in the central nervous system. Disturbance of oligodendrocyte development may result in demyelination diseases that severely affect neuronal functioning (reviewed in ref. [75]).

To study oligodendrogenesis within the "Neurosphere Assay," the number of O4+ cells/total number of cell nuclei in the migration area is counted and oligodendrocyte morphology is evaluated after 5 days of differentiation. The endpoint specific control substance chosen to inhibit oligodendrogenesis is BMP-7 (Fig. 11), because similar to other BMPs (e.g., BMP-2), BMP-7 induces astrogliogenesis (see above) and inhibits oligodendrogenesis [76, 77].

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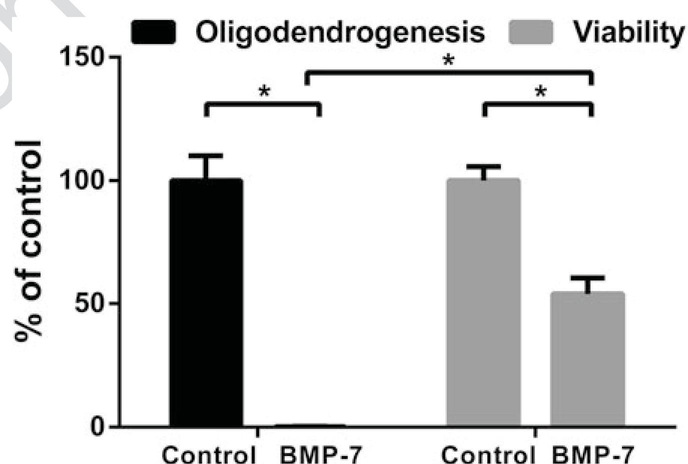

Fig. 11 Quality control of the endpoint oligodendrogenesis in human NS/PC. Human neural progenitor cells were plated onto PDL/laminin coated 8-chamber glass slides in normal differentiation medium (control) and differentiation medium with 100 ng/mL BMP-7 for 5 days. Mitochondrial activity was measured by Alamar Blue Assay. Slides were fixed and immunocytochemically stained for O4. Data are shown as mean of five independent experiments ± SEM. *p-value ≤ 0.05 (student t-test) was considered as significant

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This switch in glial differentiation fate is possibly mediated by SMAD protein signaling [78]. Comparison of oligodendrogenesis across human, mouse, and rat neurospheres revealed that after 5 days of differentiation, hNPC from all species have formed 6–7 % O4+ cells/nuclei within the migration area (Fig. 12a). However, the maturation stage of oligodendrocytes at this time in culture differs between human and rodent cultures (Fig. 12b). Human NS/PC-derived oligodendrocytes are still linear and/or lesser branched with smaller sheath-like structures than their rodent counterparts, which are more branched and form extensive myelin sheaths after 5 days of in vitro differentiation. In addition, the kinetics of oligodendrogenesis differs between species. Murine NS/PC are already differentiated to oligodendrocytes after 2 days, while human NS/PC

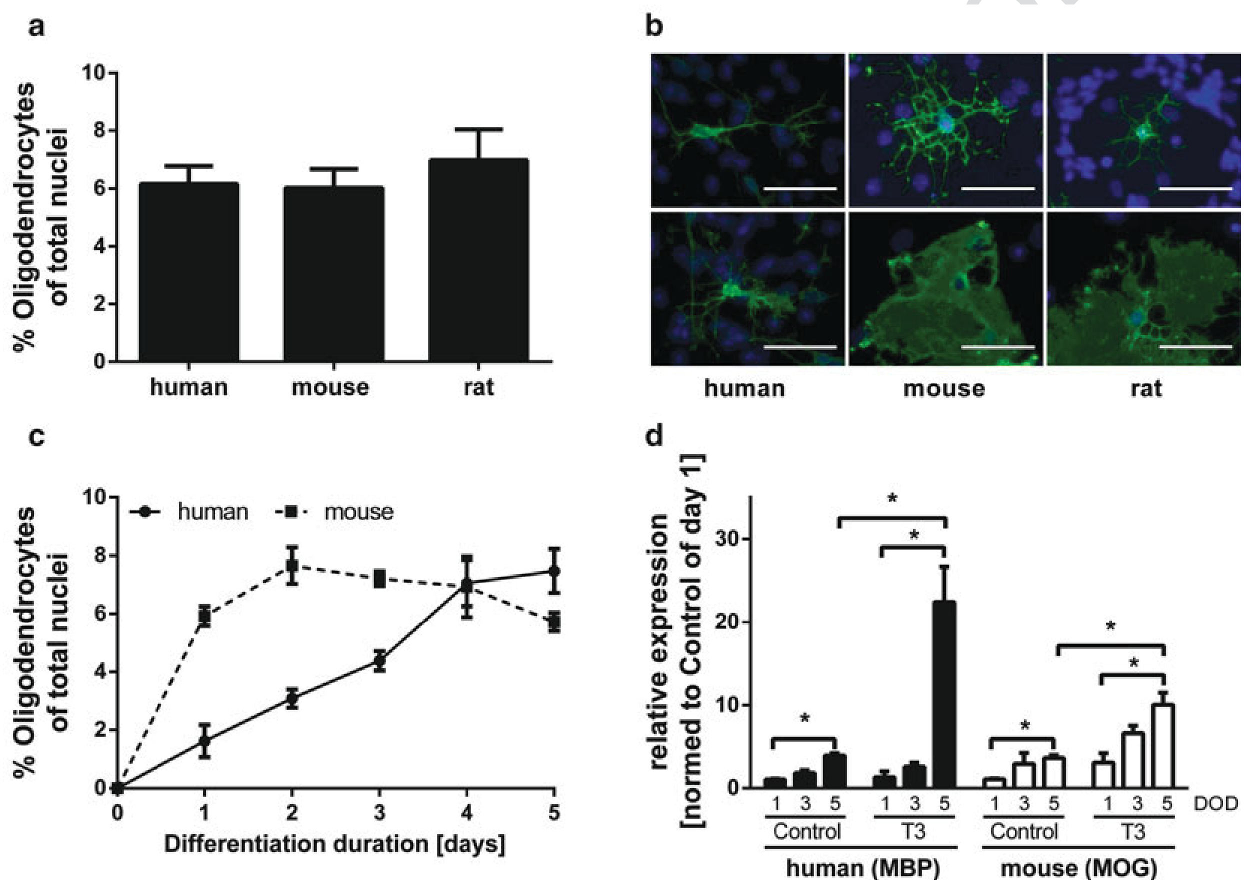

Fig. 12 (**a**, **b**) NS/PC were plated onto poly-D-lysine/laminin coated 8-chamber glass slides in differentiation medium and were differentiated for 5 days. Afterwards they were fixed and immunocytochemically stained for O4. (**a**) Percentage of oligodendrocytes in human, mouse, and rat neurospheres was evaluated in the migrating area after 5 days of differentiation ($n = 5$ each; mean ± SEM; no statistical differences by *t*-test; $p \leq 0.05$). (**b**) Stages of maturation in human, murine, and rat NS/PC after 5 days of differentiation (*green*: O4, *blue*: Hoechst), scale bar = 50 μm. (**c**) Neural progenitor cells were fixed at day 1, 2, 3, 4, and 5 of differentiation, stained for O4 and the percentage of oligodendrocytes in the migration area was calculated ($n = 4$ each; mean ± SEM). (**d**) Per condition 3 × 10 NS/PC were plated onto PDL/laminin coated 24-well plates in presence or absence of 3 nM T3. RNA was collected and isolated at day 1, 3, and 5 of differentiation (RNeasy Kit, Qiagen) and quantitative real time RT-PCR was performed. Data are shown as mean ± SEM ($n = 3$, student *t*-test, *$p \leq 0.05$, *DOD* days of differentiation

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differentiate to O4+ cells progressively over 5 days of differentiation (Fig. 12c). This characterization of oligodendrocyte maturation by morphological appearance (branches/sheath formation) over time demonstrates that murine oligodendrocytes differentiate and mature faster than their human counterparts. These in vitro observations reflect what is known in vivo: the time span between pre-oligodendrocyte formation and emergence of the first mature oligodendrocytes is 11–12 weeks in humans but only 5 days in rodents (reviewed in ref. [79]).

Molecular analyses of myelin basic protein (MBP, for hNS/PC) or myelin oligodendrocyte glycoprotein (MOG, for mNS/PC) mRNA expression support this observation (Fig. 12d). Expression of these maturation markers increases over differentiation time. Human oligodendrocytes mature between day 3 and 5, while murine oligodendrocytes mature between day 1 and 3. The in vivo relevance of oligodendrocyte differentiation from NS/PC is displayed by studying the consequence of the thyroid hormone T3 on oligodendrocyte maturation in vitro. Thyroid hormone drives oligodendrocyte maturation in vivo since hyperthyroid rodents express higher and hypothyroid rodents lower levels of oligodendrocyte maturation genes [80–82]. Similarly, T3 induces MBP and MOG in differentiated human and mouse NS/PC, respectively, with higher potency in human cultures (Fig. 12d). This stronger T3-dependent increase of oligodendrocyte maturation markers observed in human compared to murine cultures may be due to a higher T3-responsiveness of human oligodendrocyte maturation genes or an already higher maturation grade of mouse oligodendrocytes at the beginning of the differentiation process with thus limited acceleration of the process.

Besides physiological, hormonal effects on oligodendrocyte maturation (Fig. 12d) the literature provides us with the indication that in vitro studies concerning oligodendrocyte toxicity have in vivo relevance. Eschenroeder and coworkers observed that low doses of the opioid buprenorphine accelerated and increased, but high doses delayed and decreased MBP expression in oligodendrocyte cultures isolated from rat brains [83]. The same dose-dependent effects of buprenorphin on MBP expression were seen in vivo in brain lysates of pups treated on embryonic day 7 and sacrificed at PND 12, 19, or 26 [84]. A second compound affecting oligodendrocytes during the perinatal period is vanadium. This metal produces hypomyelination during the second postnatal week in rat in vivo, a period of the most intense oligodendrocyte development and myelination in this species. This hypomyelination is thought to be caused by oligodendrocyte precursor cell (OPC) apoptosis due to vanadium-induced oxidative stress because

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vanadium decreases the number of OPC in postnatal brains in vivo and causes OPC cell death in vitro. In culture, OPC are more sensitive towards vanadium-induced death than astrocytes or mature oligodendrocytes underlining the high susceptibility of OPC towards vanadium that was seen in vivo [85]. These two case studies indicate that toxicity measured in primary oligodendrocyte cultures in vitro bear a resemblance to the in vivo situation. However, isolated oligodendrocyte cultures lack interactions with other cell types like neurons or astrocytes. That this might be of relevance for in vitro testing was demonstrated by He and coworkers [86] who induced oligodendrocyte apoptosis with lipopolysaccharides (LPS) only when oligodendrocytes were cocultured with astrocytes. This example reflects that a representative in vitro system for studying DNT with regard to oligodendrocytes needs to integrate interaction of oligodendrocytes with other major brain cell types. Multiple critical pathways for oligodendrocyte survival, proliferation, and differentiation are regulated by cell-extrinsic molecules like growth factors, cytokines, hormones, and neurotransmitters, which are expressed by neighboring cells (reviewed in ref. [87]). One system for DNT testing consisting of neurons, astrocytes, oligodendrocytes and microglia are rodent brain aggregate cell cultures [88–90]. Such reaggregate cultures represent brain cell mixtures of rat brains, and thus, species specificities cannot be excluded. Moreover, they are not pure NS/PC cultures, but consist of cells of different maturation states. In contrast, the above-described NS/PC-derived human oligodendrocytes seem to recapitulate oligodendrocyte formation and maturation in coculture with neurons and astrocytes and thus might be a suitable alternative for oligodendrocyte toxicity testing. However, compounds need to be tested in a system that targets this important cell type.

3 Genetic Modification of Neurospheres: How to Create Transgenic Cells in 3D In Vitro

For basic research, transgenic animals have been of great value as they allow study of mammalian gene regulation and their physiological function in a whole organism [91]. They have been utilized to mechanistically investigate a large variety of human diseases ([91], reviewed in ref. [92]). Despite the extensive use of these models in basic and pharmaceutical sciences, enormous failure rates in drug development reflect issues of extrapolating data generated in rodents to humans [22, 93, 94]. Thus, time seems ready for the creation of in vitro transgenic human 3D cultures enabling functional studies of individual genes in a human cellular context. For this purpose, different protocols for genetic manipulation of

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human neurospheres are presented here. Such gene manipulation includes gene knockdown via siRNA or shRNA as well as specific gene overexpression.

Transfection describes a process of introducing foreign nucleic acids into eukaryotic cells (reviewed in ref. [95]) with either transient or stable expression of the foreign nucleic acid in the host cell. Transient expression systems are extremely useful for studying elements that regulate immediate gene expression or for retrieving experimental results within a short time frame. The expression of the externally introduced nucleic acid may be detected for up to 4 days depending on the specific characteristics of the introduced nucleic acid, the cell type and doubling time of cells (reviewed in ref. [95]). The most prevalent transfection method is lipofection. Lipofection has been proven to efficiently deliver molecules from small oligonucleotides to entire proteins into the cell [96]. Although lipofection is most commonly used and also is effective for transfection of human neurospheres (data not shown), we use an alternative transfection method called nucleofection for transfection of neurospheres. This method uses chemical and physical procedures to transfer exogenous material into the cell and usually results in a higher transfection efficiency than common lipofection reagents [97] and higher cell viability after transfection compared to electroporation [98]. We performed transfections with the 4D-Nucleofector® X unit (Amaxa, Lonza Cologne, GmbH) and the Nucleofector® X solutions P3 for primary cells. The correct electric pulse for the NS/PC culture was established by transfecting with the pmaxGFP® vector (Fig. 13; Amaxa, Lonza Cologne, GmbH; for detailed information of this method see 4D-Nucleofector™ instructions, Lonza), which produced observable green fluorescence several hours after transfection.

Viral vectors were originally developed as an alternative to transfection of naked DNA for molecular genetics experiments. Compared to traditional transfection, transduction can ensure that nearly 100 % of cells are infected without severely affecting cell viability [99]. Furthermore, some viruses, e.g., lentiviruses, integrate into the cell genome ensuring stable gene expression [99]. For safety reasons, lentiviral vectors never carry the genes required for their replication. To produce a lentivirus, three plasmids are transfected by calcium phosphate precipitation into a so-called packaging cell line, HEK 293FT. Two plasmids, the gag-pol and VSV-G expression vectors, which are generally referred to as packaging plasmids, encode the virus proteins. A third plasmid contains the gene of interest [100]. For NS/PC infection with viral particles, neurospheres are chopped with the McIllwain tissue chopper [29] into 0.1 mm pieces. These pieces are incubated with 10,000 lentiviral particles/cell in DMEM/F12 medium containing 100 ng/ml EGF, 50 ng/ml FGF, and 0.02 mg Polybrene. To select for transduced cells, the selection antibiotic puromycin (0.25 μg/ml)

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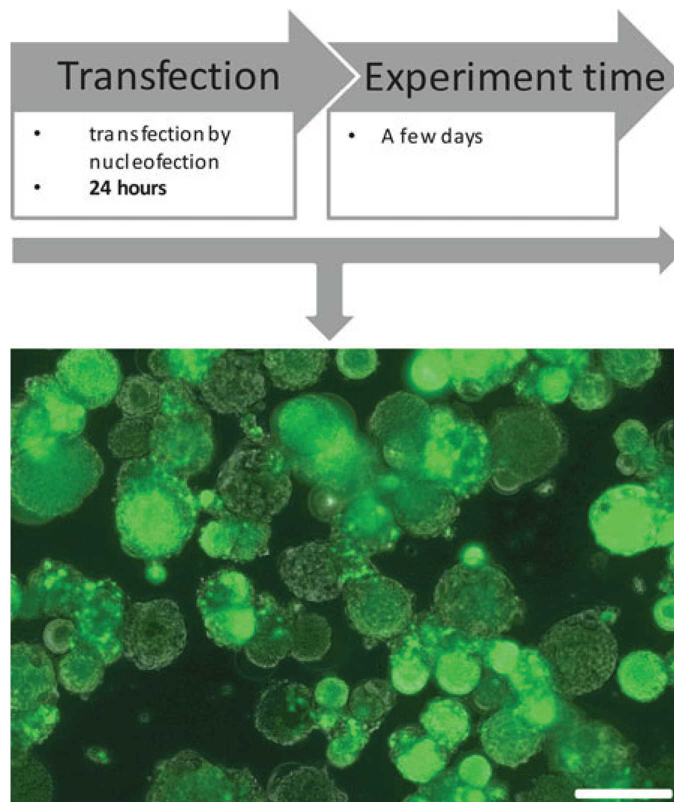

Fig. 13 Transfection of human NS/PC with the 4D-Nucleofector® X unit. The flow diagram shows the various work steps for transfecting human NS/PC by nucleofection. Furthermore the transfection of human NS/PC with green fluorescent protein (GFP) is shown. Expression of GFP is demonstrated by fluorescent microscopy 24 h after transfection, scale bar = 100 μm

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is added to the medium starting 3 days after infection. Control of the efficiency of genetic manipulation is subsequently achieved by PCR, western blotting and/or immunocytochemistry. Because of the stable insertion of nucleic acids, cells can be propagated and cultures used for experiments for several weeks (Fig. 14). 604 605 606 607 608

In contrast to lentiviruses, adenoviral DNA does not integrate into the genome and is not replicated during cell division (reviewed in ref. [101]). Nevertheless, due to their high transduction efficiency, broad host range ability to infect non-dividing cells, and potential for generating high titer virus, recombinant adenoviral vectors are one of the most efficient viral vectors for gene delivery in vivo and in vitro (reviewed in ref. [101]). For more detailed information on adenovirus production, the reader is referred to Yuan et al. [102] and Weggen et al. [103]. Again, as it is done for lentiviral transduction, human neurospheres are chopped to 0.1 mm pieces and infected with different virus concentrations (50–1000 virus particles/cell). This titration of virus particles is necessary to find the best ratio of virus particles to cells. Four hours after transduction, the medium is completely changed to normal proliferation medium (Fig. 15). Two days after infection, spheres are ready to use 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623

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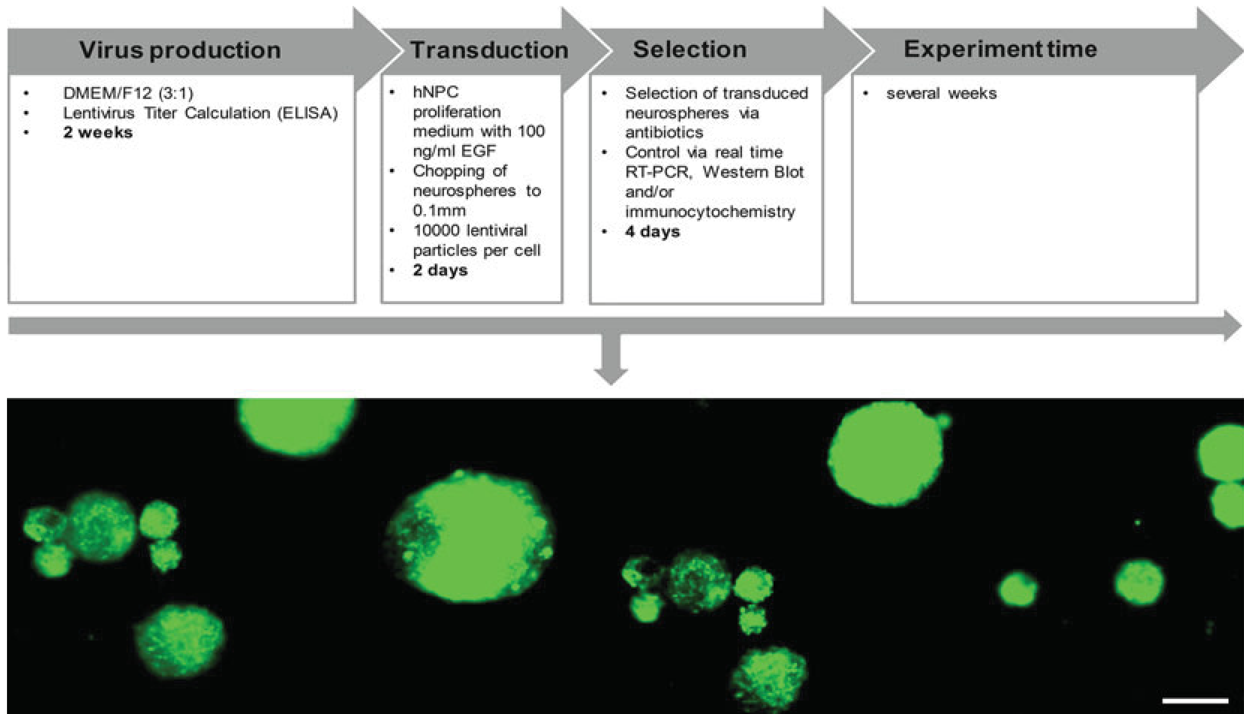

Fig. 14 Lentiviral transduction of human NS/PC. The flow diagram shows the various work steps for lentiviral transduction. Furthermore the lentiviral transduction of human NS/PC with green fluorescent protein (GFP) is shown. Expression of GFP is demonstrated by fluorescent microscopy 48 h after transduction, scale bar = 100 μm

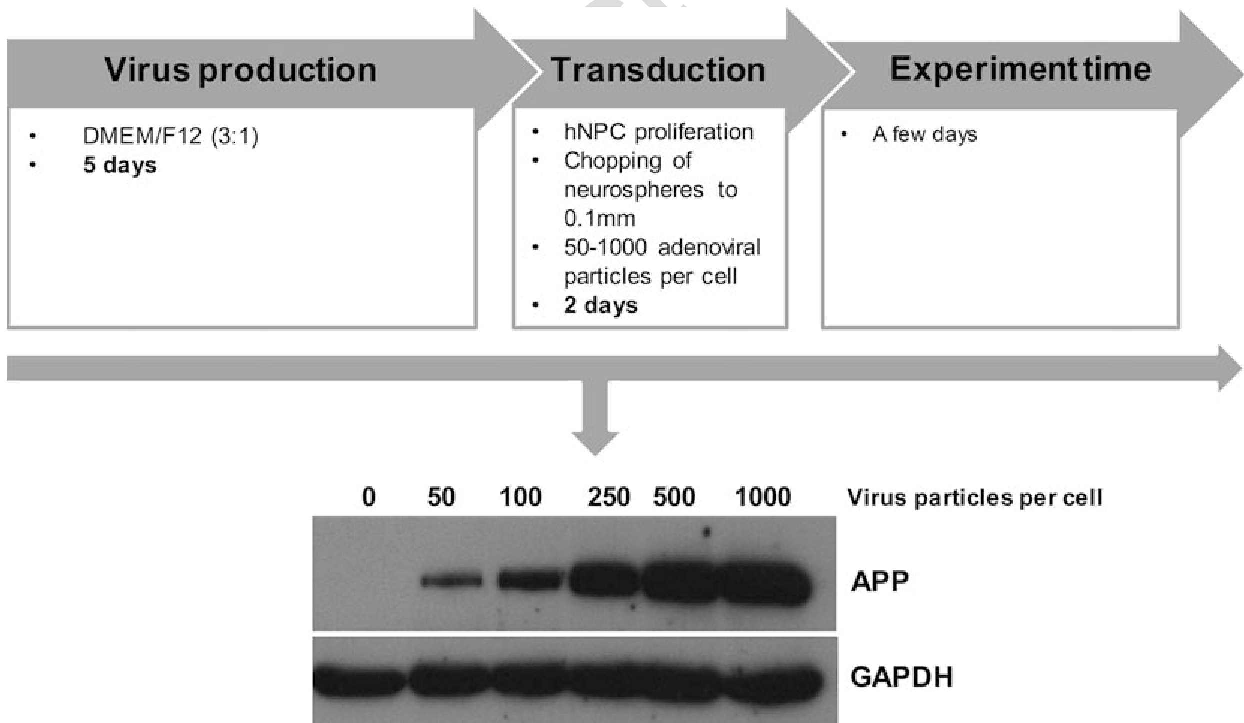

Fig. 15 Adenoviral transduction of human NS/PC. The flow diagram shows the various work steps and adenoviral transduction of human NS/PC with Amyloid Precursor Protein (APP). 48 h after transduction with different virus particle concentrations, human NS/PC lysates (20 μg protein/lane) were analyzed for APP expression by Western blot using an APP-specific antibody. A GAPDH-specific antibody was used as a loading control

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t.1 **Table 1**
Comparison of different transgenic modification methods in NS/PC in terms of experimental applicability

	Lentivirus	Adenovirus	Nucleofection
Virus production time	2 weeks	1 week	–
Time span until experimental use after transduction/transfection	1 week	2 days	1 day
Time span to perform experiments	Several weeks	A few days	A few days

for experiments and nucleic acid delivery can be monitored by western blotting and/or immunocytochemistry. Because adenoviral transduction does not lead to insertion of the foreign nucleic acid into the host genome, products get lost during cell division. This is the reason why experiments with adenoviral transduced cells can only be performed during the course of a few days. Specific times for experiments have to be tested for each nucleic acid because cellular half-life depends on the individual product. 624 625 626 627 628 629 630 631

The choice of method for gene manipulation of neurospheres depends on the experimental question asked and the transfection/transduction efficacy of the respective nucleic acid. This has to be tested individually. However, for short-term experiments, nucleofection is the fastest method for generating transient transgene neurospheres, and thus, the desired method in cases where the transfection efficacy is sufficiently high (Table 1). 632 633 634 635 636 637 638

4 Conclusions and Future Directions 639

Compared to tumor cells grown in 2D, 3D neurospheres are a fairly sophisticated cell system. They consist of primary cells, comprise or produce the four major neuroectodermal cell types of the human brain (NS/PC, neurons, astrocytes, oligodendrocytes), are self-organized, and can be cultured in two differentiation stages (proliferating and differentiating). Moreover, they can be generated from different species including humans. Species-overarching investigations of molecular and cell biological responses are of substantial importance for the translational aspect of basic science as well as for efficacy and toxicity testing in pharmacology and toxicology. Molecular species-specificities seem to be one reason for the failure of novel drug development for diseases like sepsis and ischemic stroke [93, 94]. Therefore, the data presented in this chapter focused on basic species-dependent properties of neurodevelopmental processes analyzed with the "Neurosphere Assay" in vitro. Moreover, relevance to the in vivo situation of in vitro data generated with NS/PC was presented. 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656

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With regard to the developing brain, primary human cultures are currently the closest model to the human in vivo situation. This is supported by the observations that rodent brain cells taken out of the in vivo situation placed into a tissue culture dish maintain their signaling functions or their responses towards xenobiotics (e.g., [39, 45, 44, 104, 105]), and thus, primary human cells are thought to behave similarly when removed from the in vivo to the in vitro situation. Although NS/PC generated from human embryonic (hESC) or human induced pluripotent stem cells (hiPSC) are also promising methods for human cell-based DNT testing, the in vivo-relevance of these models for neurodevelopment, especially with regard to developmental timing and cell type differentiation, i.e., formation of oligodendrocytes, remains an outstanding question.

Nevertheless, despite the advantages of such 3D in vitro systems, one still has to be aware that these are models—as are animals and other cell-based assays. Every model has its strengths and limitations. The limitations of in vitro models in conventional culture are mainly their lack of physiological cell and organ context as this creates artificial pharmacokinetics/toxicokinetics. Thus, the whole issue of absorption/distribution/metabolism/excretion (ADME) is not considered in in vitro testing situations. This can be circumvented when human in vivo target organ concentrations of compounds as well as their in vitro kinetics are known then providing the opportunity to model realistic exposure scenarios in vitro. Another approach to implement kinetics in vitro is the "Organ-on-a-Chip" technology ([106], reviewed in refs. [107, 108]). The use of human cells in this approach enables better realization of human ADME. Another limitation of the neurosphere system is the lack of some cell types. Neurospheres do not contain microglia or endothelial cells. The latter is an especially crucial factor because in conjunction with astrocytes, endothelial cells form the blood–brain barrier. This important barrier determines internal brain exposure towards compounds as well as towards endogenous mediators like hormones or cytokines. Although in these self-organized coculture models one does have interactions between different cell types—neurons, astrocytes, and oligodendrocytes—the deficiency in microglia and endothelial cells has to be taken into account. In addition, there is evidence that identical brain cell types differ in their function from one brain region to another (e.g., reviewed in ref. [109]). These brain region-specific differences can easily be studied by cell preparations from different brain areas in rodents. Such studies have identified region-dependent susceptibility of developmental astrocyte proliferation towards MeHgCl: while this compound inhibits hippocampal astrocyte proliferation, cortical, cerebellar, and brain stem astrocytes proliferation is not specifically inhibited by MeHgCl [110]. Modeling human brain region-specific cell characteristics in vitro is not possible based on current knowledge. In the future, with more

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information on the physiological factors responsible for brain region-specific cell development and function, it might be possible to also model this aspect in a dish by manipulating hESC or hiPSC accordingly. Last, these cultures cannot think, feel, and associate, so they can only mimic neurodevelopmental processes. However, with additional, complementary assays, filling the biological application domains not covered by the "Neurosphere Assay," i.e., neuronal network formation and electrical network activity measurements, a battery of tests can be created enveloping the major key events of human brain development in a dish and thus—although still not thinking—generate an in vitro scenario that is able to give a certain mechanism-based level of certainty for DNT assessment. Ideally, such a battery would be embedded into the theoretical frame of the "Adverse Outcome Pathway" (AOP) concept [111], which currently is receiving significant attention from regulatory agencies worldwide. AOPs for DNT are still sparse and thus urgently needed. The cross-species evaluation of signaling pathways, which is possible with the neurospheres presented in this chapter, is highly suited to fill the data gap on translation from rodents to humans, an important aspect within the AOP concept.

Also for basic biomedical research these cultures can be of great value, especially with the option of creating transgenic neurospheres as described above. This technique is (a) much faster and more cost-effective than first creating a transgenic animal and subsequently preparing neurospheres from the pups; and (b) is based on the species typically of greatest regulatory concern—humans. The obvious limitation of this approach is, however, that in vitro gene silencing never produces a complete knockout as in an animal model, but is restricted to a knock-down with various degrees of gene repression.

This chapter discusses the strengths and limitations of the "Neurosphere Assay" for DNT hazard assessment. In the future, this assay will be further characterized for its biological application domain with regard to human in vivo brain development. Such knowledge will provide confidence for its position in a DNT in vitro testing battery that covers major key events in neurodevelopment for regulatory implementation.

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Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations

Jenny Baumann*, **Katharina Dach*,** Marta Barenys, Susanne Giersiefer, Janette Goniwiecha, Pamela J. Lein, and Ellen Fritsche

* shared first-authorship

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2.2 BDE-99 impairs human and mouse oligodendrogenesis by species-specific modes of action

Katharina Dach, Farina Bendt, Ulrike Huebenthal, Susanne Giersiefer, Heike Heuer and Ellen Fritsche

Environmental Health Perspectives *(submitted on November 9th, 2015)*

Hintergrund: Polybromierte Diphenylether (PBDEs) sind bioakkumulierende Flammschutzmittel, die Entwicklungsneurotoxizität in Menschen und in Nagern hervorrufen. Zusätzlich wird vermutet, dass sie Thyroidhormon (TH) Disruptoren sind. Die Oligodendrogenese ist ein thyroidhormonabhängiger Prozess während der Entwicklung des zentralen Nervensystems, welcher empfindlich gegenüber PBDE Exposition ist.
Ziele: Unser Ziel war die Etablierung humaner und muriner *in vitro* Methoden, um TH Disruption auf Oligodendrozytenbildung und –reifung zu untersuchen. Weiterhin untersuchten wir, ob BDE-99 diese Prozesse durch TH Disruption stört und verglichen die Sensitivitäten sowie die involvierten Wirkmechanismen zwischen den beiden Spezies.
Methoden: Wir verwendeten humane und murine neurale Progenitorzellen (h,mNPCs), die als 3D Neurosphären kultiviert wurden, um die Effekte von TH und BDE-99 auf die spezies-spezifische Oligodendrozytenbildung und –reifung zu untersuchen.
Ergebnisse: TH beschleunigte die humane, aber induzierte die murine, Oligodendrozytenbildung, während es die Reifung von Oligodendrozyten in beiden Spezies beschleunigte. BDE-99 inhibierte die Oligodendrozytenbildung in hNPCs bereits in niedrigeren Konzentrationen als in mNPCs, wobei TH Disruption nur im murinen System involviert war. Gleichermaßen inhibierte BDE-99 die Reifung muriner Oligodendrozyten durch TH Disruption, während die humane Oligodendrozytenreifung nicht beeinflusst war. Die TH Disruption der murinen Oligodendrozytenbildung und –reifung wurde nicht durch Thyroidhormonrezeptoren (TRs) vermittelt. Während BDE-99 Effekte auf die humane Oligodendrozytenbildung keine Blockade von TH Signalwegen beinhalteten, war ein Wirkmechanismus involviert, der durch Vitamin C antagonisiert werden konnte.
Schlussfolgerung: Humane und murine Oligodendrogenese werden durch BDE-99 mit unterschiedlicher Sensitivität inhibiert und die zugrundeliegenden Wirkmechanismen sind Spezies-spezifisch. Diese Daten unterstützen das Konzept, dass *in vitro* Zellsysteme humanen Ursprungs für die Abschätzung des Gefährdungspotentials von Chemikalien wichtig sind.

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1 **BDE-99 impairs human and mouse oligodendrogenesis by species-specific modes of action**

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10 **Running title:** BDE-99 impairs NPC-derived oligodendrogenesis.

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14 **Abstract**

15

16 **Background:** Polybrominated diphenyl ethers (PBDEs) are bioaccumulating flame retardants
17 causing developmental neurotoxicity (DNT) in humans and rodents. In addition, they are
18 suspected to be thyroid hormone (TH) disruptors. One TH-dependent neurodevelopmental
19 process susceptible to PBDE exposure is oligodendrogenesis.

20 **Objectives:** We aimed at establishing human and mouse *in vitro* methods for assessing TH
21 disruption on oligodendrocyte formation and maturation. Moreover, we assessed if BDE-99
22 disturbs these processes by TH disruption and compared susceptibility as well as the underlying
23 modes of actions (MOAs) between species.

24 **Methods:** We employed human and murine neural progenitor cells (h,mNPCs) cultured as 3D
25 neurospheres to study TH and BDE-99 effects on species-specific oligodendrocyte formation and
26 maturation.

27 **Results:** TH accelerated human, but induced murine oligodendrocyte formation, while it
28 accelerated oligodendrocyte maturation in both species. BDE-99 reduced oligodendrocyte
29 formation more potently in human than in mouse NPCs with TH disruption only being involved
30 in the murine system. Likewise, BDE-99 inhibited murine oligodendrocyte maturation by TH
31 disruption, while human oligodendrocyte maturation was not affected. TH disruption of murine
32 oligodendrocyte formation and maturation by BDE-99 was not mediated by thyroid hormone
33 receptors (TRs). While BDE-99 effects on human oligodendrocyte formation did not involve a
34 blockage of proper TH signaling it involved a distinct mechanism that was antagonized by
35 vitamin C.

36 **Conclusions:** Human and murine oligodendrogenesis are inhibited by BDE-99 with different
37 susceptibilities and the underlying MOAs are species-specific. These data support the concept
38 that there is a need for human cell-based *in vitro* systems for chemical hazard assessment.
39
40 **Key words:** NPC, neurosphere, neurodevelopment, DNT, BDE-99, TH disruption,
41 neurodevelopmental toxicity

42

43 **Introduction**

44 Polybrominated diphenylethers (PBDEs) were widely used as flame retardants in consumer
45 products and although mostly banned from the markets, they remain present in the environment
46 (Law et al. 2014; Ma et al. 2013; Shaw and Kannan 2009; Yogui and Sericano 2009). Main
47 PBDE exposure sources for adults, young children, babies and fetuses comprise house dust, the
48 food chain, breast milk and cord blood, respectively (reviewed in Frederiksen et al. 2009; Sjödin
49 et al. 2003). Due to combined exposure via house dust and breast milk, toddlers present the
50 highest PBDE body burdens of all age groups (Fischer et al. 2006; Rose et al. 2010; Toms et al.
51 2009). Exposure during development is of high concern as PBDEs are developmentally
52 neurotoxic for humans (reviewed in Grandjean and Landrigan 2014).
53 Thyroid hormones (THs) play a fundamental role in brain development as they guide e.g.
54 migration, differentiation, myelination and synaptogenesis in rodents (reviewed by Bernal 2005).
55 PBDEs decrease L-thyroxine (T4) and increase thyroid stimulating hormone (TSH) blood levels
56 thus disrupting rodent TH homeostasis (reviewed in Costa et al. 2014). In addition, *in utero*
57 PBDE-exposed animals present similar behavioral abnormalities (reviewed in Costa and
58 Giordano 2007) as hypothyroid animals (Negishi et al. 2005). However, whether TH disruption

59 is indeed involved in PBDE-induced developmental neurotoxicity (DNT) in rodents remains
60 elusive. In humans, high PBDE measures were correlated with decreased, increased or
61 unchanged T4 and increased or decreased TSH blood levels (reviewed by Costa et al. 2014)
62 indicating an inconclusive PBDE effect on TH homeostasis in humans.
63 This study aimed to elucidate if disturbance of TH signaling by BDE-99 interferes with the
64 neurodevelopmental process of oligodendrogenesis *in vitro* and thus might be a potential of
65 action (MOA) of PBDE-induced DNT. We first demonstrated that human and murine neural
66 progenitor cells (h,mNPCs) are an appropriate *in vitro* systems to study TH disruption since they
67 functionally express important TH signaling components. In addition, we identified
68 oligodendrocyte formation/maturation as species-specific, TH-dependent processes *in vitro*.
69 Moreover, we observed pronounced species-specific effects of BDE-99 on oligodendrocyte
70 formation and maturation: it reduced oligodendrocyte formation in hNPCs and mNPCs with
71 different potencies and only in mNPCs this reduction involved TH disruption. Furthermore,
72 BDE-99 affected only murine oligodendrocyte maturation by interfering with TH action,
73 whereas human oligodendrocyte maturation was not compromised by BDE-99. As a MOA for
74 human disturbed oligodendrocyte formation by BDE-99, a reactive oxygen species (ROS)-
75 independent mechanism that was antagonized by vitamin C was identified.

76 **Methods**

77 ***Chemicals.***
78 Brominated diphenyl ether (BDE)-99 was kindly provided by U. Strähle from the Karlsruhe
79 Institute of Technology, thyroid hormones (THs) L-3,3´,5- triiodothyronine (T3) and L-3,3´,5,5´-
80 tetraiodothyronine (T4), L-ascorbic acid (vitamin C) and Trolox (vitamin E analog) were
81 purchased from Sigma Aldrich (Steinheim, Germany). Chemicals were dissolved as follows: 1,

82 2, 6.66, 10 mM stocks of BDE-99 in dimethyl sulfoxide (DMSO; Carl Roth GmbH, Karlsruhe,
83 Germany), 300 µM T3 or T4 in a 1:1 dilution of 96% ethanol and 1 M HCl (both Carl Roth
84 GmbH) ("EtOH/HCl"), 100 mM L-ascorbic acid in H_2O, 200 mM Trolox in DMSO. Solvent
85 concentrations used in the experiments were 0.1% (co-treatment experiments) or 0.3% (dose-
86 response experiments) DMSO for BDE-99, 0.01% EtOH/HCl (T3, T4), 0.05% DMSO (Trolox),
87 0.1% or 0.5% H_2O (L-ascorbic acid) or the respective combination in the co-treatments. For
88 antagonization experiments, IC_{50} concentrations for BDE-99 (2 µM and 10 µM (approx.) for
89 hNPCs and mNPCs, respectively - 14 µM BDE-99 are not medium-soluble with a DMSO
90 concentration of 0.1%) were chosen.

91 ***Neurosphere Culture.***

92 Normal human neural progenitor cells (hNPCs, gestational week (GW) 16-20) were purchased
93 from Lonza Verviers SPRL (Belgium). Developmental time-matched murine NPCs from
94 postnatal day (PND) 1 (Workman et al. 2013) were prepared as described previously for rat
95 NPCs (Baumann et al. 2014) with modifications. Briefly, only the murine forebrain was used and
96 digested with papain for only 7 min. Human and murine NPCs were cultured as neurospheres in
97 proliferation medium consisting of DMEM (Life Technologies, Darmstadt, Germany) and Hams
98 F12 (Life Technologies) (3:1) supplemented with 2% B27 (Life Technologies), 20 ng/ml
99 epidermal growth factor (EGF, Life Technologies), 20 ng/mL (hNPCs) or 10 ng/ml (mNPCs)
100 recombinant human fibroblast growth factor (FGF, R&D Systems, Wiesbaden, Germany), and
101 1% penicillin and streptomycin (Pan-Biotech, Aidenbach; Germany). Neurospheres were
102 maintained at 37°C with 5% CO_2 and passaged mechanically with a tissue chopper to 0.2 mm
103 once a week. Half of the medium was replaced thrice a week (for detailed information
104 see Baumann et al. 2014).

105 ***Differentiation of NPCs.***

106 NPCs were chopped to 0.2 mm 1-3 days (hNPCs) or 3-5 days (mNPCs) before plating the
107 experiment in proliferation medium. Neurospheres of 0.3 mm diameter were washed in
108 differentiation medium and plated into poly-D-lysine (PDL)/laminin (Sigma Aldrich) coated 8-
109 chamber glass cover slides. In each chamber, five neurospheres were plated in 500 µl
110 differentiation medium [DMEM (Life Technologies), Hams F12 (Life Technologies) 3:1
111 supplemented with 1% of N2 (Life Technologies) and 1% penicillin and streptomycin (Pan-
112 Biotech)] containing the respective exposure. In all murine experiments 1% hormone-free fetal
113 calf serum (FCS; Biochrom, Berlin, Germany) was added to the differentiation medium.
114 Neurospheres were differentiated for 5 days or in the time course experiment for 1, 2, 3, 4 and 5
115 days, respectively. Half of the exposure solution was refreshed at day 2 of differentiation.

116 ***Viability assay.***

117 Cell viability was determined using the AlamarBlue assay (CellTiter-Blue assay, Promega), as
118 previously described (Baumann et al. 2014). Briefly, in every experiment mitochondrial
119 reductase activity was assessed for 2 hours in the same chambers where the neurospheres were
120 cultured. As background control chambers with the corresponding medium but without cells
121 were used.

122 ***Immunocytochemistry/Fluorescence microscopy.***

123 After differentiation and viability measurement neurospheres were fixed with 4%
124 paraformaldehyde (PFA, Merck, Darmstadt, Germany) at 37°C for 30 min and stored in
125 phosphate buffered saline (PBS; Biochrom, Berlin, Germany) at 4°C until the
126 immunocytochemical staining for O4 (1:200 mouse IgM anti-O4 antibody (R&D Systems) and

127 1:250 Alexa Fluor 488 anti-mouse IgM (Life Technologies)) was performed. The percentage of
128 oligodendrocytes ($O4^+$ cells)/nuclei was determined as described earlier (Baumann et al. (2014).

129 ***Quantitative RT-PCR (qRT-PCR).***

130 To evaluate gene expression in proliferating neurospheres, triplicated samples of 50
131 neurospheres with a 0.3 mm diameter were collected 2 (hNPCs) or 3 (mNPCs) days after
132 chopping and lysed. For differentiating experiments, three wells with each ten 0.3 mm
133 neurospheres were used per exposure condition: Neurospheres were differentiated in
134 PDL/laminin coated wells of a 24 well plate in 1 ml differentiation medium containing the
135 respective exposure. In 3 or 5 days differentiation experiments half of the exposure medium was
136 refreshed at differentiation day 2. Cells were lysed at day 1, 3 or 5 of exposure, RNA was
137 isolated (RNeasy Mini Kit, Qiagen, Hilden, Germany) and cDNA was transcribed (Quantitect
138 Reverse Transcription Kit, Qiagen) according to the manufacturer instructions. qRT-PCR was
139 performed using QuantiFast SYBR Green PCR Kit (Qiagen) in the Rotor Gene Q cycler
140 (Qiagen). Expression levels were normalized to the expression of *β-actin*. The detailed PCR
141 protocol and primer sequences are given in the Supplemental Material.

142 ***Statistics.***

143 For dose-response curves a sigmoidal curve fit (variable slope) was applied using Graphpad
144 Prism 6.0 (La Jolla, California). Data was pooled from at least three independent experiments
145 and was analyzed with the same software using OneWay ANOVA for dose-response
146 experiments, TwoWay ANOVA for co-exposure and time-course experiments or a student´s t-
147 test for comparison of wildtype and knockout mice within the same treatment. As post-hoc tests
148 Bonferroni's (OneWay ANOVA) or Tukey's (TwoWay ANOVA) multiple comparison test were
149 used. T-tests were performed with Welsh’s correction assuming different standard deviations for

150 the treatments. Significance threshold was established at $p<0.05$ except in gene expression
151 experiments where it was fixed at $p<0.1$.

152 **Results**

153 ***Expression and functionality of TH signaling components.*** Human and murine NPCs express
154 genes encoding for thyroid hormone receptors (TRs) α and β (Fig. S1 A-D). Although, the
155 expression of the gene encoding for human TRβ1 (*THRB1*) was significantly induced by the
156 receptor active thyroid hormone L-triiodothyronine (T3), the low copy number change from 3 to
157 7 copies/10,000 *β-actin* makes a biological significance of this increase highly unlikely.
158 Functionality of TRs was demonstrated by T3-induced *hairless* expression (Fig. S1 E,F).
159 *Hairless* is a TH-inducible gene in fetal brains *in vivo* (Potter et al. 2002; Thompson and Potter
160 2000) that is TRα-dependent (Ramos and Weiss 2006). Furthermore, T3 induced its own
161 metabolism by stimulating *deiodinase 3* expression (Fig. S1 G,H).
162 ***BDE-99 effects on human and murine oligodendrocyte formation.*** Human and murine NPCs
163 form around 6% oligodendrocytes/nuclei in the migration area after 5 days of differentiation
164 (Baumann et al. 2015). BDE-99 reduced this percentage of $O4^+$ cells/nuclei in the migration area
165 of human and murine NPCs concentration-dependently after 5 days of differentiation without
166 reducing viability (Fig. 1A,C). Human oligodendrocyte formation was found to be 7 times more
167 sensitive towards the BDE-99 treatment than the murine process (IC_{50}: 1.85 μM and 13.64 μM
168 BDE-99; $IC_{20:}$ 0.862 μM and 6.85 μM). Significant BDE-99-induced disturbances of human
169 oligodendrogenesis started in the nM range (LOEAC 862 nM). Fluorescence microscope images
170 illustrate the BDE-99-mediated reduction in oligodendrocytes after 5 days of differentiation (Fig.
171 1B,D).

172 ***TH effects on basal and BDE-99-reduced mouse and human oligodendrocyte formation.*** T3
173 and T4 only induced oligodendrocyte formation in mNPCs, but not in hNPCs after 5 days of
174 differentiation (Fig. 2). In line with these results, 3 nM T3 or T4 antagonized the BDE-99-
175 induced reduction in oligodendrocyte formation only in in mNPCs, but not in hNPCs (Fig.
176 2B,E). Time-course experiments revealed that human oligodendrocytes form during the first 4
177 days of differentiation. Treatment of the cells with T3 accelerated, but did not induce human
178 oligodendrocyte formation. Moreover, T3 did not antagonize the inhibitory BDE-99 effect on
179 human oligodendrocyte formation at any time point (Fig. 2C). In murine cultures,
180 oligodendrocytes formed within the first 1-2 days and T3 induced their formation. Furthermore,
181 T3 antagonized the BDE-99-dependent reduction of murine oligodendrocyte formation (Fig. 2F).
182 ***TH effects on basal and BDE-99-reduced mouse and human oligodendrocyte maturation.*** For
183 investigating oligodendrocyte maturation, expression of the oligodendrocyte-specific markers
184 human myelin basic protein (*MBP*) and murine myelin oligodendrocyte glycoprotein (*Mog*) were
185 analyzed (Fig. 3). These different markers were used, because human oligodendrocytes do not
186 express the late marker *MOG* after 5 days of differentiation due to their immaturity, while for
187 murine oligodendrocytes - due to their high maturation stage – *Mbp* was already highly
188 expressed on day 1 of differentiation and did not increase over differentiation time (data not
189 shown). We also confirmed this species-dependent difference in oligodendrocyte maturation
190 stage by morphological analysis as branched, sheet-forming oligodendrocytes found in murine
191 oligodendrocyte culture represent phenotypically a more mature state than the more linear
192 oligodendrocytes observed in human cultures (Fig. 3C). Both, human *MBP* (h*MBP*) and murine
193 *Mog* (m*Mog*) expression increased significantly during the 5 days of differentiation indicating
194 oligodendrocyte maturation (Fig. 3A,D). During this time period, T3 significantly induced gene

195 copy numbers of NPCs' *MBP* (human) and *Mog* (mouse), while BDE-99 reduced basal and T3-
196 induced h*MBP* as well as m*Mog* expression. To evaluate if this reduced h*MBP* and m*Mog*
197 expression is a consequence of reduced oligodendrocyte formation or rather a consequence of a
198 compromised maturation, h*MBP* and m*Mog* expression was normalized to the number of
199 oligodendrocytes in the respective cultures (Fig. 3B,E). The calculation of this maturation
200 quotient (Q_M)

201 $$Q_M = \textit{MBP} \text{ or } \textit{Mog} \text{ expression/\%oligodendrocytes}$$

202 clearly revealed that 3 nM T3 significantly increased the maturation stage of oligodendrocytes in
203 both species (human: 4.4-fold, mouse 1.8-fold). In contrast, treatment of the cells with 2 µM
204 BDE-99 (IC_{50} concentration for inhibition of human oligodendrocyte formation, Fig. 1) did not
205 reduce human Q_M, neither alone nor in combination with T3 (Fig. 3B), indicating that the
206 reduction in h*MBP* expression is solely due to the lower number of oligodendrocytes in the
207 BDE-99-treated cultures. Because oligodendrogenesis in hNPCs is TH-independent, h*MBP*
208 reduction by BDE-99 is a process not involving TH disruption. In contrast, 10 µM BDE-99
209 (approx. IC_{50} inhibition of murine oligodendrocyte formation, Fig. 1) given alone as well as in
210 combination with T3 reduced murine Q_M to 30% of the respective solvent control value (Fig.
211 3E). BDE-99 concentrations that alone did not affect oligodendrocyte maturation (1-100 nM),
212 significantly lowered T3-induced murine Q_M indicating disrupted TH signaling (Fig. 3F).

213 ***Role of TRs in T3-induced and BDE-99-reduced murine oligodendrocyte***
214 ***formation/maturation.*** The involvement of TRs in T3 and BDE-99 effects on murine
215 oligodendrocyte formation and maturation was studied using neurosphere cultures prepared from
216 PND1 TRα and β knockout mouse brains. Oligodendrocyte formation as well as *Mog* gene
217 expression/%oligodendrocytes (Q_M) were normalized to the respective genotype control because

218 raw values did not distinguish significantly for the different genotypes (data not shown). T3
219 significantly induced oligodendrocyte formation (Fig. 4A) and maturation (Fig. 4B) in TRβ, but
220 not TRα knockout neurospheres, confirming the specific involvement of TRα in T3-induced
221 oligodendrocyte formation and maturation. On the contrary, BDE-99 effects on murine
222 oligodendrocyte formation and maturation were not directly mediated by TRs because BDE-99
223 reduced the number of differentiated oligodendrocytes as well as *Mog* expression/
224 %oligodendrocytes in TR knockout neurospheres in a similar way as in wildtype cells (Fig.
225 4A,B). However, in contrast to wild type cultures, T3 treatment did not antagonize the BDE-99
226 effects on oligodendrocyte formation in absence of TRs, while T3 rescued the BDE-99 effect in
227 wildtype mNPCs (Fig. 4A). For oligodendrocyte maturation, 10 µM BDE-99 (approximate IC_{50}
228 concentration for inhibition of mouse oligodendrocyte formation, Fig. 1) reduced the T3-induced
229 *Mog* expression in all genotypes to the gene expression levels of BDE-99 alone (Fig. 4B)
230 indicating that BDE-99 acts on mouse oligodendrocyte maturation independent of TRs. That
231 BDE-99 indeed exerts TH disruption on mouse oligodendrogenesis – although not through TRs –
232 becomes obvious by co-treatment of mNPCs with T3 and BDE-99 at BDE-99 concentrations not
233 reducing basal *Mog* expression in absence of T3 (≥10 nM BDE-99), that completely antagonized
234 TH-induced gene expression (Fig. 3F).

235 To further elucidate the underlying mechanisms by which BDE-99 interferes with TH signaling,
236 expression of *TRs*, *deiodinase 3* and *hairless* were evaluated. BDE-99 did not influence *TR*
237 mRNA expression (Fig. S1 A-D) or TRα-mediated transcription of *hairless* alone or in
238 combination with T3 (Fig. S1 E,F). Furthermore, BDE-99 did not alter basal or T3-induced
239 *deiodinase 3* mRNA expression (Fig. S1 G,H). BDE-99 does not seem to interfere with transport

240 of T3 into the cell either because application of T3 induced *hairless* and *deiodinase 3 mRNA*
241 expression indicating a proper T3-TR interaction.
242 ***Involvement of ROS in BDE-99-dependent reduced human oligodendrocyte formation***. Since
243 our studies revealed that TH disruption was not the predominant mechanism by which BDE-99
244 compromised human oligodendrocyte formation we considered alternative pathways. (Fig.
245 2B,C). In particular, we hypothesized that the production of reactive oxygen species (ROS)
246 might be involved in BDE-99-induced toxicity on oligodendrocyte formation. One indicator of
247 ROS production is a change in gene expression of antioxidative defense genes like *heme*
248 *oxygenase 1*, *catalase*, *superoxide dismutase* and *glutathione peroxidase*. 2 µM BDE-99 did not
249 alter the expression of these genes after 24 hours of BDE-99 exposure suggesting that BDE-99 is
250 not producing an excess of ROS in hNPCs (Fig. 5A). However, application of vitamin C (50 and
251 100 µM) antagonized the BDE-99-dependent reduction in human oligodendrocyte formation
252 (Fig. 5B). As Trolox, a water-soluble derivative of vitamin E and effective ROS-scavenger, did
253 not exert comparable antagonizing effects in presence of BDE-99 (Fig. 5C), it is likely that
254 vitamin C rescues BDE-99-reduced oligodendrocyte formation by a mechanism other than ROS
255 scavenging. In contrast to human oligodendrocytes, mouse oligodendrocyte formation was not
256 rescued by vitamin C against BDE-99 (Fig. 5E). Also Trolox did not rescue murine
257 oligodendrocytes against BDE-99-induced toxicity (Fig. 5F). It is to note, that for murine
258 oligodendrogenesis, lower Trolox concentrations (10 and 20 µM) were used than for human
259 oligodendrogenesis since in mouse NPCs, 50 and 100 µM Trolox reduced the culture viability
260 and oligodendrocyte formation (data not shown).

261 **Discussion**

262 PBDEs were classified as human DNT compounds because human exposure during pregnancy
263 and early childhood is associated with lower IQs and hyperactivity in children at school age
264 (Eskenazi et al. 2013; Herbstman et al. 2010; Roze et al. 2009). The molecular mechanisms
265 underlying such disturbances of brain development by PBDEs remain elusive. However, PBDEs
266 alter TH homeostasis in rodents and interfere with TH signaling *in vitro* (reviewed in Costa et al.
267 2014). In addition, BDE-99, one of the most abundant PBDE congeners in human tissue (Costa
268 et al. 2008; McDonald 2005), reduced oligodendrocyte differentiation of human NPCs
269 (Schreiber et al. 2010). Because a crucial role of TH for oligodendrogenesis *in vivo* was
270 described earlier (Baumann and Pham-Dinh 2001; Farsetti et al. 1991; Ibarrola and Rodríguez-
271 Peña 1997; Marta et al. 1998; Rodriguez-Peña et al. 1993; Schoonover et al. 2004), we
272 investigated in this study if disruption of TH signaling in hNPCs and mNPCs might be an
273 underlying molecular mechanism for BDE-99-induced interference with oligodendrocyte
274 formation and maturation.

275 BDE-99 reduced human and murine oligodendrocyte formation in a concentration-dependent
276 manner (IC_{50} 1.8 µM and 13.64 µM, respectively and IC_{20} 0.862 µM and 6.85 µM, respectively)
277 with human NPCs being 7-times more sensitive towards BDE-99 exposure than their murine
278 counterparts (Fig. 1A,C). These data are in accordance with two other publications dealing with
279 PBDE effects on oligodendrogenesis: our previous study with an IC_{50} for inhibiting human
280 oligodendrogenesis around 1 µM BDE-99 (Schreiber et al. 2010) and inhibition of murine NSC
281 differentiation into oligodendrocytes with an IC_{25} for BDE-47 of 10 µM (Li et al. 2013).

282 The IC_{20} value for inhibiting human oligodendrocyte formation lies in the upper nanomolar
283 range (862 nM; approximately 431 ng/ml) and seems to be of relevance with regards to human

284 exposure towards PBDEs. Breast milk fed children are assumed to reach a daily PBDE exposure
285 up to 306 ng/kg/day (Costa and Giordano 2007; Schecter et al. 2006) and the serum
286 concentration of the five most abundant PBDEs reached 480 ng/g lipid weight (lw; approx. 960
287 nM in serum lipids) for Californian toddlers (Fischer et al. 2006). Measurements in wildlife birds
288 revealed a 1.5-fold accumulation of PBDEs in brain lipids compared to the corresponding serum
289 lipid samples (Voorspoels et al. 2006). Assuming similar accumulation in human brain lipids,
290 toddler PBDE brain lipid concentrations could reach 720 ng/g lw (approx. 1440 nM in brain
291 lipids), which is higher than our observed human IC_{20} (862 nM). However, it is obvious that this
292 is only a rough kinetic estimation containing a variety of uncertainties.
293 Next, we tested the hypothesis if TH disruption might be the underlying molecular mechanism
294 for BDE-99-dependent inhibition of oligodendrogenesis. Therefore, we first characterized TH
295 effects on human and mouse oligodendrogenesis. TH alone accelerated, but did not induce
296 oligodendrocyte formation from hNPCs (Fig. 2A-C). In contrast, we were not able to measure a
297 TH-dependent acceleration of oligodendrocyte formation in murine cultures (Fig. 2D-F). Most
298 likely, in our neurosphere assays, murine oligodendrocytes mature much faster than human cells
299 and thus they are generated within the first day of NPC differentiation (Fig. 2F). In addition, TH
300 induced mRNA expression of the oligodendrocyte maturation markers, h*MBP* (Fig. 3A) and
301 m*Mog* (Fig. 3D), and enhanced oligodendrocyte phenotypic maturation appearance (Fig. 3C) of
302 both species supporting the notion that TH accelerates oligodendrocyte maturation. Quickening
303 of maturation becomes obvious when copy numbers of myelin-related genes were linked to the
304 percentage of formed oligodendrocytes in the same cultures (Fig. 3B,E). This quotient Q_M
305 reflecting gene copy numbers/%oligodendrocytes demonstrates that human and mouse
306 oligodendrocyte maturation are guided by TH. TH is also guiding murine oligodendrogenesis *in*

307 *vivo:* Farsetti et al. (1991) observed that hypothyroid mice expressed lower levels of *Mbp* than
308 euthyroid mice.
309 For humans, there is much less data available when it comes to TH actions on
310 neurodevelopmental processes. However, the Allan-Herndon-Dudley-syndrome (AHDS), a
311 devastating disease caused by a mutation in the X-linked TH transporter *MCT8* gene, leads to
312 brain hypothyroidism due to defective TH uptake across the blood-brain-barrier and possibly into
313 certain developing brain cells. One pathological feature of patients with AHDS is delayed
314 myelination (Namba et al. 2008; Rodrigues et al. 2014; Tonduti et al. 2013) which might be due
315 to either a delayed oligodendrocyte formation or maturation or even a combination of both
316 (López-Espíndola et al. 2014). This *in vivo* phenotype is reflected *in vitro* by accelerated
317 oligodendrocyte formation and maturation by TH hNPCs. Concerning the speed of
318 oligodendrocyte formation and maturation we found that these processes happen much faster in
319 mouse than in human *in vitro* cultures (Fig. 2C,F; 3A,D). Also *in vivo* oligodendrocytes form and
320 maturate much faster in rodents than in humans: the time span between pre- oligodendrocyte
321 formation and emergence of the first mature oligodendrocytes is 11–12 weeks in humans but
322 only 5 days in rodents (Barateiro and Fernandes 2014).
323 BDE-99 reduced both, human and murine, oligodendrocyte formation, but this reduction was
324 only TH dependent in mNPCs. To test if BDE-99 acts as a TH disruptor on oligodendrocyte
325 maturation and if there are species differences between human and mouse cells, we assessed the
326 effects of this compound on human and mouse maturation quotients Q_M. The Q_M demonstrated
327 that BDE-99 did not affect human (Fig. 3B), but inhibited murine oligodendrocyte maturation by
328 TH disruption (Fig. 3F). These data indicate that there are species differences in BDE-99 action

329 on oligodendrogenesis and thus suggest the necessity of using human cells for human hazard
330 characterization of TH disruption on the developing brain.
331 Next, we studied the mechanisms of the observed species-specific effects of TH and BDE-99 on
332 oligodendrocyte development. Neurospheres prepared from TRα knockout mice indicated that
333 TH-dependent induction of murine oligodendrocyte formation and maturation was TRα-
334 dependent (Fig. 4A,B). The TRα involvement in murine oligodendrocyte formation is in
335 accordance with data from the literature showing that newborn TRα, but not TRβ, knockout mice
336 have less oligodendrocytes at birth than wildtype animals (Billon et al. 2002). In contrast, the
337 BDE-99 effect on murine oligodendrocyte formation and maturation were not directly mediated
338 by TRs because BDE-99 reduced number of differentiated oligodendrocytes as well as *Mog*
339 expression/%oligodendrocytes in TR knockout neurospheres in a similar manner as in wildtype
340 neurospheres (Fig. 4A,B). These findings are in accordance with our observation that BDE-99
341 did not inhibit T3-induced expression of *hairless* (Fig. S1 E,F), a gene that is regulated by TRα
342 in mice (Ramos and Weiss 2006). Since BDE-99 did neither alter TR expression (Fig. S1 A-D),
343 nor compromised T3-induced *deiodinase 3* expression (Fig. S1 G,H), its interference with
344 murine TH signaling takes place either downstream of TR-mediated gene transcription or by
345 non-genomic TH signaling pathways (reviewed by Horn and Heuer 2010).
346 BDE-99 inhibited human oligodendrocyte formation independently of TH disruption (Fig. 2B,C)
347 and did not interfere with TH-dependent h*MBP* (Fig. 3B) or *hairless* (Fig. S1E) expression.
348 Thus, it does not disrupt TH-dependent oligodendrogenesis. As another possible MOA we
349 postulated that the formation of reactive oxygen species (ROS) might be involved in BDE-99
350 toxicity on oligodendrogenesis. ROS formation by PBDEs is discussed controversially in
351 literature depending on the congener, cell type and method used (reviewed by Costa et al. 2014).

352 BDE-99 did not up-regulate ROS-related genes in hNPCs and mNPCs (Fig. 5A,D) and the
353 antioxidant Trolox did not antagonize the BDE-99 effect on oligodendrocyte formation (Fig.
354 5C,F). Interestingly, vitamin C (50-100 µM) rescued BDE-99-reduced oligodendrocyte
355 formation in human, but not in mouse NPCs. A high vitamin C concentration (500µM) strongly
356 induces human, but not murine, basal oligodendrocyte formation and maturation (Fig. S2).
357 Vitamin C enables hydroxylation of proline to hydroxyproline (Pinnell 1985), a major
358 component of the extracellular matrix protein collagen promoting rodent myelination of dorsal
359 root ganglion neurons by Schwann cells in the peripheral nervous system (Carey and Todd 1987;
360 Eldridge et al. 1987; Podratz et al. 2004). The effects of vitamin C on central nervous system
361 oligodendrogenesis are not known. Thus, the mechanism by which vitamin C antagonizes BDE-
362 99-dependent reduction in human oligodendrocyte formation remains enigmatic but might
363 involve collagen production.

364 **Conclusions**

365 We developed a test system to identify human- and rodent-specific TH disruptors of
366 oligodendrocyte maturation by assessing the Q_M. This Q_M showed that BDE-99 inhibits murine
367 oligodendrocyte maturation by TH disruption, but does not affect human oligodendrocyte
368 maturation. The BDE-99 effect on murine, but not human oligodendrocyte formation is TH
369 dependent. On the contrary, vitamin C rescues BDE-99 effects on human, but not mouse
370 oligodendrocyte formation. This study shows the need to consider species differences in
371 susceptibility and MOA analyses of chemicals for human hazard characterization.

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506

507 **FIGURE LEGENDS**

508

509 **Fig. 1: BDE-99 effects on human and murine oligodendrocyte formation.** Human (A,B) and
510 murine (C,D) NPCs were differentiated with increasing concentrations of BDE-99 for 5 days and
511 an Alamar-Blue assay was performed two hours prior fixation. Oligodendrocytes were
512 immunocytochemically stained with O4 antibody and nuclei were counterstained with Hoechst
513 33258. A,C) Data for viability and the percentage of oligodendrocytes/nuclei in the migration
514 area were normalized to the solvent control (mean ± SEM, min n=3). A sigmoidal curve fit was
515 applied and for oligodendrogenesis IC_{20} and IC_{50} values were calculated. *, $p<0.05$ compared to
516 solvent treatment. B,D) Illustration of human and murine oligodendrocytes visualized by O4
517 immunofluorescence staining after treatment with solvent or the respective IC_{50} concentration of
518 BDE-99 for five days (scale bar: 50 µm).

519

520 **Fig. 2: TH effects on basal and BDE-99-reduced mouse and human oligodendrocyte**
521 **formation.** Human (A-C) and murine (D-F) NPCs were differentiated for 5 days (A,B,D,E) or 1-
522 5 days (C,F) under exposure to BDE-99 and/or TH (T3 or T4). An immunocytochemical staining
523 for oligodendrocytes ($O4^{+}$) and a counterstaining with Hoechst 33258 for nuclei were performed.
524 The percentage of $O4^{+}$-cells/nuclei is shown (in C and F) while in A,B,D and E values were
525 normalized to the respective solvent control values. A,D) Dose response curves for increasing
526 TH (T3 or T4) concentrations. Data are shown as mean ± SEM (n=3 (mouse), n=4 (human)).
527 B,E). Results of the co-exposure experiments to BDE-99 and T3 or T4 presented as mean ± SEM
528 (n=3 (human), n=4 (mouse)). C,F) Time-course results for BDE-99, T3 and co-exposure (mean ±
529 SEM (n=3 (human), n=4 (mouse)). Significant difference ($p<0.05$) from the solvent control (of
530 the respective day in C and F) is indicated as * and from BDE-99 treatment as #.
531

Fig. 3: TH effects on basal and BDE-99-reduced mouse and human oligodendrocyte maturation. Human (A-C) and murine (C-F) NPCs were differentiated with BDE-99 and/or T3 for 1,3 or 5 days (A,D) or 5 days (B,C,E,F). *MBP* (hNPCs) or *Mog* (mNPC) expression was determined by real-time RT-PCR. Copy numbers of the genes were normalized to the expression of *β-actin*. A,D) Show mean ± SEM of *MBP* or *Mog* expression for 1,3 and 5 days differentiated NPCs (n=3). $p<0.1$ was considered as significant. * indicates significance differences ($p<0.1$) from the respective solvent control. # indicates significance differences ($p<0.1$) from the same treatment at day 1. B,E,F) *MBP* or *Mog* expression at day 5 was normalized to the percentage of oligodendrocytes expressing the gene. Data are shown as mean ± SEM (n=3-4) (significant vs. * control or # T3 treatment, $p<0.1$). C) Representative fluorescence microscope pictures of 5 days differentiated human and murine oligodendrocytes in absence or presence of 3 nM T3.

544 **Fig. 4: Role of TRs in T3-induced and BDE-99-reduced murine oligodendrocyte**
545 **formation/maturation.** Neurospheres prepared from wildtype, TRα-/- and TRβ-/- mouse brains
546 were differentiated for 5 days in the presence of 10 µM BDE-99 and/or T3. A)
547 Immunocytochemical staining of oligodendrocytes with O4 antibody was performed and nuclei
548 were counterstained with Hoechst 33258. The percentage of $O4^{+}$-cells/nuclei is shown
549 normalized to the respective genotype solvent control (absolute percentage of oligodendrocytes
550 does not differ between non-treated wildtype and TR knockout neurospheres) (mean ± SEM,
551 n=4). B) *Mog* copy numbers determined by real time RT-PCR were first normalized to the *β-*
552 *actin* expression, then divided by the percentage of oligodendrocytes and finally normalized to
553 the *Mog* expression/%oligodendrocytes of the respective solvent control (maturation in solvent
554 treated samples of wildtype and knockout mice did not differ.) Data are shown as mean ± SEM,
555 n=3 (wildtype), n=4 (knockout). $p<0.05$ (A) or $p<0.1$ (B) was considered as significantly
556 different from the respective solvent control (*), BDE-99 treatment (#) or the respective wildtype
557 treatment (').
558

559 **Fig. 5: Involvement of ROS in BDE-99-dependent reduced human oligodendrocyte**
560 **formation.** Human (A-C) and murine (D-F) NPCs were differentiated for five days in presence
561 BDE-99 and/or antioxidant (vitamin C or Trolox). Immunocytochemical oligodendrocyte
562 stainings (B,C,E,F) or gene expression studies by real-time RT-PCR (A,D) were performed.
563 A,D) Expression of the ROS related genes *heme oxygenase-1* (*HMOX-1/Hmox-1*), *catalase*
564 (*CAT/Cat*), *superoxide dismutase* (*SOD/Sod*) and *glutathione peroxidase* (*GPX/Gpx*) was
565 evaluated with the ΔΔCT method. Results are shown as $2^{-\Delta\Delta CT}$ (mean ± SEM, n=3). B,C,E,F)
566 Percentage of $O4^{+}$ cells/nuclei normalized to the solvent control (mean ± SEM, n=3-4). $p<0.05$
567 (B,C,E,F) or $p<0.1$ (A,D) was considered significantly different from solvent control (*) or from
568 BDE-99 treatment (#).

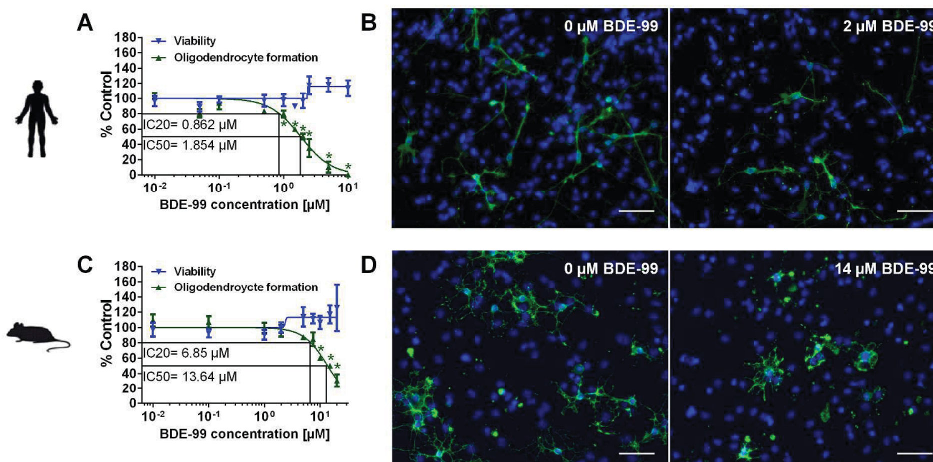


Fig. 1: BDE-99 effects on human and murine oligodendrocyte formation. Human (A,B) and murine (C,D) NPCs were differentiated with increasing concentrations of BDE-99 for 5 days and an Alamar-Blue assay was performed two hours prior fixation. Oligodendrocytes were immunocytochemically stained with O4 antibody and nuclei were counterstained with Hoechst 33258. A,C) Data for viability and the percentage of oligodendrocytes/nuclei in the migration area were normalized to the solvent control (mean ± SEM, min n=3). A sigmoidal curve fit was applied and for oligodendrogenesis IC20 and IC50 values were calculated. *, $p<0.05$ compared to solvent treatment. B,D) Illustration of human and murine oligodendrocytes visualized by O4 immunofluorescence staining after treatment with solvent or the respective IC50 concentration of BDE-99 for five days (scale bar: 50 µm).
265x133mm (300 x 300 DPI)

Fig. 2: TH effects on basal and BDE-99-reduced mouse and human oligodendrocyte formation. Human (A-C) and murine (D-F) NPCs were differentiated for 5 days (A,B,D,E) or 1-5 days (C,F) under exposure to BDE-99 and/or TH (T3 or T4). An immunocytochemical staining for oligodendrocytes (O4+) and a counterstaining with Hoechst 33258 for nuclei were performed. The percentage of O4+-cells/nuclei is shown (in C and F) while in A,B,D and E values were normalized to the respective solvent control values. A,D) Dose response curves for increasing TH (T3 or T4) concentrations. Data are shown as mean ± SEM (n=3 (mouse), n=4 (human)). B,E). Results of the co-exposure experiments to BDE-99 and T3 or T4 presented as mean ± SEM (n=3 (human), n=4 (mouse)). C,F) Time-course results for BDE-99, T3 and co-exposure (mean ± SEM (n=3 (human), n=4 (mouse)). Significant difference ($p<0.05$) from the solvent control (of the respective day in C and F) is indicated as * and from BDE-99 treatment as #.

289x147mm (300 x 300 DPI)

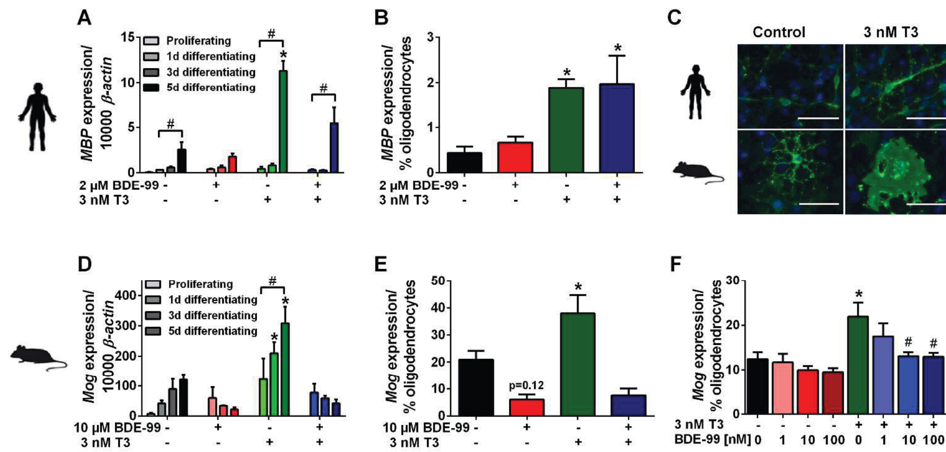


Fig. 3: TH effects on basal and BDE-99-reduced mouse and human oligodendrocyte maturation. Human (A-C) and murine (C-F) NPCs were differentiated with BDE-99 and/or T3 for 1,3 or 5 days (A,D) or 5 days (B,C,E,F). MBP (hNPCs) or Mog (mNPC) expression was determined by real-time RT-PCR. Copy numbers of the genes were normalized to the expression of β-actin. A,D) Show mean ± SEM of MBP or Mog expression for 1,3 and 5 days differentiated NPCs (n=3). $p<0.1$ was considered as significant. * indicates significance differences ($p<0.1$) from the respective solvent control. # indicates significance differences ($p<0.1$) from the same treatment at day 1. B,E,F) MBP or Mog expression at day 5 was normalized to the percentage of oligodendrocytes expressing the gene. Data are shown as mean ± SEM (n=3-4) (significant vs. * control or # T3 treatment, $p<0.1$). C) Representative fluorescence microscope pictures of 5 days differentiated human and murine oligodendrocytes in absence or presence of 3 nM T3.
298x146mm (300 x 300 DPI)

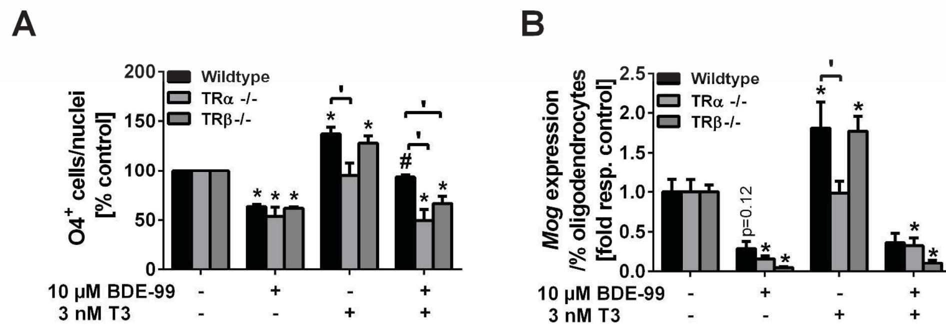


Fig. 4: Role of TRs in T3-induced and BDE-99-reduced murine oligodendrocyte formation/maturation. Neurospheres prepared from wildtype, TRα-/- and TRβ-/- mouse brains were differentiated for 5 days in the presence of 10 µM BDE-99 and/or T3. A) Immunocytochemical staining of oligodendrocytes with O4 antibody was performed and nuclei were counterstained with Hoechst 33258. The percentage of O4+-cells/nuclei is shown normalized to the respective genotype solvent control (absolute percentage of oligodendrocytes does not differ between non-treated wildtype and TR knockout neurospheres) (mean ± SEM, n=4). B) Mog copy numbers determined by real time RT-PCR were first normalized to the β-actin expression, then divided by the percentage of oligodendrocytes and finally normalized to the Mog expression/%oligodendrocytes of the respective solvent control (maturation in solvent treated samples of wildtype and knockout mice did not differ.) Data are shown as mean ± SEM, n=3 (wildtype), n=4 (knockout). $p<0.05$ (A) or $p<0.1$ (B) was considered as significantly different from the respective solvent control (*), BDE-99 treatment (#) or the respective wildtype treatment (').
293x108mm (300 x 300 DPI)

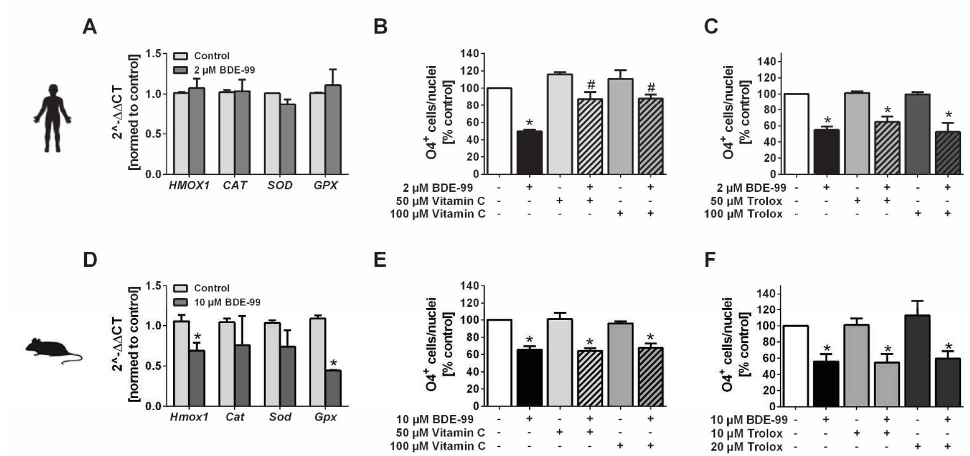


Fig. 5: Involvement of ROS in BDE-99-dependent reduced human oligodendrocyte formation. Human (A-C) and murine (D-F) NPCs were differentiated for five days in presence BDE-99 and/or antioxidant (vitamin C or Trolox). Immunocytochemical oligodendrocyte stainings (B,C,E,F) or gene expression studies by real-time RT-PCR (A,D) were performed. A,D) Expression of the ROS related genes heme oxygenase-1 (HMOX-1/Hmox-1), catalase (CAT/Cat), superoxide dismutase (SOD/Sod) and glutathione peroxidase (GPX/Gpx) was evaluated with the ΔΔCT method. Results are shown as 2-ΔΔCT (mean ± SEM, n=3). B,C,E,F) Percentage of O4+ cells/nuclei normalized to the solvent control (mean ± SEM, n=3-4). $p<0.05$ (B,C,E,F) or $p<0.1$ (A,D) was considered significantly different from solvent control (*) or from BDE-99 treatment (#).
297x156mm (300 x 300 DPI)

SUPPLEMENTAL MATERIAL

BDE-99 impairs human and mouse oligodendrogenesis by species-specific modes of action

Katharina Dach, Farina Bendt, Ulrike Huebenthal, Susanne Giersiefer, Heike Heuer and Ellen Fritsche

Fig. S1: Expression of TH signaling components. RT-PCR was performed for non-exposed proliferating or BDE-99, T3 or co-exposed differentiating human (A,C,E,G) and murine (B,D,F,H) NPCs. Gene expression of thyroid hormone receptors: human A) THRA1 and C) THRB1 (n=5) and murine B) Thra1 and D) Thrb1 (n=4), (E+F) *hairless* (*HR/Hr*; n=4) and G+H) *deiodinase 3* (*DIO3/Dio3;* n=3 (human), n=4 (mouse)) is shown as mean ± SEM. Gene copy numbers are shown normalized to *β-actin*. Significantly different from * the solvent control of the respective differentiation day or # between the same treatments at different differentiation days ($p<0.1$).

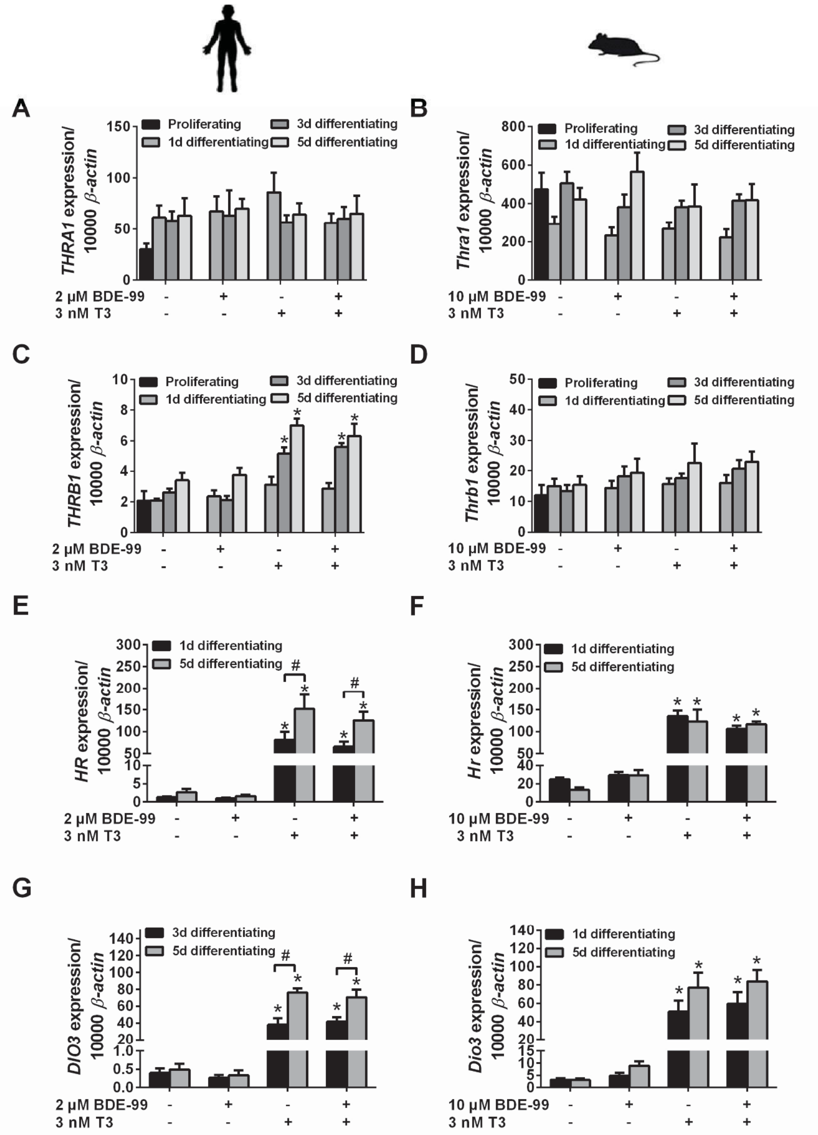
Fig. S2: Vitamin C effects on human and murine oligodendrocyte formation and maturation. Human (A) and murine (B) NPCs were differentiated with solvent or 500 µM vitamin C for 5 days and cells were fixed. Oligodendrocytes were immunocytochemically stained with O4 antibody and nuclei were counterstained with Hoechst 33258. Representative fluorescence microscope pictures of human (A) and murine (B) oligodendrocytes differentiated with solvent or 500 µM vitamin C (scale bar: 50 µm).

PCR procedure

The PCR mix consisted of 7.5 µl of PCR Master Mix SybrFAST (Qiagen, Hilden, Germany), 2.5 µl solutions of each primer (stock concentration 4 µM) and 2.5 µl of cDNA (1:2.5 diluted). The application started with an initial incubation step of 7 min at 95°C to activate the DNA polymerase. The conditions for PCR amplifications were 47 cycles of 10 sec at 95°C for denaturation, 35 sec at 60 °C for primer annealing, elongation and fluorescence detection. We evaluated gene expression using the cycle threshold (Ct) value from each sample. For determination of absolute copy numbers, we used product-specific standards amplified from cDNA to generate standard curves.

Table 1: Primer sequences.

	Species	**Forward primer**	**Reverse primer**
β-actin	human	CAGGAAGTCCCTTGCCATCC	ACCAAAAGCCTTCATACATCTCA
	mouse	CACCCGCCACCAGTTCGCCA	AGCACAGGGTGCTCCTCAGGG
catalase	Human (*CAT*)	CGTGCTGAATGAGGAACAGA	AGTCAGGGTGGACCTCAGTG
	Mouse (*Cat*)	ATGACAACCAGGGTGGTGCCC	GCGGGCCCCATAGTCAGGGT
deiodinase	Human (*DIO3*)	AAGTGCCCAGACCTTCCAAA	AAAGAAACCCTTGCAGCCTTC
	Mouse (*Dio3*)	ATCCGTGTTTTCCCTCTCGTC	GCTGGAAGGCCATTTTCTGTC
glutathione peroxidase	Human (*GPX*)	TCTGTTGCTCGTAGCTGCTGC	GGGGTCAAGAGGAGGAGAGA
	Mouse (*Gpx*)	TCGGACACCAGGAGAATGGCAA	GCGGCACACCGGAGACCAAA
hairless	Human (*HR*)	CCGCTTTCTCCAGATGGTGTG	AGAGGAAGTGCTGAGTGACG
	Mouse (*Hr*)	AATACTGTGCCCACCAAGGG	TGAGTTCACACCATAGGCCG
heme oxygenase-1	Human (*HMOX1*)	GCCATGAACTTTGTCCGGTG	GGATGTGCTTTTCGTTGGGG
	Mouse (*Hmox1*)	CTCTGTCCAATGTGGCCTTCT	CACTGGCTGGATGTGCTTTTG
myelin basic protein	Human (*MBP*)	CAGAGCGTCCGACTATAAATCG	GGTGGGTTTTCAGCGTCTA
myelin oligodendrocyte glycoprotein	Mouse (*Mog*)	TCCATCGGACTTTTGATCCTCA	CGCTCCAGGAAGACACAACC
superoxide dismutase	Human (*SOD*)	GGCCGATGTGTCTATTGAAGA	GGGCCTCAGACTACATCCAA
	Mouse (*Sod*)	TCGAGCAGAAGGCAAGCGGTG	TCTCTTCATCCGCCGGGCCA
thyroid hormone receptor α1	Human (*THRA1*)	GCTGCTAATGTCAACAGA	CCCCGATCATGCGGAGGTCA
	Mouse (*Thra1*)	GCGAAAATTCCTGCCGGATG	GATCTGGTCTTCGCAAGGCA
thyroid hormone receptor β1	Human (*THRB1*)	AAGTGCCCAGACCTTCCAAA	AAAGAAACCCTTGCAGCCTTC
	Mouse (*Thrb1*)	ATCCGTGTTTTCCCTCTCGTC	GCTGGAAGGCCATTTTCTGTC



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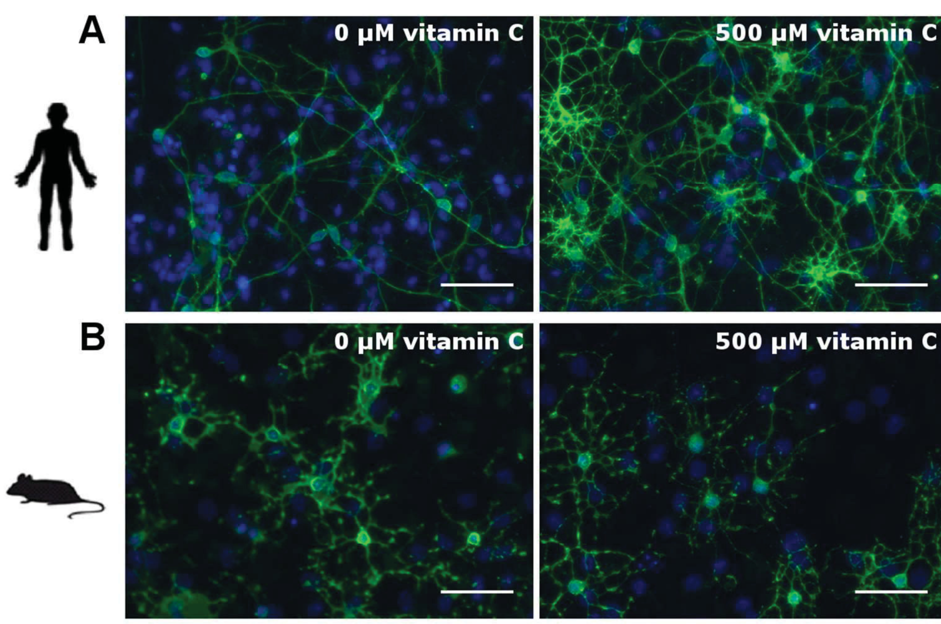

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BDE-99 impairs human and mouse oligodendrogenesis by species-specific modes of action

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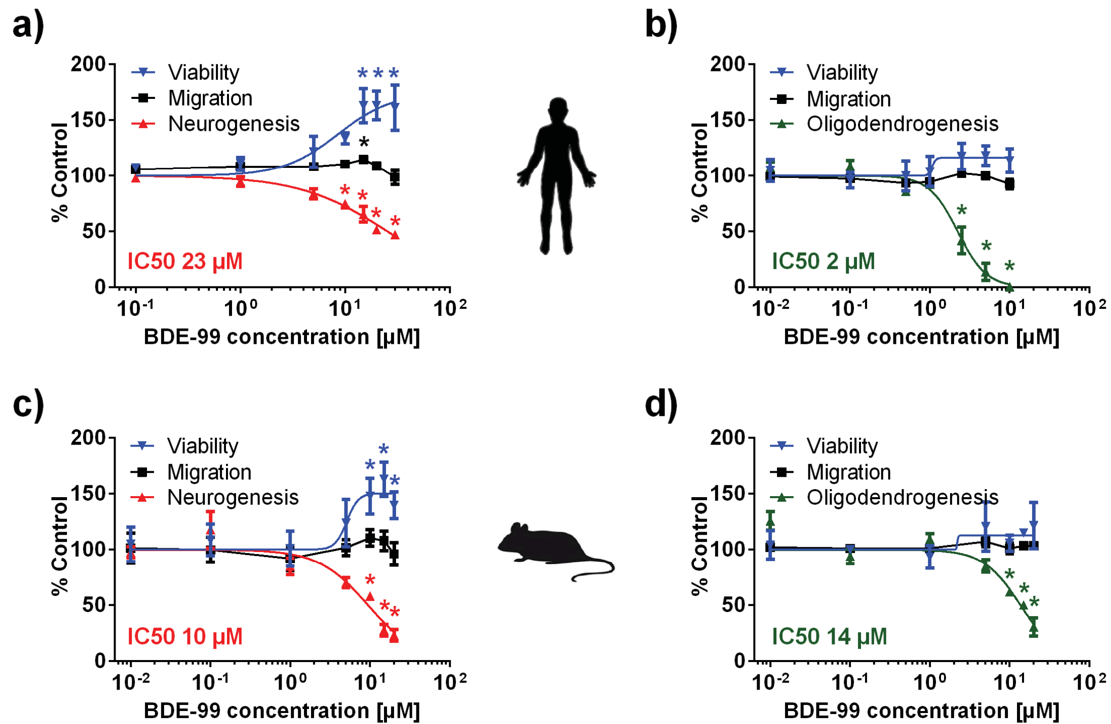

Figure A: BDE-99 dose-response curves for human and murine neurogenesis and oligodendrogenesis. Human (a+b) and murine (c+d) NPCs were differentiated with increasing concentrations of BDE-99 for 3 days (neurogenesis) or 5 days (oligodendrogenesis). In neurogenesis experiments neurospheres were pre-treated with BDE-99 under proliferative conditions for 2 days before plating the differentiation experiment. Migration distance was measured in oligodendrogenesis experiments after 2 days of differentiation and in neurogenesis experiments at day 3 of differentiation. Prior to fixation an Alamar-Blue Assay was performed. Cells were immunocytochemically stained with βIII-tubulin (neurons) or O4 (oligodendrocytes) antibody and nuclei were counterstained with Hoechst 33258. Data for viability, migration and the percentage of neurons per nuclei (a+c) or the percentage of oligodendrocytes/nuclei (b+d) in the migration area are shown normalized to the solvent control (mean ± SEM, n=3). A sigmoidal curve fit was performed and IC_{50} values were calculated for neurogenesis and oligodendrogenesis. * denotes significance versus the solvent control ($p<0.05$, One Way Anova with Bonferroni's multiple comparison test).

Effects of T3 and T4 (0.003-30 nM) on NPC viability, migration and differentiation

Human and murine NPCs were differentiated for 5 days in oligodendrogenesis experiments in absence or presence of TH (T3 or T4). Migration was measured at day 2 of differentiation and viability was assessed by a Cell-Titer Blue Assay measuring mitochondrial activity prior to fixation. Oligodendrocytes were immunocytochemically stained with O4 antibody and nuclei were counterstained with Hoechst. The percentage of oligodendrocytes/nuclei was evaluated.

In neurogenesis experiments proliferative NPCs were pre-treated with solvent or TH (T3 or T4) for 2 days and were then differentiated for 3 days with the same treatment. Migration was measured and viability was assessed by a Cell-Titer Blue Assay prior to fixation. Neurons were immunocytochemically stained with βIII-tubulin antibody and nuclei were counterstained with Hoechst. The percentage of neurons/nuclei was evaluated.

Significant ($p<0.05$; OneWay Anova with Bonferroni's multiple comparison test) TH effects in respect to the control are shown in Table 1 (for T3) and Table 2 (for T4).

Table 1: Overview of T3 effects on relevant endpoints in the 'Neurosphere Assay' (in % solvent control).

	differentiation duration	hNPC	mNPC
Viability	**3 days (N)**	3 nM 80% * 30 nM 70% *	not performed
	5 days (O)	no effect	no effect
Migration	**2 days (O)**	no effect	no effect
	3 days (N)	3 nM 80% * 30 nM 80% *	not performed
Oligodendrogenesis	**5 days**	no effect	**max induction for 3 nM 140%**
Neurogenesis	**3 days**	no effect	not performed

* T3 reduces the proliferation of NPCs. Therefore, the T3 treated neurospheres plated after 2 days of pretreatment under proliferative conditions (neurogenesis conditions) were smaller and contained less NPCs than the solvent treated ones resulting in reduced viability and migration.

Table 2: Overview of T4 effects on relevant endpoints in the 'Neurosphere Assay' (in % solvent control).

	differentiation duration	hNPC	mNPC
Viability	**3 days (N)**	no effect	no effect
	5 days (O)	no effect	no effect
Migration	**2 days (O)**	no effect	no effect
	3 days (N)	no effect	no effect
Oligodendrogenesis	**5 days**	no effect	**0.3 – 30 nM 130-170%**
Neurogenesis	**3 days**	no effect	3 nM 60% (n=2) 30 nM 40% (n=2)

(N) : endpoint assessed within the neurogenesis experiment;
(O): endpoint assessed within the oligodendrogenesis experiment

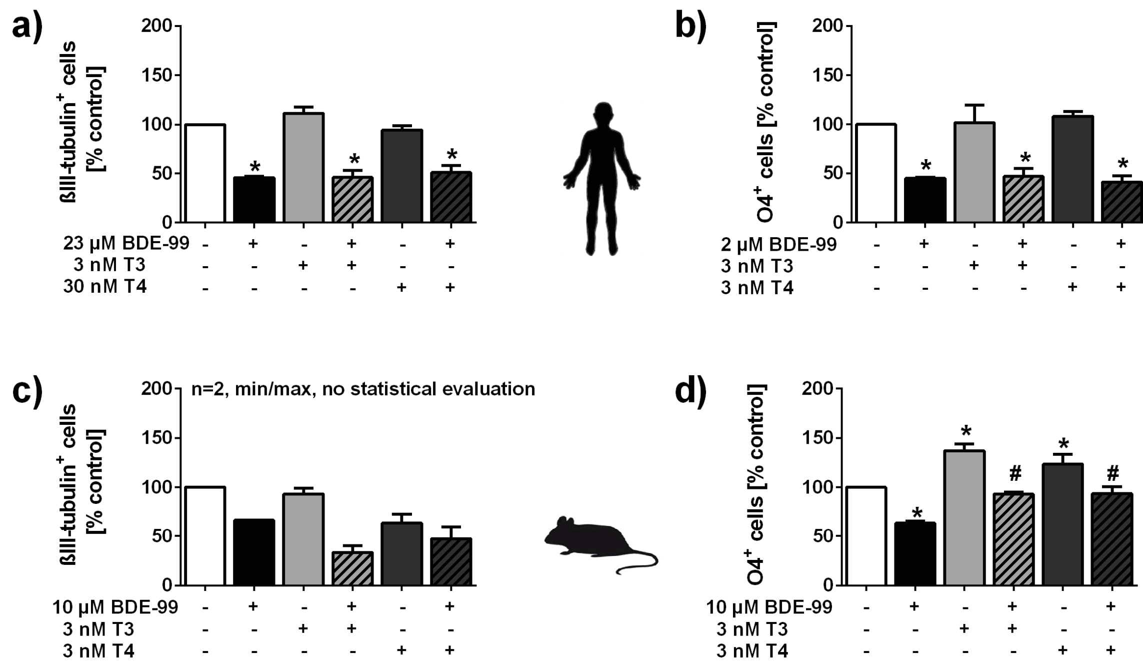

Figure B: Co-treatment of BDE-99 effects with T3 or and T4. Human (a+b) and murine (c+d) NPCs were differentiated with BDE-99, THs or the respective co-treatment for 3 days (neurogenesis) or 5 days (oligodendrogenesis). In neurogenesis experiments neurospheres were pre-treated with the respective treatment under proliferative conditions for 2 days before plating the differentiation experiment. Cells were immunocytochemically stained with βIII-tubulin (neurons) or O4 (oligodendrocytes) antibody and nuclei were counterstained with Hoechst 33258. Data for the percentage of neurons per nuclei (a+c) or the percentage of oligodendrocytes/nuclei (b+d) in the migration area are shown normalized to the solvent control (mean ± SEM, n=3 to 4). Significance is denoted by * versus the solvent control and by # versus BDE-99 treatment ($p<0.05$; TwoWay Anova with Tukey's multiple comparison test).

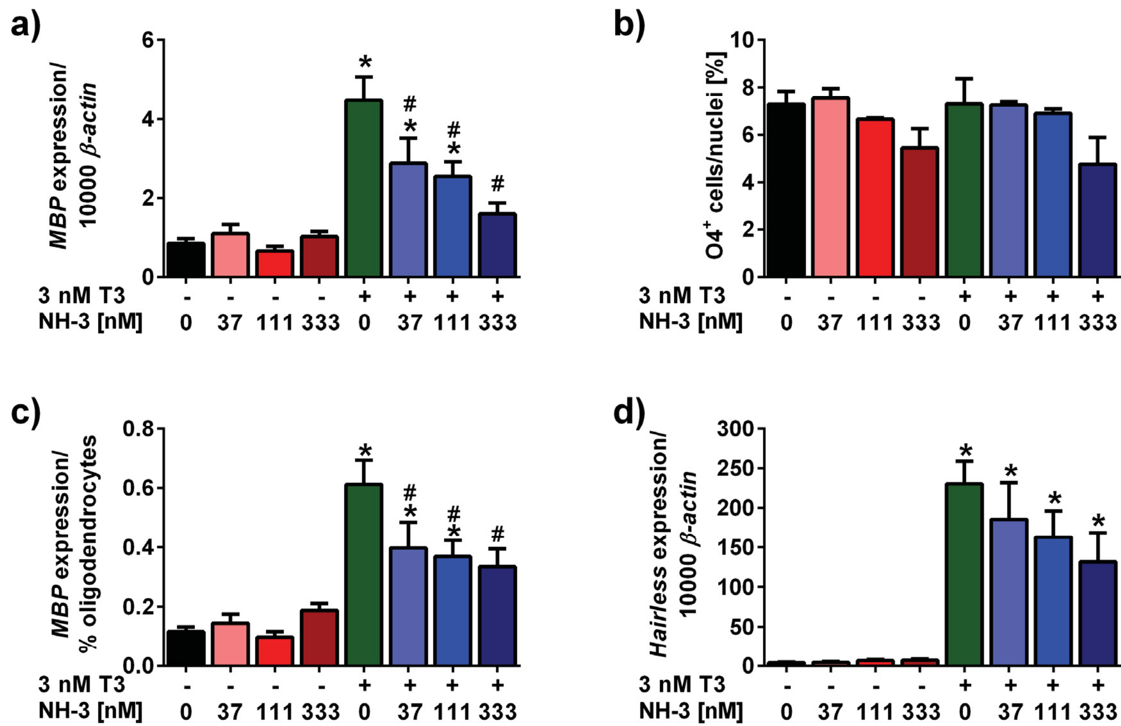

Figure C: Effects of TR antagonist NH-3 on human oligodendrocyte formation and maturation as well as TR-mediated transcription of *hairless*. Human NPCs were differentiated with respective treatments (NH-3, T3 or co-treatment) for 5 days for RT-PCR (a,d) or oligodendrocyte staining (b). a) *MBP* expression was determined by reverse-transcribed RT-PCR. *MBP* copy numbers were normalized to the expression of *β-actin*. Data are shown as mean ± SEM (n=5). $p<0.1$ was considered as significant. b) Cells were immunocytochemically stained with oligodendrocyte marker O4 and counterstained with Hoechst 33258. The percentage of oligodendrocytes/nuclei in the migration area is shown as mean ± SEM (n=5). $p<0.05$ was considered as significant. c) *MBP* expression was normalized to the percentage of oligodendrocytes expressing the gene. Data are shown as mean ± SEM (n=5). $p<0.1$ was considered as significant. d) *hairless* expression is shown normalized to *β-actin* expression as mean ± SEM (n=5). $p<0.1$ was considered as significant. * denotes significance versus the respective solvent control and # versus T3 treatment. For statistical evaluations TwoWay Anova with Tukey's multiple comparison test was performed.

3. Discussion

3.1 The ‘Neurosphere Assay’ as an *in vitro* model for studying species-specific DNT mechanisms

Developmentally neurotoxic compounds cause neurological deficits like lower IQs, learning disability and mental retardation in children resulting in high costs for society (Bellanger et al. 2013; Landrigan et al. 2002). Current DNT testing is performed in animal studies, which are on the one hand extremely time- and cost-intensive (Crofton et al. 2012) and on the other hand are not necessarily predictive for human risk assessment (Leist and Hartung 2013). Therefore, stakeholders agreed on the demand to integrate *in vitro* models into DNT testing strategies, which consider not only species differences but also allow to study critical toxicity pathways (Bal-Price et al. 2015a; Coecke et al. 2007; Crofton et al. 2012). A promising *in vitro* model in this regard is the ‘Neurosphere Assay’ described in paragraph 1.2.3.

Over the last decade human NPCs were intensively characterized and interference of exogenous noxae with relevant endpoints was described by our group (Fritsche et al. 2011; Gassmann et al. 2010, 2014; Moors et al. 2007, 2009; Schreiber et al. 2010). To enable a species comparison within the ‘Neurosphere Assay’ a rat neurosphere culture was established. The rat is the most commonly used animal in animal models and this approach allows comparing generated rat *in vitro* data with existing *in vivo* data from literature. The book chapter Baumann et al. (2014) describes in detail how the ‘Neurosphere Assay’ for human and rat NPCs is performed. The relevance of studying species differences was demonstrated in Baumann et al. (2015) for the first time comparing substance effects in rat and human NPCs: The effects of six positive and three negative DNT compounds on viability, migration and neuronal differentiation were tested in human and rat NPCs and the ‘Neurosphere Assay’ grouped most of the chemicals correctly. Within this study species differences in susceptibility and within the most sensitive endpoint towards a certain chemical were observed between human and rat demonstrating the need of species comparisons.

The involvement of signaling pathways *in vivo* is currently studied in knockout mice. This is attributed to the fact that a higher number of knockout mouse models exists in comparison to rat models, that knockout mice are easier to generate and that the mouse models are far better understood (Jacob et al. 2010; Peters et al. 2007; Nobel Prize 2007 for the generation

of knockout mice). Therefore, in order to investigate signaling pathways, also in regard to AOP development, one goal of this thesis was to establish a murine neurosphere culture and to implement the use of neurospheres prepared from knockout mice. TR knockout murine neurospheres were used in mechanistic studies included in manuscript 2.2 to investigate whether TRs are involved in BDE-99 or T3 effects on oligodendrogenesis.
In general, primary murine cultures need more complex culture conditions (more medium supplements and fetal calf serum for differentiation) compared to primary human or rat cultures (Rogers 2003). Using human/rat culture conditions for the murine culture led to a high sphere-to-sphere variability in the percentage of neurons under control conditions (0 - 30% neurons/nuclei). This made a statistical evaluation in neuron change after chemical treatment impossible. The rodent neurosphere preparation protocol routinely used for rat preparations had to be optimized for murine neurosphere preparation by only using forebrain and a shorter digestion time. Using only forebrain for the murine neurosphere preparation lowered the sphere-to sphere variability in neurons significantly compared to using whole brains for the preparations. There is evidence in the literature, that neural stem cells derived from different brain regions retain their regional character and that cells from different brain regions might inhibit each other in the ability to form neurons (Klein et al. 2005). We found no neurons in murine neurospheres prepared from only cerebellum under standard differentiation conditions, what might explain the high variability in murine whole brain neurosphere cultures. Furthermore, the FGF concentration in the proliferating murine culture was halved compared to the human culture, because murine neurospheres recovered better from passaging with the lower FGF concentration. During differentiation, 1% fetal calf serum (FCS) is now added to prevent high apoptosis rates and thereby prolong the possible differentiation duration. After establishment of the mouse neurosphere culture it was characterized (manuscript 2.1) and used within this project for investigation of TH signaling and PBDE effects.

The manuscript 2.1 “Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations” is the first publication of our group characterizing the endpoints of the ‘Neurosphere Assay’ for murine NPCs. The included murine data were generated within this PhD project. The manuscript describes how neurodevelopmental processes are analyzed within the ‘Neurosphere Assay’ for *in vitro* DNT testing. We compared proliferation, migration and differentiation into neurons, oligodendrocytes and astrocytes between all three species and observed that NPCs from the three species distinguish in their proliferation capacity, migration speed and the amount of formed neurons. Although all three species showed the same percentage of oligodendrocytes after 5 days of differentiation, murine oligodendrocytes formed much faster than human ones and were more mature. These *in*

vitro observations are in accordance with *in vivo* literature: the time span between pre-oligodendrocyte formation and emergence of the first mature oligodendrocytes is 11–12 weeks in humans but only 5 days in rodents (Barateiro and Fernandes 2014). We included endpoint specific positive controls because showing that substances modulating an endpoint *in vivo* also modulate it in the used *in vitro* system is crucial to guarantee that the *in vitro* system reflects the basic biology of the endpoint. As endpoint specific control for proliferation withdrawal of growth factors EGF and FGF is used. For migration src kinase inhibitor PP2 is used, which reduces migration to 60% (Moors et al. 2007). EGF treatment reduces differentiation into neurons (Ayuso-Sacido et al. 2010) and bone morphogenetic protein (BMP)-7, like other BMPs, induces astrocyte and inhibits oligodendrocyte differentiation (Gross et al. 1996). Furthermore, for each endpoint we compared substance effects on primary rodent cells with rodent *in vivo* literature and present the relevance of *in vitro* neural stem/progenitor cells for the *in vivo* situation.

As explained before, the neurosphere model mimics many relevant processes of early fetal brain processes and has clear advantages towards other *in vitro* models (human origin, culture as 3D aggregates, presence of the three main cell types of the brain and therefore cross-talk between different cell types, species-comparison within the same *in vitro* system), it enables to study toxicity pathways and contributes to the creation of adverse outcome pathways (AOPs).

But the ‘Neurosphere Assay’ as well as all other *in vitro* systems has its limitations. It lacks physiological cell and organ surrounding, which is necessary for pharmaco- and toxicokinetics. Therefore absorption/distribution/metabolism and excretion (ADME) cannot be studied *in vitro.* Furthermore, the neurosphere system lacks brain cell types like microglia and endothelial cells and there is evidence that the function of identical cell types varies between brain regions (Hewett 2009). Those region-specific differences can be studied with rodent stem/progenitor cells isolated from different brain regions but not with human whole brain homogenates.

Although the ‘Neurosphere Assay’ only covers early fetal brain development and has its limitations, it is an appropriate candidate to be included into DNT strategies consisting of several cell systems covering different stages of development (see Introduction Figure 9).

3.2 Species differences in susceptibility towards PBDEs

First, BDE-99 effects on viability, migration and differentiation into neurons and oligodendrocytes were studied performing concentration-response experiments with human and murine neural progenitor cells (additional data Figure A). The used BDE-99 concentrations did not inhibit human or murine NPCs' viability or migration. However, BDE-99 reduced human and murine neuro- and oligodendrogenesis concentration-dependently with human oligodendrogenesis as the most sensitive endpoint. Human oligodendrogenesis was 10x more sensitive than human neurogenesis as the least sensitive endpoint (IC_{50}: 2 µM and 23 µM). Sensitivity of murine neurogenesis and oligodendrogenesis was in the same range (IC_{50}: 10 µM and 14 µM). These data show that human NPCs are more sensitive towards BDE-99 exposure than the mouse for the endpoint oligodendrogenesis, while the susceptibility for neurogenesis is vice-versa.
Studies on PBDE effects in neural stem/progenitor cells are rare and focus on proteome analyses or gene expression studies (Du et al. 2015; Song et al. 2014) as well as Ca^{2+} signaling (Gassmann et al. 2014), but not on the investigation of individual processes of brain development. Another study found reduced viability and differentiation into neurons and astrocytes, but induced apoptosis in *in vitro* neurosphere cultures derived from embryos of *in vivo* BDE-209 treated rats (Chen et al. 2014). However, the rat neurospheres in this study were prepared from embryos at gestational day (GD) 14 and thereby do not resemble the developmental stage of NPCs used in our study. Furthermore, repeated application was performed each day over 14 days (GD1-GD14) resulting in an accumulation of BDE-209. Moreover, the applied concentrations, ranging from 10-50 mg/kg/day (10-50 µM per day), by far exceed the exposure of breast fed children (306 ng/kg/day) (Costa and Giordano 2007; Schecter et al. 2006). Additionally, in those studies different PBDE congeners were used and effects vary for different congeners (Hamers et al. 2006).
The used BDE-99 concentrations (additional data Figure A; human: up to 30 µM, mouse: up to 20 µM) did not reduce NPC viability. Schreiber et al. (2010) reported that up to 10 µM BDE-99 did not affect viability of hNPCs over 14 days. In opposite to Tagliaferri et al. (2010) who reported that 33 µM BDE-99 reduced viability to 50% of control after 24 h in human neuroblastoma cells, no effect on hNPC viability after 30 µM treatment for 3 days was observed (additional data Figure A). While Huang et al. (2010) found that 24 h treatment with 10 µM BDE-99 reduced viability to 50% of the control in mouse cerebellar granule neurons, up to 20 µM BDE did not reduce mNPC viability after 5 days of differentiation (additional data Figure A). These data suggest that BDE effects on viability depend on the applied cell system and the exposure duration. That neuroblastoma cells are more sensitive

towards apoptosis-inducing agents than primary hNPC was shown earlier (Moors et al. 2009).

PBDE effects on migration have not been studied *in vitro* so far and the 'Neurosphere Model' is unique in assessing this endpoint. While BDE-99 did not affect human or murine NPC migration (additional data Figure A), *in vivo* proteomic analysis suggest that BDE-99 exposure might cause disturbances in cell motility in mouse cerebral cortex (Alm et al. 2008). Although seen on the proteome level, no histopathological evaluation of PBDEs on migrational processes *in vivo* have been performed that could confirm the proteome data on the organ level.

BDE-99 reduced human and murine NPCs' differentiation into neurons and oligodendrocytes (additional data Figure A). Similar to PBDE effects on neural migration, effects of PBDE exposure on differentiation are rarely studied *in vitro* (Li et al. 2013; Schreiber et al. 2010). However, several studies *in vivo* suggest that developmental BDE-99 exposure might interfere with neuritogenesis/synaptogenesis in mice and rats by altering expression of proteins involved in those processes like GAP-43, stathmin and neuromodulin (Alm et al. 2006, 2010). Our calculated IC_{50} concentration for BDE-99 inhibiting human oligodendrocyte formation (2 µM) and the IC_{20} concentration for BDE-99 inhibiting murine oligodendrocyte formation (7 µM) are in accordance with the other two publications studying PBDE effects on oligodendrogenesis where an IC_{50} BDE-99 concentration of around 1 µM was found for human oligodendrocyte formation (Schreiber et al. 2010) and an IC_{25} BDE-47 concentration of 10 µM for inhibiting murine NSC differentiation into oligodendrocytes (Li et al. 2013).

Since human oligodendrogenesis is the most sensitive endpoint BDE-99, effects on oligodendrocyte formation and maturation in human and murine NPCs were studied in more detail (manuscript 2.2).

3.3 Characterization of the TH responses in the 'Neurosphere Assay' and establishment of the oligodendrocyte maturation assay for studying TH disruption

THs, T3 and T4, are known to play a crucial role in many processes of brain development like proliferation, migration, differentiation, myelination and synaptogenesis (reviewed in Anderson (2001); Bernal and Nunez (1995) and Bernal (2005)) and TH deficiency causes mental retardation (Boyages and Halpern 1993; Cao et al. 1994; DeLong et al. 1985). Because PBDE-treated animals show similar behavioral abnormalities (Costa and Giordano 2007) than animals, which suffer from hypothyroidism during brain development (Negishi et

al. 2005), endocrine disruption of TH signaling is discussed as a mechanism underlying PBDE-induced DNT (Costa et al. 2014; Sharlin 2015). However, species-differences remain an issue in toxicology (Hartung 2009; Leist and Hartung 2013), which makes the relevance of the classification of human endocrine disruptors in rodents questionable. Therefore, within this thesis, a test assay for the species-specific identification of TH disruptors for humans and mice based on NPCs was developed. By using this assay the question was addressed whether BDE-99 effects on oligodendrogenesis are mediated by interaction with TH signaling.

In a first step, the ‘Neurosphere Assay’ was characterized for its TH responses and thus challenged for its ability to study TH signaling and disruption. Therefore, the effects of the THs T3 and T4 (0.003-30 nM) on the endpoints studied within the ‘Neurosphere Assay’, migration and differentiation into neurons and oligodendrocytes, were evaluated. Furthermore, viability was measured (additional data Table 1 and Table 2). The expression and TH-dependent induction of important TH signaling components (TRs and metabolizing enzymes) was investigated and compared between species.
Viability and migration of human and murine NPCs were not affected by T3 or T4 treatment. TH, T3 or T4, treatment had no effect on human neuron or oligodendrocyte formation. In opposite, 3 nM T3 and all T4 concentrations induced murine oligodendrocyte formation (T3: 140% of control; T4: 130-170% of control). Two experiments indicate that T4 reduces murine neurogenesis (40-60% of control, n=2).
In summary, the only endpoint induced by THs was murine, but not human, oligodendrocyte formation. A TH concentration of 3 nM was chosen for further experiments since human total T3 and T4 levels in serum and cerebrospinal fluid are in the low nanomolar range (Anckarsäter et al. 2007; Hagen and Elliott 1973; Johansson et al. 2013; Malin et al. 1989; Nishikawa et al. 1981) and this concentration achieved the maximal induction of murine oligodendrocyte formation by T3.
Especially for oligodendrogenesis the crucial role of THs is well-known. THs are important for the maturation of rodent oligodendrocytes *in vitro* (Bhat et al. 1979; Shanker et al. 1985) and *in vivo:* hypothyroid animals express higher and hyperthyroid animals lower levels of oligodendrocyte maturation markers (Farsetti et al. 1991; Ibarrola and Rodríguez-Peña 1997; Marta et al. 1998; Rodriguez-Peña et al. 1993). Furthermore, THs determine the timing of oligodendrocyte formation and the amount of oligodendrocytes that is formed *in vitro* (Barres BA, Lazar MA 1994; Billon et al. 2001; Ibarrola et al. 1996; Koper et al. 1986). In humans a mutation in the X-linked TH transporter gene *MCT8* (Allan-Herndon-Dudley-syndrome, AHDS) leads to a hypothyroid situation in the brain. One pathological feature of

brains from children which this devastating disease is delayed myelination (Namba et al. 2008; Rodrigues et al. 2014; Tonduti et al. 2013).

Proliferating and differentiating human and murine NPCs express genes encoding the TH metabolizing enzyme deiodinase 3 (D3) and TRs, TRα1 and β1. (Fig. S1, manuscript 2.2). Expression levels of *thyroid hormone receptors* were higher in murine than in human NPCs. Human TRβ1 was the only receptor which expression was induced by T3 treatment. However, the low copy number change from 3 to 7 copies/10,000 *β-actin* makes a biological significance highly unlikely. The functionality of TRs was shown in both species since T3 induced the transcription of the gene *hairless* (Fig. S1, manuscript 2.2). *Hairless* is a T3-inducible in fetal brains *in vivo* (Potter et al. 2002; Thompson and Potter 2000) which T3 induction is TRα-mediated (Ramos and Weiss 2006). Furthermore, T3 probably induces its own metabolism as it strongly stimulated *deiodinase 3* expression in both species (Fig. S1, manuscript 2.2). TH transport into the cell must be present since THs triggered TR-dependent gene expression (Fig. S1, manuscript 2.2).

Next, the ability of BDE-99 to disturb NPC development by TH disruption was studied. Therefore, time-course experiments for oligodendrocyte formation, the most sensitive endpoint for BDE-99 treatment, were performed in presence of T3, BDE-99 or a combination of both (Fig. 2, manuscript 2). In addition, gene expression of oligodendrocyte maturation markers were studied over time (Fig. 3, manuscript 2.2). T3 accelerated human oligodendrocyte formation after 3 days of differentiation, but after 5 days of differentiation T3 treated NPCs had the same percentage of oligodendrocytes in the migration area than untreated NPCs. In contrast, T3 induced oligodendrocyte formation of mNPCs over the whole duration of 5 days. The observations in the mouse cultures are in accordance with previously published work that T3 is important for the timing of oligodendrocyte formation and the amount of formed oligodendrocytes *in vitro* (Barres BA, Lazar MA 1994; Billon et al. 2001; Ibarrola et al. 1996; Koper et al. 1986). To the best of my knowledge, oligodendrocyte formation has not been studied for humans so far.

In addition to oligodendrocyte formation, their maturation was studied by quantification of oligodendrocyte maturation markers via real time RT-PCR (Fig. 3, manuscript 2.2). This added a new endpoint to the 'Neurosphere Assay' (manuscript 2.2). Oligodendrocyte maturation was assessed by normalizing the gene expression (derived from real time RT-PCR experiments) of oligodendrocyte maturation markers human myelin basic protein (*MBP*) or murine myelin oligodendrocyte glycoprotein (*Mog*) to the percentage of oligodendrocytes in the migration area resulting in the maturation quotient Q_M (Q_M = *MBP* or *Mog* expression/ %oligodendrocytes). The use of the different markers is attributed to the different maturation stages of oligodendrocytes in the two species. Human and murine oligodendrocytes matured over a differentiation period of 5 days. Opposite to murine

oligodendroyctes, human oligodendrocytes did not mature between differentiation day 1 and 3, but between day 3 and 5. Furthermore, murine oligodendrocytes were more mature after 5 days of differentiation than human ones: they were already branched and displayed sheets, while human ones where still predominantly linear (Fig. 3, manuscript 2.2). Moreover, murine NPCs expressed *Mog*, a gene which is only expressed in myelinating oligodendrocytes (Baumann and Pham-Dinh 2001), which was not expressed by hNPCs. *Mbp*, an earlier marker, which is already expressed by non-myelinating oligodendrocytes (Baumann and Pham-Dinh 2001), was expressed by murine oligodendrocytes from the beginning and its expression did not increase over differentiation time. These observations are in accordance with *in vivo* literature that human oligodendrocytes mature slower than rodent ones (Barateiro and Fernandes 2014).
T3 accelerated human and murine oligodendrocyte maturation (Fig. 3, manuscript 2.2) what is in accordance with rodent *in vitro* (Bhat et al. 1979; Shanker et al. 1985) and *in vivo* literature (Farsetti et al. 1991; Ibarrola and Rodríguez-Peña 1997; Marta et al. 1998; Rodriguez-Peña et al. 1993). In humans the AHDS leading to a hypothyroid situation in the brain causes delayed myelination (Namba et al. 2008; Rodrigues et al. 2014; Tonduti et al. 2013) which can be due to less formed oligodendrocytes or their delayed maturation (López-Espíndola et al. 2014). This *in vivo* phenotype is reflected in the 'Neurosphere Assay' by accelerated oligodendrocyte formation and maturation with T3.
To shed light on the mechanism how T3 acts on murine oligodendrogenesis, NPCs generated from TRα and TRβ were included in the study. These cells revealed that the T3 induction of murine oligodendrocyte formation and maturation was mediated by TRα, but not TRβ (Fig. 4, manuscript 2.2). This TRα involvement in murine oligodendrocyte formation is in accordance with the literature showing that TRα mediates the effect on the timing of oligodendrocyte formation as newborn TRα, but not TRβ knockout mice display less oligodendrocytes at birth than wildtype animals (Billon et al. 2002). In further studies we plan to knockdown TR receptors in hNPCs to elucidate whether TRα is also mediating the T3 induced maturation in hNPCs.
These results show that neurospheres express important TH signaling components and that TH effects on endpoints of the 'Neurosphere Assay' are in accordance with the *in vitro* and *in vivo* literature, at least to the extent of information published. This makes the 'Neurosphere Assay' an appropriate tool for studying TH signaling and TH disruption.

BDE-99 and TH co-treatment experiments for human and murine neuron and oligodendrocyte formation revealed that TH only antagonizes the BDE-99-dependent decrease in murine oligodendrocyte formation. The reduction of mouse neuron as well as human neuron and oligodendrocyte formation by BDE-99 remained unaffected by THs

indicating that BDE-99 distrurbed these endpoints by mechanism(s) other than TH disruption (additional data Figure B).

Time course experiments over 5 days clarified that in both species BDE-99 treatment inhibited oligodendrocyte formation rather than inducing oligodendrocyte-specific cell death (Fig. 2, manuscript 2.2). The BDE-99 effect on murine oligodendrocyte formation was antagonized by T3 over the whole differentiation period. On the contrary, the BDE-99 effect on human oligodendrocyte formation was not antagonized by T3 at any time point, even not at those days when T3 accelerated this process.

After oligodendrocyte maturation was found to be the only endpoint, which was induced by T3 in both species, this endpoint was chosen to discriminate species-specific TH disruptors. BDE-99 inhibited oligodendrocyte maturation in murine NPCs by endocrine disruption since low concentrations not affecting maturation alone, concentration-dependently reduced oligodendrocyte maturation in combination with T3. In contrast, BDE-99 was not an endocrine disruptor of human oligodendrocyte maturation since it did not reduce human oligodendrocyte maturation, neither alone nor in combination with T3 (Fig. 3, manuscript 2.2).

For both species interference of BDE-99 with TH signaling was investigated. By using murine neurospheres prepared from TRα or TRβ knockout mice it was shown that neither of both receptors was involved in the BDE-99 effect on murine oligodendrocyte formation or maturation (Fig. 4, manuscript 2.2). Furthermore, BDE-99 did not change expression of genes encoding TRα1 or TRβ1 in human or murine differentiated neurospheres and did not interfere with T3-induced TRα-mediated transcription of *hairless* (Fig. S1, manuscript 2.2), a gene which is induced by TH in fetal brains (Potter et al. 2002; Thompson and Potter 2000) by a TRα-dependent mechanism (Ramos and Weiss 2006). In the literature contradictory results were found for BDE-99 interference with TR-mediated transcription: Ibhazehiebo et al. (2011) observed no suppression of TR-mediated transcription by BDE-99, but Blanco et al. (2011) reported that BDE-99 deregulated TH-mediated transcription of important genes for brain development as well as of TR isoforms in rat cerebellar granule neurons. In our experiments BDE-99 did not affect T3-induced expression of *deiodinase 3*, a thyroid hormone metabolizing enzyme. A study dealing with PBDE effects on brain cells reported that BDE-99 reduced deiodinase 2 activity in human glioma cells (Roberts et al. 2015), yet not on the transcriptional level.

Because BDE-99 effects on human oligodendrocyte formation were TH independent, involvement of reactive oxygen species (ROS) was investigated (Fig. 5, manuscript 2.2). In literature ROS formation by PBDEs is discussed controversially depending on the used congener, cell system and method (reviewed in Costa et al. 2014). BDE-99 did not up-regulate ROS related genes in human and murine NPCs and the antioxidant Trolox did not

antagonize the BDE-99 caused reduction of human oligodendrocyte formation. Interestingly, vitamin C (50 and 100 µM) rescued BDE-99-reduced oligodendrocyte formation in human, but not murine NPCs. A high vitamin C concentration (500 µM) strongly induced human, but not murine, oligodendrocyte formation and maturation (Fig. S2, manuscript 2.2). Vitamin C enables hydroxylation of proline to hydroxyproline (Pinnell 1985), a major component of the extracellular matrix protein collagen promoting rodent myelination of dorsal root ganglion neurons by Schwann cells in the peripheral nervous system (Carey and Todd 1987; Eldridge et al. 1987; Podratz et al. 2004). The effects of vitamin C on central nervous system oligodendrogenesis are not known. Therefore, the mechanism by which vitamin C rescues BDE-99-dependent reduction of oligodendrocyte formation in hNPCs remains enigmatic, but might involve collagen production.

In summary, BDE-99 inhibited mouse oligodendrocyte formation and maturation via TH disruption, yet independent of TRs. In contrast, BDE-99 effects on human oligodendrocyte formation were not TH dependent and BDE-99 did not inhibit human oligodendrocyte maturation. ROS formation is not involved in the BDE-99 effect on human oligodendrocyte formation either, but vitamin C is able to antagonize this BDE-99 effect – only in human, not in mouse NPCs. This study shows the need for taking species-specificities into consideration when studying toxicity pathways.

To test the functionality of the oligodendrocyte maturation assay also for human NPCs the TR antagonist NH-3 was used (Nguyen et al. 2002; additional data Figure C). As expected, NH-3 reduced T3-induced TRα-mediated transcription of *hairless* and inhibited T3-induced, but not basal human oligodendrocyte maturation, in a concentration-dependent manner. These data indicate that the oligodendrocyte maturation assay developed within this thesis can detect TH disruptors. For a proper assay validation, however, more potential endocrine disruptors like polychlorinated biphenyls (PCBs) (Fritsche et al. 2005; Zoeller 2000), OH-PCBs (Iwasaki et al. 2002), OH-PBDEs (Butt et al. 2011), bisphenol A (BPA) (Moriyama et al. 2002), tetrabromo- and tetrachloro BPA (TBBPA,TCBPA) (Butt et al. 2011; Kitamura et al. 2002), cocaine (Kovalevich et al. 2012) or perfluoroalkyl compounds as perfluorohexane sulfonates (PFHXS) (Vongphachan et al. 2011) need to be tested in this assay.

3.4 Development of AOPs for thyroid hormone disruption

The AOP concept integrates molecular mechanisms into risk assessment (Ankley et al. 2010). Thereby, an AOP describes the adverse outcome (AO) of toxicants on individuals or the population starting from a single molecular initiating event (MIE) by causally linking related key events (KEs) on different stages of biological organization (OECD 2013; section 1.2.2, Figure 6).

The aim of the implementation of AOPs into current testing strategies is to achieve a more predictive hazard characterization and better chemical classification. Within the AOP species-specific toxicity pathways are considered. Thus, this translational aspect from rodents to humans is supposed to contribute to reducing uncertainty in hazard assessment (Bal-Price et al. 2015a). For the description of full AOPs the MIE, the linked KEs and the AO need to be known. Each AOP is independent of the chemical, but starts with a single, defined MIE. Different AOPs can cross and overlap when they share one or more KE(s) or have the same AO leading to the formation of complex AOP networks. Thus, one single MIE can cause multiple AOs and the same AO can be caused by more than one MIE.

In case of DNT the AO in many cases is known, but there is especially a lack of knowledge on MIEs. Cellular KE for DNT include neural stem/progenitor cell proliferation, apoptosis, migration, differentiation into neurons and glia cells, neurite outgrowth, myelination and dendrite and axon formation (Bal-Price et al. 2015a). Those endpoints can be assessed with *in vitro* models like the 'Neurosphere Assay'.

Within this thesis an AOP scheme was developed how in general a TH disrupting chemical might lead to the adverse outcome of mental retardation and it was focused on studying the cellular KE oligodendrocyte formation and maturation in human and murine NPCs (Figure 11). As putative TH disruptors BDE-99 in both species (manuscript 2.2) as well as the TR antagonist NH-3 (Nguyen et al. 2002) in hNPCs (additional data Figure C) were tested.

MIEs for TH disruption can cover a brought spectrum due to the complexity of TH signaling (reviewed by Horn and Heuer 2010) and will lead to multiple AOPs for TH disruption. Putative molecular initiating events for the AO mental retardation are shown in the AOP scheme (Figure 11). Those are based on interference of compounds with classical TH signaling like decreased TH transport across the membranes, the inhibition of TH metabolism by deiodinases or the blockade of the TR binding site for THs by binding of the compound to TRs. In addition, MIEs might also happen on targets of non-classical TH signaling (section 1.1.2, Figure 4).

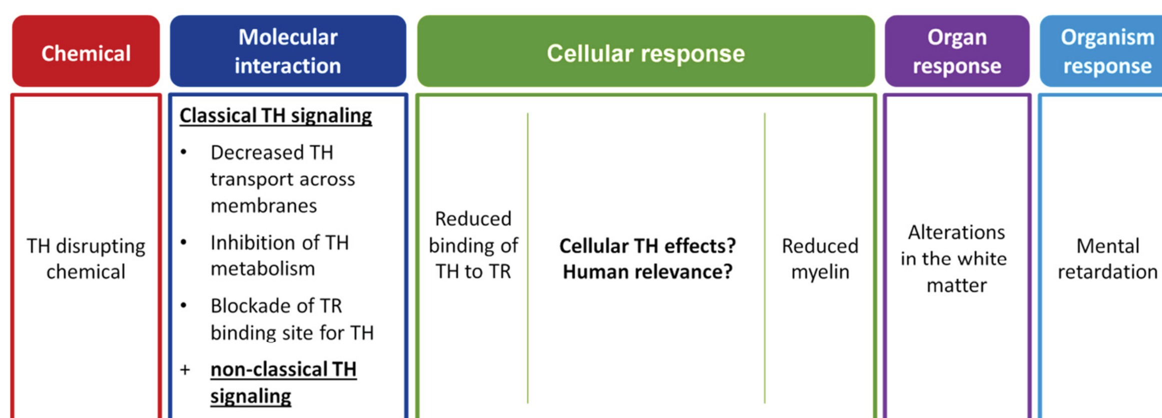

Figure 11: Putative AOP scheme how TH disrupting chemicals can lead to the adverse outcome of mental retardation. This scheme contains multiple AOPs since each MIE is at the beginning of an individual AOP. Molecular initiating events might be classical interactions with TH signaling like decreased TH transport into the cell, inhibition of TH metabolism or blockade of the TR binding site for TH. But MIEs might also be interferences of compounds with non-classical TH signaling. Cellular effects lead to reduced myelin causing alteration in the white matter which are correlated to the adverse outcome of mental retardation.

These events have in common that they cause a reduction of the KE ‘binding of THs to TR in the cell nucleus’ (=common KE; Bal-Price et al. 2015a). One outcome of reduced brain TH signaling during development is disturbance of the white matter (López-Espíndola et al. 2014; Namba et al. 2008; Rodrigues et al. 2014) representing a KE on the organ level. The data of this thesis bridges the gap between reduced cellular TH signaling and white matter disturbance as it identifies species-specific TH actions on oligodendrocyte formation and maturation. With regards to the AO, white matter alterations have been associated with psychiatric disorders like schizophrenia, chronic depression and posttraumatic stress as well as with neurodevelopmental cognitive and emotional disorders like autism and attention deficit hyperactivity disorder (reviewed by Fields 2008).

For murine NPCs the BDE-99 effect on oligodendrocyte formation was TH dependent and BDE-99 inhibited oligodendrocyte maturation via endocrine disruption. However, the molecular initiating event causing this interference with TH signaling was not identified so far. As discussed before, TH transport into the cell was not altered by BDE-99 since it did not interfere with cellular TH signaling. BDE-99 did not inhibit T3-induced *deiodinase 3* expression and did not interfere with T3-induced TRα-mediated transcription of the gene *hairless*. Therefore, BDE-99 might act on a pathway downstream of TR-mediated transcription or its effect might be mediated by interference with non-classical TH actions (reviewed by Horn and Heuer 2010).

Since BDE-99 was not found to be a TH disruptor in human NPCs, the TR antagonist NH-3 (Nguyen et al. 2002) was used to test the human oligodendrocyte maturation assay as an *in vitro* test for identifying human TH disruptors in developing brain cells (additional data Figure C). NH-3 concentration-dependently reduced T3-induced TRα-mediated *hairless* expression.

In addition, it disrupted T3-dependent oligodendrocyte maturation showing that the human oligodendrocyte maturation assay indeed detects disturbances of TH signaling.
The oligodendrocyte maturation assay was established to identify TH disruptors in both species. Testing of BDE-99 in these assays identified this flame retardant as a TH disruptor in mouse, but not human NPCs. These data clearly demonstrate the need to implement species-specific pathway analyses in AOP development and supports the previous notion that human cells should be used for hazard assessment of TH disruption in the developing brain.

3.5 Risk assessment of PBDEs

It was clearly shown that PBDEs are developmental neurotoxic for humans reducing the IQ and inducing hyperactivity (Chao et al. 2007; Eskenazi et al. 2013; Roze et al. 2009; Shy et al. 2011). In rodents they also exert DNT potential by inducing behavioral abnormalities, learning and memory deficits and hyperactivity (e.g. Branchi et al. 2002; Dufault et al. 2005; Gee et al. 2008; Kodavanti et al. 2010; Suvorov et al. 2009; Viberg et al. 2003, 2004). However, the mechanisms of PBDE-induced DNT still remain elusive (reviewed by Costa et al. (2014)).
Human oligodendrogenesis was the most sensitive endpoint towards BDE-99 within the endpoints studied (cell death, NPC proliferation, migration, neurogenesis, oligodendrogenesis and oligodendrocyte maturation). It was shown to be 7-times more sensitive than murine oligodendrogenesis (IC_{50}: 2 µM and 14 µM, respectively). In contrast, human neurogenesis was significantly less sensitive than murine neurogenesis (IC_{50}: 23 µM and 10 µM, respectively). NPC viability, proliferation and migration were not affected by BDE-99 at all. These data show that species do not only differ in their susceptibility, but also in their most sensitive endpoint (MSE) towards BDE-99. These observations were recently made for a variety of other compounds tested in the neurosphere assay including arsenic, valproic acid and methylazoxymethanol (Baumann et al. 2015). For DNT hazard assessment purposes, more KEs have to be tested to identify the MSE. In addition to the ‘Neurosphere Assay’ endpoints specific for neurodevelopmental processes within the embryonic stage of development as well as more complex processes like neuronal subtype specification, synapse and network formation need to be included in a proposed DNT testing strategy (Baumann et al. 2015). After identification of the MSE, the data can then be evaluated in the parallelogram approach. For BDE-99, however, no *in vivo* data on rodent oligodendrocyte

toxicity is available. Such data gaps limit AOP building which is needed for novel concepts of risk assessment.
Despite the lack of data on BDE-99 toxicodynamics and toxicokinetics (mouse versus human and *in vivo* versus *in vitro*) a risk analysis is attempted that might allow interpretation of human neurosphere *in vitro* results. The IC_{20} value for inhibiting human oligodendrogenesis lies in the upper nanomolar range (862 nM, corresponding to approximately 430 ng/ml). Breast milk fed children are assumed to reach a daily total PBDE exposure up to 306 ng/kg/day (Costa and Giordano 2007; Schecter et al. 2006) and in serum lipids the total concentration of the five most abundant congeners reaches 480 ng/g lipid weight (lw; corresponding to approximately 960 nM in serum lipids) for Californian toddlers (Fischer et al. 2006). In wildlife birds the sum of PBDE congener concentrations in brain lipids were observed to be 1.5-times higher than in the corresponding serum lipids (Voorspoels et al. 2006). This demonstrates that PBDEs enter the blood brain barrier (BBB) and are able to accumulate in fatty tissues like the brain. Assuming a similar accumulation in human as in bird brain lipids, toddlers' PBDE brain lipid concentrations might reach 720 ng/g lw (approximately 1440 nM in brain lipids), which is higher than our observed human IC_{20} (862 nM). However, this is only a rough kinetic estimation with a variety of uncertainties, e.g. this study only dealt with BDE-99 and not with a congener mixture and no kinetic *in vivo-in vitro* extrapolation or human-specific kinetics were taken into consideration.
This case study with BDE-99 demonstrated that a substance can be an endocrine disruptor in rodent, but not in human NPCs and thus illustrates the need to consider species differences in hazard and risk assessment not only in the susceptibility to compounds, but also in the involved toxicity pathways.

4. Abstract

Polybrominated diphenyl ethers (PBDEs) were widely used as flame retardants in consumer products. Due to their lipophilicity they rapidly accumulate in the environment and in human tissues. This is of high concern because PBDEs were classified as developmentally neurotoxic compounds for humans. Thyroid hormones (THs) play a crucial role in many processes of brain development and TH deficiency during development is thus one cause for mental retardation in humans. Since PBDE-treated animals show behavioral abnormalities comparable to animals suffering from hypothyroidism during brain development, TH disruption is discussed as a mechanism for PBDE-induced developmental neurotoxicity (DNT). However, the molecular mechanisms of PBDE-induced DNT are so far elusive.

The major aim of this thesis was to evaluate whether BDE-99, one of the most abundant congeners in human tissue, interferes with basic processes of fetal brain development like migration or differentiation into neurons and oligodendrocytes by TH disruption. This aim was pursued by using human and murine neural progenitor cells (h,mNPCs) grown as neurospheres. To reach this goal, first, the mouse neurosphere culture was instituted and characterized. Second, TH signaling was characterized in hNPCs and mNPCs. Third, the oligodendrocyte maturation assay as a test for studying TH disruption in NPCs was developed. Based on these establishments, species-specific modes of action of BDE-99 on oligodendrogenesis were studied.

BDE-99 treatment did not reduce NPC migration, but concentration-dependently inhibited human and mouse neuron and oligodendrocyte formation with human oligodendrocyte formation as the most sensitive endpoint. Murine oligodendrocyte formation was induced by TH via thyroid hormone receptor (TR)α and BDE-99 disturbed this endpoint by TH disruption independently of TRs. In addition, oligodendrocyte maturation was induced by TH in both species and was therefore chosen as the endpoint for studying TH disruption. BDE-99 interfered with oligodendrocyte maturation by disrupting TH signaling in murine NPCs, but did not affect human oligodendrocyte maturation.

In summary, within this thesis the mouse neurosphere culture was established, TH-dependent endpoints were identified in hNPCs and mNPCs and the oligodendrocyte maturation assay was established as a test for studying TH disruption in NPCs. The results of the mechanistic study with BDE-99 clearly indicate species-specific modes of action for this compound acting as a TH disruptor only in mouse, but not in human NPCs. Thus, these data support the notion that human *in vitro* models should be chosen for chemical hazard assessment and mode of action analyses.

5. Zusammenfassung

Polybromierte Diphenylether (PBDEs) fanden breite Verwendung als Flammschutzmittel in verschiedensten Haushaltsprodukten. Aufgrund ihrer Lipophilie akkumulieren sie schnell in der Umwelt und in menschlichen Geweben. Dies ist von hoher Relevanz, weil PBDEs als entwicklungsneurotoxische Substanzen für den Menschen klassifiziert wurden. Thyroidhormone (THs) spielen eine wichtige Rolle bei einigen Prozessen der Gehirnentwicklung und daher führt TH Mangel während der Entwicklung zu mentaler Retardierung. Da PBDE behandelte Tiere ähnliche Verhaltensauffälligkeiten zeigen wie Tiere, die während der Gehirnentwicklung unter Hypothyreose leiden, wird TH Disruption als ein Mechanismus für die von PBDE hervorgerufene Entwicklungsneurotoxizität (ENT) diskutiert. Die molekularen Mechanismen, durch welche PBDEs ENT hervorgerufenen, sind jedoch weitestgehend unbekannt.

Das Hauptziel dieser Arbeit war aufzuklären, ob BDE-99, eines der am häufigsten in menschlichem Gewebe vorkommenden PBDE Kongenere, grundlegende Prozesse der fetalen Gehirnentwicklung wie Migration oder Differenzierung in Neurone und Oligodendrozyten durch TH Disruption stört. Hierfür wurden humane und murine neurale Progenitorzellen (h,mNPCs) verwendet, welche als Neurosphären kultiviert wurden. Zum Erreichen dieses Ziels wurde erstens die Mausneurosphärenkultur etabliert und charakterisiert, zweitens wurden die zellulären TH Signalwege in hNPCs und mNPCs charakterisiert und drittens wurde ein Test zur Untersuchung von TH Disruption basierend auf NPCs entwickelt. Aufbauend auf diesen Etablierungsarbeiten wurden die Mechanismen untersucht, die der toxischen Wirkung von BDE-99 auf die Oligodendrogenese zugrunde liegen.

Eine Behandlung von NPCs mit BDE-99 beeinflusste die Migration von NPCs nicht, inhibierte jedoch konzentrationsabhängig die humane und murine Bildung von Neuronen und Oligodendrozyten. Dabei stellte die humane Oligodendrozytenbildung den sensitivsten Endpunkt dar. TH induzierte die murine Oligodendrozytenbildung mittels Thyroid-hormonrezeptor (TR)α und BDE-99 störte diesen Endpunkt durch TH Disruption, jedoch unabhängig von TRs. Zudem induzierte TH die Oligodendrozytenreifung in beiden Spezies, welche deshalb als Endpunkt für die Untersuchung von TH Disruption gewählt wurde. BDE-99 inhibierte die Oligodendrozytenreifung in mNPCs durch TH Disruption, während es die Reifung von humanen Oligodendrozyten nicht beeinträchtigte.

Zusammengefasst wurde in dieser Arbeit die Mausneurosphärenkultur etabliert, wurden TH-abhängige Endpunkte in hNPCs und mNPCs identifiziert und der „Oligo-dendrozytenreifungsassay“ wurde etabliert, um TH Disruption in NPCs zu untersuchen. Die Ergebnisse der anschließenden mechanistischen Studie zeigen Spezies-spezifische Wirkmechanismen für BDE-99, welcher nur in murinen, aber nicht in humanen NPCs als TH Disruptor wirkt. Daher unterstützen diese Ergebnisse die Ansicht, dass humane *in vitro* Systeme für die Abschätzung des Gefährdungspotentials von Chemikalien sowie die Aufklärung von Wirkmechanismen genutzt werden sollten.

Abbreviations

2D	*two-dimensional*
3D	*three-dimensional*
3R	refine, reduce, replace
(Q)SAR	(Quantitative) structure–activity relationship
AO	adverse outcome
AOP	adverse outcome pathway
BBB	blood brain barrier
BrdU	bromodeoxyuridine
D2	deiodinase 2 (enzyme)
D3	deiodinase 3 (enzyme)
DNA	deoxyribonucleic acid
DNT	developmental neurotoxicity
EGF	epidermal growth factor
EPA	Environmental Protection Agency
ESC	embryonic stem cell
EST	embryonic stem cell test
FCS	fetal calf serum
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GW	gestational week
HTS	high-throughput screening
KE	key event
MAPK	mitogen-activated protein kinases
MBP/Mbp	gene encoding human/murine myelin basic protein
MEA	Multielectrode Array
MIE	molecular initiating event
MOA	mode of action
MOG/Mog	gene encoding human/murine myelin oligodendrocyte glycoprotein
MSE	most sensitive endpoint
NCC	neural crest cell
NEP	neuroepithelial precursor
NO	nitric oxide
NPC	neural progenitor cell
NRC	National Research Council
NSC	neural stem cell

OECD	Organization for Economic Co-operation and Development
PBDE	polybrominiated diphenyl ether
PDL	poly-L-lysine
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PND	postnatal day
RXR	retinoid X receptor
SRA	Systems Research and Applications Corporation
T3	L-triiodothyronine
T4	L-thyroxine
TH	thyroid hormone
THRA/Thra	gene encoding human/mouse TRα isoforms
THRB/Thrb	gene encoding human/mouse TRβ isoforms
TR	thyroid hormone receptor (protein)
TSH	thyroid-stimulating hormone
TTR	transthyretin
US(A)	United States (of America)

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Eidesstattliche Erklärung/Declaration

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit „Mechanistische Studien zur Entwicklungsneurotoxizität Polybromierter Diphenylether (PBDE) in 3D Modellen von Maus und Mensch in vitro" selbständig verfasst und ausschließlich die von mir angegebenen Hilfsmittel verwendet habe. 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 "Mechanistic studies on the developmental neurotoxicity of polybrominated diphenyl ethers (PBDEs) in human and murine 3D in vitro models" 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 not used in the same or in a similar version to achieve an academic grading elsewhere.

Katharina Dach

Düsseldorf, November 2015