Polybrominated Diphenyl Ethers Disturb Neural Development in Mice and Men

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Polybromierte Diphenylether stören die Gehirnentwicklung von Mäusen und Menschen

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SUMMARY

Polybrominated diphenyl ethers (PBDEs) are persistent and bioaccumulative flame retardants that are of concern as they are ubiquitous and potentially toxic; and they have been found at rapidly rising levels in humans during the past few decades. The greatest concern of PBDEs for potential adverse health effects relates to their developmental neurotoxicity (DNT). Various PBDE congeners caused behavioral alterations like hyperactivity and disrupted performance in learning and memory tests in perinatally exposed mice and rats.

To facilitate hazard assessment for humans, the impact of PBDEs on human neurodevelopment *in vitro* and the mechanisms underlying these changes were investigated. For these analyses, two of the most prominent congeners found in human tissues, the tetra-brominated BDE-47 and the penta-brominated BDE-99, were used. The effects of these PBDEs on endpoints that are specific for developmental neurotoxicity, proliferation, migration and differentiation, as well as on cell viability were investigated in a human model that mimics brain development *in vitro*. PBDEs did not disturb human neural progenitor cell (hNPC) viability and proliferation, but decreased migration distance of hNPCs. Moreover, they caused a reduction of differentiation into neurons and oligodendrocytes. Simultaneous treatment with the thyroid hormone receptor (THR) agonist T_3 rescued these effects on migration and differentiation, while the THR antagonist NH-3 did not exert an additive effect. Thus, PBDEs disturb development of hNPCs *in vitro* via endocrine disruption of cellular thyroid hormone signalling at concentrations that might be of relevance for human health.

Besides these endocrine disrupting properties, PBDEs are known to cause disturbances in calcium homeostasis. Thus, the effects of BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 on intracellular Ca^{2+} $[Ca^{2+}]_i$ -homeostasis in hNPCs were investigated. Acute exposure of hNPCs to BDE-47 or 6-OH-BDE-47 resulted in a significant increase in $[Ca^{2+}]_i$. Using the mitochondrial uncoupler FCCP and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-inhibitor Thapsigargin (TG) to empty intracellular Ca^{2+}-stores, it was discovered that the increase in $[Ca^{2+}]_i$ depends on intracellular Ca^{2+}-release from the ER as well as from mitochondria. Additionally, some currents seem to be caused by influx of extracellular Ca^{2+}. The hydroxylated metabolite 6-OH-BDE-47 was more potent in disturbing Ca^{2+}-homeostasis in hNPCs than its parent compound BDE-47. These findings show that oxidative metabolism of PBDEs could increase their neurotoxic potential for human neural progenitor cells.

In order to extrapolate the human *in vitro* data to results obtained in rodents *in vivo*, the effects of PBDEs on murine neural progenitor cell (msNPC) development were investigated. PBDEs did not disturb viability and proliferation of murine neurospheres. Furthermore, in contrast to

the effects on human cells, PBDEs did not disturb migration and neurogenesis in msNPCs. However, BDE-99 caused a reduction of differentiation into oligodendrocytes, which was also seen in hNPCs. Although the effects of PBDEs on neural development could be rescued by co-administration of thyroid hormone (T_3) in both species, hNPCs showed a higher sensitivity to T_3. These discrepancies in the effects of PBDEs and T_3 were due to different expression patters of thyroid hormone receptors. Accordingly, human neural progenitor cells show an enhanced sensitivity towards PBDE-induced neurotoxicity in comparison to their murine counterparts. This species-specific difference is the result of diversely developed thyroid hormone systems *in vivo* in rodents and humans during development.

The data obtained in this study give mechanistic support to recent epidemiologic observations that PBDEs cause neurobehavioral disturbances in children.

ZUSAMMENFASSUNG

Polybromierte Diphenylether (PBDE) sind persistente und bioakkumulierende Flammschutzmittel, die ubiquitär in der Umwelt vorhanden und potentiell toxisch sind. Des Weiteren wurde in den letzten Jahrzehnten ein starker Anstieg der PBDE-Konzentration in menschlichem Gewebe registriert. Die wohl größte Gefahr für die menschliche Gesundheit geht von den entwicklungsneurotoxischen Eigenschaften der PBDE aus. So wurde in aktuellen Studien nachgewiesen, dass verschiedene PBDE-Kongenere zu Verhaltensauffälligkeiten wie Hyperaktivität und Störungen des Lernens und des Gedächtnisses bei Mäusen und Ratten führen.

Um das Gefährdungspotential für den Menschen abschätzen zu können, wurden die Auswirkungen von PBDE auf die humane neurale Entwicklung *in vitro* untersucht. Außerdem wurden die molekularen Mechanismen erforscht, die den beobachteten Veränderungen zugrunde liegen. Für diese Analysen wurden zwei der am häufigsten in humanem Gewebe gefundenen Kongenere verwendet, das tetra-bromierte BDE-47 und das penta-bromierte BDE-99. Der Einfluss dieser PBDE auf die für Entwicklungsneurotoxizität spezifischen Endpunkte, Proliferation, Migration und Differenzierung, sowie auf die Viabilität wurde mit Hilfe eines humanen Modells untersucht, welches die basalen Prozesse der Gehirnentwicklung *in vitro* widerspiegelt. PBDE wirkten sich weder auf die Viabilität noch auf die Proliferation humaner neuraler Progenitorzellen (hNPCs) aus, führten jedoch zu einer Verkürzung der Migrationsstrecke. Zudem verursachten sie eine Inhibition der Differenzierung von hNPCs zu Neuronen und Oligodendrozyten. Eine simultane Exposition mit dem Thyroidhormonrezeptor (THR)-Agonisten T_3 hob die Effekte auf die Migration und die Differenzierung auf, indes zeigte der THR-Antagonist NH-3 keine additiven Effekte. Folglich bewirken PBDE eine Störung der Entwicklung von hNPCs *in vitro* durch endokrine Disruption des Thyroidhormon-Signalweges.

Neben ihren Eigenschaften als endokrine Disruptoren ist bekannt, dass PBDE die Kalzium-Homöostase beeinträchtigen. Aus diesem Grund wurden die Effekte von BDE-47 und seines Metabolits 6-OH-BDE-47 auf die intrazelluläre Ca^{2+}-Homöostase in hNPCs untersucht. Die Belastung der Zellen mit BDE-47 oder 6-OH-BDE-47 führte zu einem akuten Anstieg der intrazellulären Ca^{2+}-Konzentration $[Ca^{2+}]_i$. Mit Hilfe des mitochondrialen Entkopplers FCCP und des SERCA-Inhibitors Thapsigargin konnte geklärt werden, dass der Anstieg seinen Ursprung im endoplasmatischen Retikulum (ER) sowie in den Mitochondrien hat. Ein weiterer Teil des Kalziumstroms beruhte auf einem Einstrom von extrazellulärem Ca^{2+}. Auffällig war, dass der hydroxylierte Metabolit 6-OH-BDE-47 einen stärkeren Effekt auf die Kalzium-Homöostase zeigte als die Ursprungssubstanz BDE-47. Diese Untersuchungen zeigen, dass der oxidative Metabolismus das neurotoxische Potential der PBDE für humane Zellen erhöhen kann.

Um zu klären, ob PBDE auch eine Gefährdung für die menschliche Gesundheit *in vivo* darstellen, und um die Unsicherheiten, die mit der Extrapolation von Ergebnissen aus Tierversuchen auf den Menschen einhergehen, zu verringern, wurden die Auswirkungen von PBDE auf murine neurale Progenitorzellen (msNPC) untersucht. PBDE beeinflussten weder die Viabilität noch die Proliferation von murinen Neurosphären. Im Gegensatz zu den Effekten in humanen Zellen führte eine Exposition auch nicht zu einer Inhibition der Migration oder der Neurogenese in msNPCs. BDE-99 hingegen führte zu einer signifikanten Reduktion der Oligodendrozyten, ein Effekt, der auch in humanen Neurosphären zu beobachten war. Obwohl die Effekte der PBDE auf die neurale Entwicklung in beiden Spezies durch eine Ko-Applikation von Thyroidhormon (T_3) aufgehoben werden konnten, zeigten humane Zellen eine stärkere Reaktion auf die Stimulation mit T_3. Die beobachteten Unterschiede in der Reaktion auf PBDE und T_3 ließen sich auf unterschiedliche Expressionsmuster der verschiedenen Isoformen des Thyroidhormon-Rezeptors zurückführen. Dementsprechend zeigen humane neurale Progenitorzellen eine erhöhte Sensitivtät gegenüber PBDE-induzierter Neurotoxizität im Vergleich zu murine Zellen. Diese spezies-spezifischen Unterschiede spiegeln sich auch *in vivo* durch unterschiedlich entwickelte TH-Systeme in Nagetieren und dem Menschen während der Entwicklung wider.

Folglich geben diese Daten Aufschluss über die den entwicklungsneurotoxischen Eigenschaften der PBDE zugrundeliegenden Mechanismen und unterstützen somit aktuelle epidemiologische Studien, denen zufolge eine erhöhte PBDE-Belastung während der Entwicklung mit Verhaltensauffälligkeiten bei Kindern korreliert.

1. INTRODUCTION

1.1. DEVELOPMENTAL NEUROTOXICITY (DNT)

1.1.1. BRAIN DEVELOPMENT

The mammalian brain is a laminated structure that includes a complex diversity of neurons and shows rich local and extrinsic connectivity. During prenatal life, it develops from single cells along the dorsal ectoderm of the fetus into a highly regulated organ. The mature brain consists of billions of precisely located, interconnected and specialized cells[1].

Human brain development. The central nervous system (CNS) begins as a strip of cells consisting of neural stem cells (NSC) known as neuroepithelial cells (NEC). These cells proliferate and form the neural plate, and as the edges of this sheet fold outwards, the neural tube is formed, which gives rise to the different sections of the brain. NECs are radially elongated and contact both the apical (ventricular) and the basal (pial) surface. By the onset of neurogenesis, the NECs are replaced by radial glia cells (RG)[2], which generate neurons, oligodendrocytes and astrocytes, the three major cell types of the brain[3].

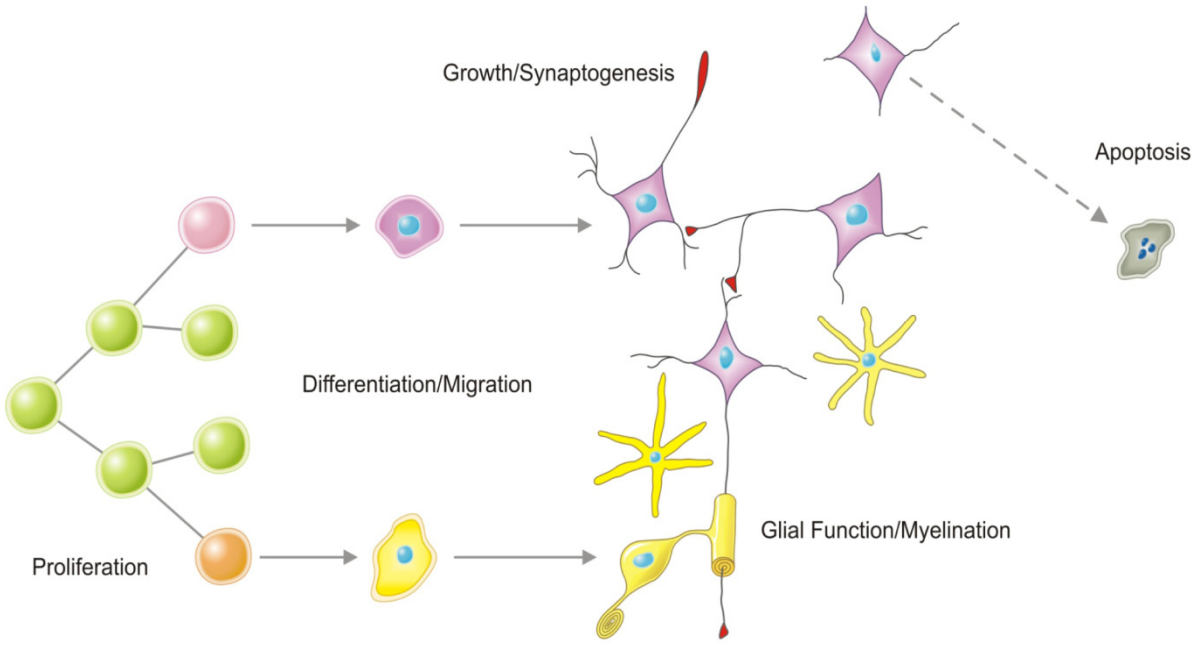

Figure 1 | **Scheme of cellular processes during brain development.** The figure shows the proliferation of neural progenitor cells (green) and their maturation to neuronal (pink) and glial precursors (orange). These more restricted precursors differentiate to functional neurons (purple), astrocytes and oligodendrocytes (both in yellow), while they migrate to their final position. Afterwards, neurons start to prune and build synapses, while insufficient connected neurons undergo apoptosis (with courtesy from Bill Mundy, U.S. EPA**).**

This generation of neural cell types passes through three general phases. During the first phase, the expansion one, the number of neural progenitor cells (NPCs) rapidly increases by symmetric cell division. Subsequently, the neurogenic phase begins, in which neurons and

neuroblasts are generated directly from precursors by symmetric and asymmetric division. Finally, macroglia cells emerge, which marks the gliogenic phase[4].

During neurogenesis, the radial processes of RG support migration of neurons and guide them to their target location. Once neurons reach their final destination at about the 16th fetal week, they arborize and branch in an attempt to establish appropriate connections[5]. Subsequent to the initial phase of innervations, approximately 50% of all neurons are eliminated by apoptosis[6,7]. Oligodendrocyte and astrocyte differentiation follows the initial phase of neurogenesis, and their birth often lags behind that of neurons[8,9]. Afterwards, glial cells start to exert their function by ensuring the ionic and trophic balance of the extracellular milieu in the case of astrocytes, whereas oligodendrocytes insulate and myelinate axons of neurons.

Species-specific differences. Although the knowledge of differences in brain development between humans and rodents is rather limited and often neglected, they might indicate important evolutionary steps that are of considerably functional relevance. Apparently, there are entire quantitative differences that might simply relate to the expansion of the forebrain. However, there are some unique human characteristics that are more crucial, such as the early expression of GFAP in radial glia at the onset of neurogenesis[10-12], the existence of non-dividing radial glia[13], the particularly distinctive subgranular layer and the existence of entirely new classes of neurons, such as fusiform neurons[14], precocious predecessor cells[15] and the uniquely hominid features of adult human astrocytes[16].

Besides the molecular and cellular differences in brain development, perhaps the most distinctive feature of the human brain are the enormous cerebral hemispheres, which are evolutionarily correlated with increasing behavioral and cognitive capacities. This expansion of size is mirrored in the prolonged development of the human brain and the length of pregnancy. Therefore, the developmental stage of the embryo needs to be considered for comparisons of rodent and human *in vitro* and *in vivo* data. Unfortunately, there is no simple mathematical relationship between animal and human development. Whereas the start of neurogenesis in humans takes place at gestational week (GW) 5[17], reaches its peak at GW13[5] and ends at GW22[18], the murine neurogenesis starts at embryonic day (ED) 10[17], reaches its peak at ED14[19,20] and ends at ED18[20]. The six-layered cortex is developed in mice on ED15[21] and in humans on GW14[17], while gliogenesis starts at GW18 in humans[18] and at ED17 in mouse[22]. All species-specific temporal characteristics of brain development were tried to be depicted on a common timeline in Figure 2. This timeline indicates that the developmental stage of human neurospheres used in this study (GW16) corresponds to the developmental stage of E16 in mice. Contrarily, using a mathematical model comparing similarities and relative differences in the timing of neural events, GW16 in humans correlates with PND3 in mice[23].

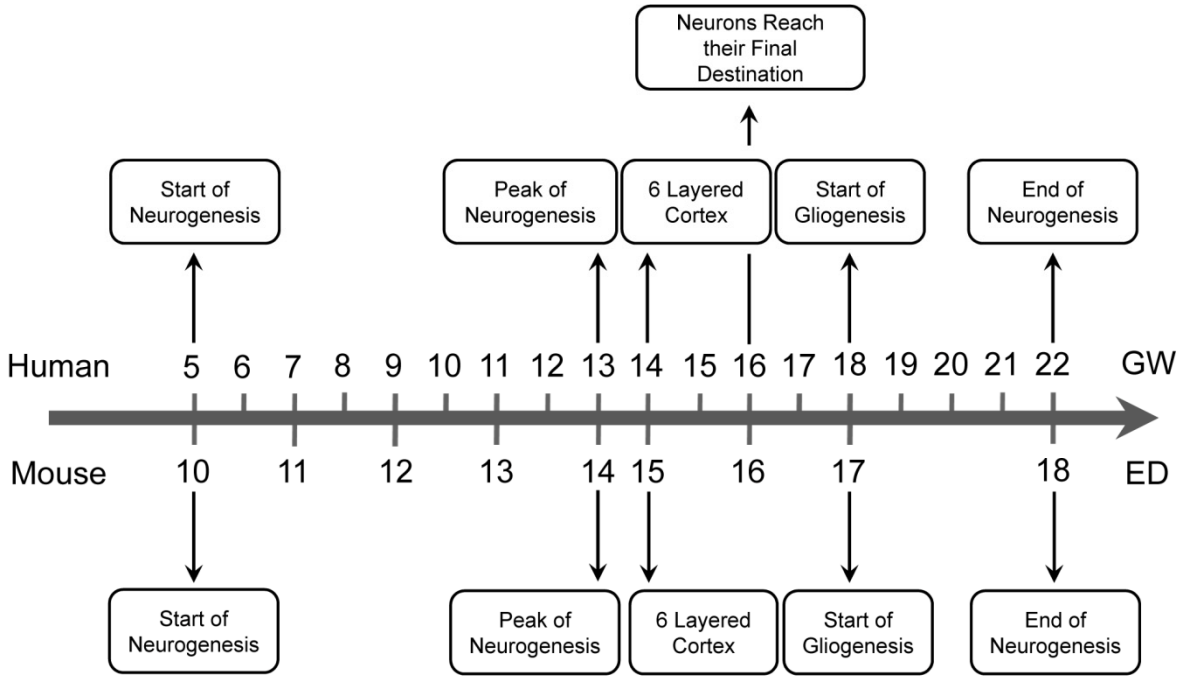

Figure 2 | **Timeline of human and murine brain development.** The timeline shows specific marks for human brain development from gestational week (GW) 5 to 22 and for murine brain development from embryonic day (ED) 10 to 18.

Thyroid hormones in brain development. The thyroid hormones (THs), thyroxine (T_4) and the more active form triiodothyronine (T_3), are amino acids produced and secreted by the thyroid gland, which exert major effects on developmental and physiological processes[24]. During development, especially the human brain is an important target of TH, and TH deficiency during fetal and postnatal periods causes irreversible mental retardation and neurological deficits[25]. Three stages of TH dependent neurological development can be distinguished[26]. The first stage occurs in humans at GW 16-20; as the fetus has not started its own thyroid hormone synthesis yet, TH is provided solely by the mother[27-29]. TH influences neuronal proliferation and migration of neurons in the cerebral cortex, hippocampus and medial ganglionic eminence at this stage[30-33]. The second stage takes place after onset of fetal thyroid function in the second trimester. THs are applied from both the fetus and the mother to the developing brain[27-29]; and neurogenesis, neuronal migration, axonal outgrowth, dendritic branching, synaptogensis, the initiation of glial cell differentiation and migration as well as the onset of myelination are sensitive to THs[34-36]. During the third stage, the postnatal one, TH is derived only from the child. Here, TH dependent processes include migration of granule cells in the hippocampal dentate gyrus and cerebellum, pyramidal cells in the cortex and Purkinje cells in the cerebellum, and TH-dependent gliogensis and myelination continue[34-36].

All these processes are mediated by thyroid hormone receptors (THRs), which are nuclear proteins containing several functional domains like the ligand- and the DNA-binding domains

(Figure 3)[37,38]. There are two receptor genes, designated THRα and THRβ, located on different chromosomes. They encode nine protein products, of which only three contain the ligand- and the DNA-binding domains: α_1, β_1 and β_2[25]. Although THRα_1 accounts for the largest fraction of the total receptor in brain and therefore presumably mediates most TH effects, the THRβ gene is also expressed in the brain. The prevalent view is that the receptor isoforms are mostly equivalent in their biological activity, and that the different physiological roles of each receptor depend on their particular patterns of temporal and regional expression[25].

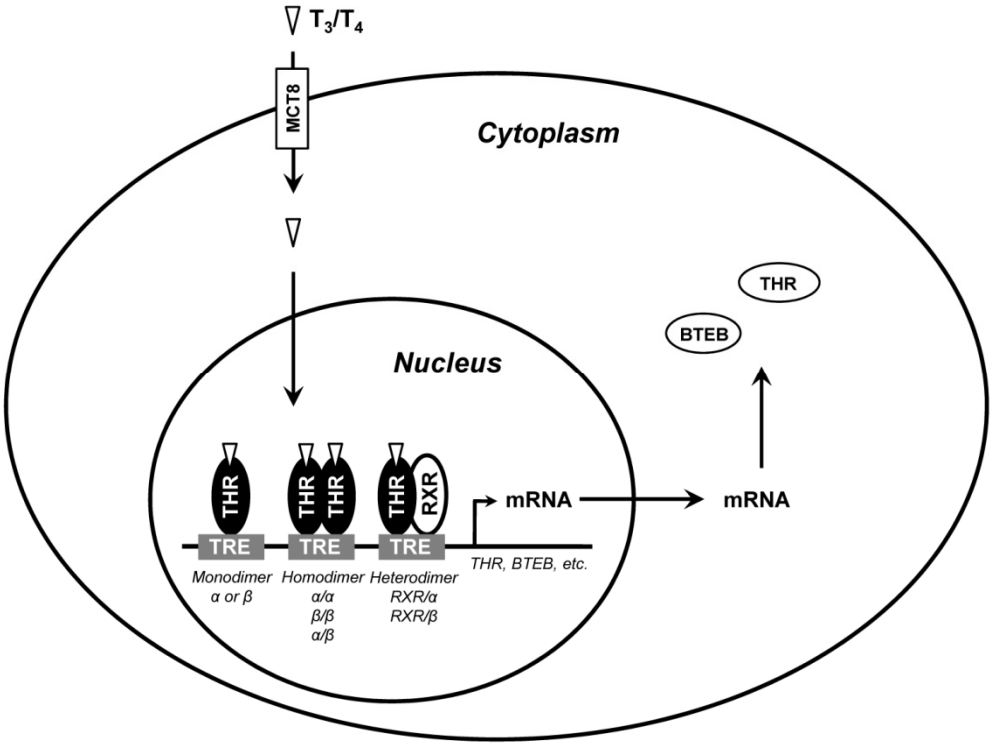

Figure 3 | **The THR signal transduction.** T_3 or T_4 is transported into THR expressing neural cells, possibly through the MCT8 transporter. T_3 and T_4 bind to the nuclear receptor complex, resulting in transcriptional transactivation. THR can bind DNA as a monomer or homodimer, or it functions as a heterodimer with the retinoic X receptor (RXR).

It seems that TH plays a key role in brain development especially in humans as they are born with a fully mature thyroid hormone system, whereas rodents are born with a less developed thyroid system[39].

Calcium in brain development. Calcium (Ca^{2+}), an early-response second messenger, plays an important role in a number of physiological processes, including cell proliferation, differentiation and apoptosis[40-42]. The complex spatial and temporal properties of intracellular Ca^{2+}-signals are responsible for a wide variety of specific neuronal processes as well, including dendritic spine growth, synaptic plasticity and neurotransmission. Ca^{2+}-influx as well as Ca^{2+}-release from intracellular Ca^{2+}-stores such as the endoplasmic reticulum (ER) or mitochondrial stores define the magnitude, time course and spatial spread of the Ca^{2+}-signal[43,44]. Besides its part during

development, Ca^{2+} is also a key compound in the adult brain as it plays a central role with regard to signal transduction, transmitter release, long-term potentiation, synaptic plasticity and several other physiological brain functions[45]. Ca^{2+}-waves can be observed in astrocytes as well, although their physiological role is not fully understood yet[46].

1.1.2. SUSCEPTIBILITY OF THE DEVELOPING BRAIN

One in every six children has a developmental disability, and in most cases, these disabilities affect the nervous system[47]. The discipline that describes such functional and morphological effects of exogenous substances that lead to pathological changes in the developing brain in the perinatal period is the developmental neurotoxicology. During vulnerable periods, the developing nervous system reacts more sensitive to environmental insults as it is dependent on regional emergence of critical developmental processes (i.e. proliferation, migration, differentiation, synaptogenesis, myelination and apoptosis). All these processes have to take place within a fixed time frame, in which each developmental stage has to be reached on schedule and in the correct sequence (Figure 4).

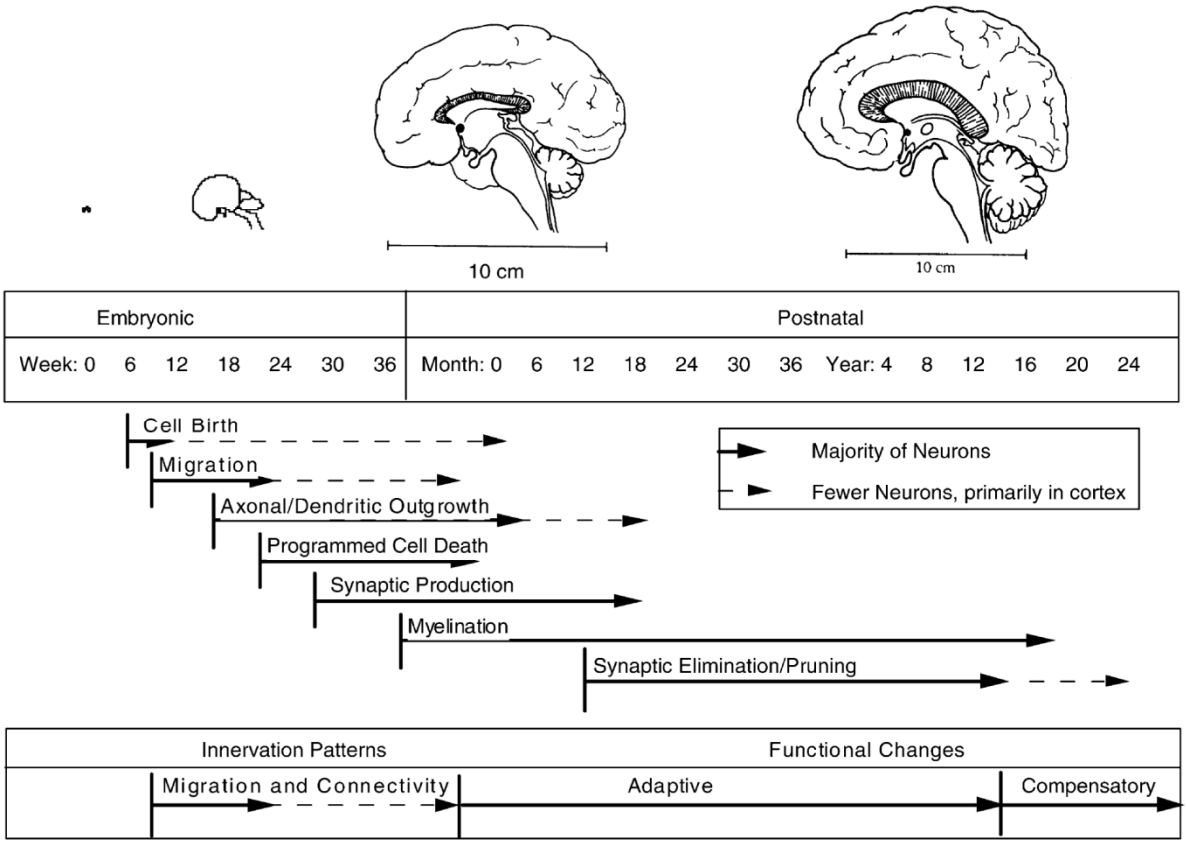

Figure 4 | **The stages of human brain development (top) and different windows of vulnerability (bottom).** The figure shows the developmental processes dependent of time, which occur in phases and thus set the stage for potential periods of vulnerability (top). Neurotoxic substances received early in life (bottom) will be interfering with innervation patterns, whereas at later stages, insult will cause functional changes[48].

This extraordinary complexity of human brain development leads to a unique susceptibility to toxic interference that has no counterpart in the mature brain[18,48-50]. Therefore, the developing brain is inherently much more susceptible to injury caused by toxic agents than the brain of an adult[51].

Although the placenta offers some protection against chemical exposure during fetal development, it is not an effective barrier against environmental pollutants[52]. Additionally, the prenatal sensitivity to DNT is enhanced due to the undeveloped blood-brain barrier that protects the adult brain. Therefore, even ions and hydrophilic noxae are able to enter the fetal brain much easier than the adult one[49]. The susceptibility of infants and children to industrial chemicals is further extended by their increased exposures, augmented absorption rates and diminished ability to detoxify many exogenous compounds, relative to that of adults[53,54].

1.1.3. TESTING FOR DNT

The current guidelines for chemical testings from the OECD and the U.S. EPA prescribe animal experiments to determine developmental neurotoxicity of chemicals[55,56]. To this end, motoric and sensoric abilities as well as learning and memory are investigated[57]. According to the OECD guideline 426, the neuropathologic endpoints for DNT are weight and form of the brain, as well as degeneration, necrosis, gliosis and hyperplasia[49]. This guideline stipulates an exposure from gestational day (GD) 6 to postnatal day (PND) 10 at three concentrations. This exposure scheme bears some disadvantages as it does not include all developmental stages of the brain[49]. For example, dichlorodiphenyltrichloroethane (DDT) is developmentally neurotoxic only applied after PND10[58]. Besides these limitations of animal testings according to the existing guidelines, such a DNT *in vivo* testing strategy implies the use of 140 dams and 1000 pups and is thus extremely time and cost intensive[59].

Nevertheless, animal testings will continue to be an important factor for our knowledge of DNT properties of chemicals as an *in vitro* testing cannot substitute for a whole organism with its abilities of absorption, distribution, metabolism and excretion. Notwithstanding these features, species-specific differences are a major problem in toxicology and lead up to 40% false classification[60], which is why testings in the 'right' species are so important and not to be neglected. New opportunities and technologies for studying the developing human brain and DNT in humans will be essential for risk assessment. Therefore, alternative testing strategies are needed that refine, reduce and (maybe) replace animal testings, creating affordable, sensitive and mechanism-based methods suitable for high- and medium-throughput screenings[61].

There are some potential *in vitro* models for toxicity testings that mimic some of the basal processes of brain development in the culture dish. The most promising models for DNT testings are based on three-dimensional animal or human cell culture systems: the embryonic stem cell test (EST), the whole embryo culture (WEC) and human neural progenitor cells (hNPCs), grown as neurospheres.

Embryonic stem cell test (EST). The EST is the only validated *in vitro* model for developmental toxicity testing (embryotoxicity) so far[62]. It is based on the capacity of murine embryonic stem cells (cell line D3) to differentiate to contracting cardiomyocytes, and it is able to assess the embryotoxic potential of test compounds[63]. The used stem cell lines also bear the potential to differentiate into neurons and astrocytes as well as to form embryoid bodies; thus, the EST might be a tool to investigate DNT besides embryotoxicity as well[64]. Like in all non-human systems, the use of the EST for DNT testings is limited due to species-specific differences.

Whole embryo culture (WEC). The method of WEC is based on the findings from Nicholas and Rudnick, who demonstrated for the first time that rat embryos can grow outside the maternal organism[65]. Today, WECs can be performed with fish, amphibian and avian as well as with mammalian embryos from mice, rats, hamsters and rabbits[66]. The most interesting topic with regard to industrial application is the suitability of the WEC system for screening new agents with a possible teratogenic potency. According to the ECVAM Scientific Advisory Committee (ESAC) statement in 2001, the WEC test is a scientifically validated test, which is ready to be considered for use in assessing the embryotoxic potential of chemicals for regulatory purposes[67]. Due to the developing brain in the cultured embryo, the WEC is also applicable for DNT testings. However, the WEC bears the same limitations for human risk assessment as the EST, which are mainly the species-specific differences.

Human neural progenitor cells (hNPCs). The main focus of neurosphere research has concentrated on their application for neuroregeneration[68-71]. Apart from that, our laboratory established the 'neurosphere assay' for DNT testing purposes as it identifies effects of exogenous noxae on basic processes of brain development such as proliferation, migration, differentiation, neurite outgrowth and apoptosis (Figure 5)[72]. Besides our group, which established this alternative model for DNT testing systematically, other groups used neurospheres for specific toxicological questions as well.

Thereby, cell proliferation in neurospheres has been assessed by using several different methods, namely BrdU incorporation, expression of proliferation markers, counting the number of cells in dissociated spheres or measuring the increase in sphere diameter over time. Employing these methods, several groups investigated the influence of lead and ethanol on rodent and human neurosphere proliferation[73-78].

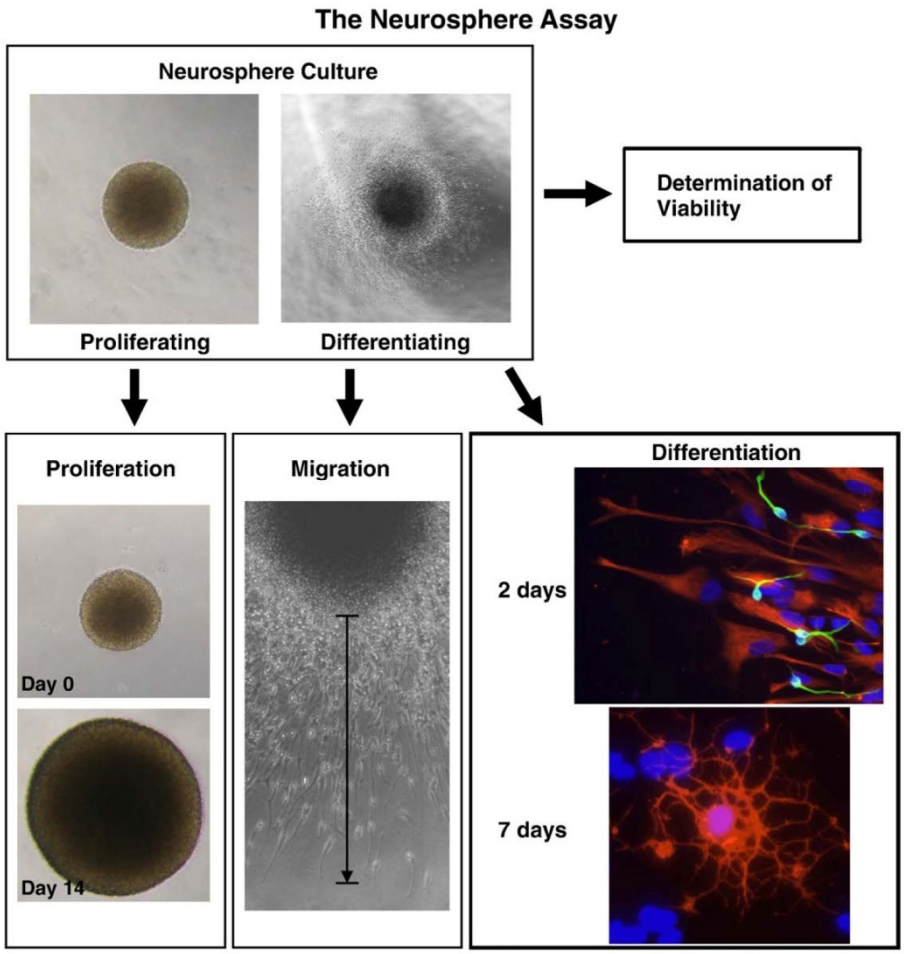

Figure 5 | **The neurosphere assay.** Proliferation is assessed via increase in single sphere diameter or indirectly via increase in metabolic activity. After mitogen withdrawal and in presence of an extracellular protein matrix, NPCs migrate radially out of the sphere. Migration distance over time is measured as indicated by the arrow. Specific immunocytochemical staining of NPCs indicates the presence of β(III)-tubulin (green) and GFAP (red) after 2 days and O4 (red) after 7 days; nuclei are stained with DAPI (blue). Viability of proliferating and differentiating neurospheres is assessed to differentiate between general toxicity and specific developmental neurotoxicity of compounds[72].

After mitogen withdrawal, cells migrate radially out of the sphere, and their migration distance over time can be measured. Two neurodevelopmental toxicants (ethanol and methylmercury) have been studied for their potential to interfere with normal migration in rodent and human neurospheres[79,80]. During migration, NPCs start to differentiate and give rise to the three major cell types of the brain, neurons, astrocytes and oligodendrocytes. Differentiation of post-mitotic cells has also been assessed after exposure to lead, methylmercury, ethanol and PCBs[74,78,81-83]. In addition to these studies performed in primary brain neurospheres, another work was performed using neurospheres generated from the human immortalized embryocarcinoma cell line Ntera2/clone D1, which also shows the ability to proliferate, migrate and differentiate under certain conditions[84]. However, this tumor-derived cell line may contain abnormal characteristics and thus shows an increased chance of genomic instability with increasing passage number.

Taken together, a number of studies indicates that the three dimensional neurosphere system is able to serve as a cell model for DNT testing, which mimics the basic processes of brain development. So far, several studies by others and us described effects of ethanol, lead, methylmercury and polychlorinated biphenyls (PCBs), which represent four of the six chemicals identified as developmentally neurotoxic for humans (see 1.1.4). In the future, a direct comparison of toxic effects on neurospheres generated from different species is needed to decrease the uncertainty associated with animal to human extrapolation.

1.1.4. DEVELOPMENTALLY NEUROTOXIC SUBSTANCES

In 1981, approximately 100.000 chemicals were registered in the European Union (EU)[85]. From these 100.000 chemicals, over 1.000 are known to be neurotoxic in experiments, and 201 of these are also known to be neurotoxic in human beings. The largest groups of neurotoxic compounds are metals, solvents and pesticides. However, only six of these chemicals are known to be toxic for human neurodevelopment[1,86,87]. The industrial chemicals that are recognized to cause neurodevelopmental disorders are lead, methylmercury, PCBs, polybrominated diphenyl ether (PBDEs), arsenic and solvents (e. g. toluene and ethanol).

Lead. Knowledge about the neurotoxic potential of lead in adults stretches back three millennia to the Roman Empire. The developmentally neurotoxic effects in children have been known for more than 100 years as the first description of epidemic lead poisoning in young Australian children was published in 1904[88]. Lead was largely used in petrol, paints, ceramic glazes and many other industrial products through the 20th century. The direct toxic actions of lead include apoptosis, excitotoxicity, influences on neurotransmitter storage and release processes, on mitochondria, second messenger, cerebrovascular endothelial cells and both astrocytes and oligodendrocytes[89]. The symptoms of severe lead poisoning in children are widespread subclinical neurobehavioral deficits, including problems with concentration, memory and cognition as well as lethargy and clumsiness[90-93]. However, a recent epidemiological meta-analysis suggests that the current effects of lead exposure on human brain development might be even greater than previously thought[94].

Methylmercury. The neurotoxicity of high levels of methylmercury (MeHg) is known both in humans and experimental animals. DNT of organic mercury became evident by catastrophic episodes of poisoning, e. g. in Minamata and Niigata, Japan, where an epidemic of spasticity, blindness and profound mental retardation was found in infants. In retrospective, the consumption of fish from waters that were severely polluted with MeHg from local industrial discharge was verified as the source of poisoning[89]. Another outbreak of poisoning occurred in

Iraq in the early 1970s, when people ate bread made from grain that had been treated with an organomercury fungicide[95]. The high affinity of MeHg to thiol groups makes proteins and peptides bearing cysteins susceptible to structural changes and modifications. Therefore, it is known that exposure to MeHg leads to induction of apoptosis, modifications of cytoskeleton, disturbances in Ca^{2+}-homeostasis and to oxidative stress, and it influences neurotransmitter release as well[95]. The U.S. National Academy of Science concluded that there is strong evidence for fetal neurotoxicity of MeHg, even at low exposures[96].

Polychlorinated biphenyls. DNT of PCBs was first reported in the 1970s, when children were exposed to high PCB concentrations during two poisonings in Asia[97]. PCBs led to growth impairment, slow development, lack of endurance, clumsy movement and very low IQs[98]. However, the mechanistic basis of PCB neurotoxicity remains unclear. Interactions with endocrine systems, particularly the thyroid and estrogen/androgen systems, are possible explanations[82,99]. PCBs had been widely applied in electrical equipment as insulators, until its production was banned in the late 1970s because of their presumed carcinogenicity[100].

Polybrominated diphenyl ethers. PBDEs are structurally similar to PCBs. Due to their application in this study, they will be extensively discussed in the next chapter.

Arsenic. The developmentally neurotoxic potential of arsenic was reported for the first time in 1955, when the consumption of powdered milk contaminated with arsenic led to over 12.000 cases of poisoning and 131 deaths[101]. A follow-up study showed that the exposure to arsenic caused mental retardation, poor school records, emotional disturbances and abnormal or borderline encephalograms[100,102]. Nevertheless, evidence for subclinical DNT is less well established than for lead or MeHg.

Solvents. Toluene and ethanol belong to the group of solvents, whose neurotoxicity in adults is well known from acute poisoning cases and occupational studies. Toluene has been abused by sniffing, and prenatal exposure leads to significant DNT known as fetal solvent syndrome (FSS)[103]. Ethanol is a well-documented developmental toxicant causing physiological and mental dysfunctions in children after prenatal exposure, a syndrome called fetal alcohol syndrome (FAS). These abnormalities include CNS dysfunctions such as microencephaly, brain malformations, mental retardation and behavioral abnormalities[104,105]. The molecular mechanisms of ethanol toxicity are disturbances of neural migration, loss of neurons and glial cells as well as altered cell proliferation induced by e. g. apoptosis, blockade of NMDA glutamate receptors, hyperactivation of $GABA_A$ receptors and oxidative stress[106].

There are some more candidate substances for DNT, like pesticides, manganese, fluoride and perchlorate, which deserve particular attention. However, documentation of their

developmentally neurotoxic effects in human beings is poor, and the proof for DNT is still missing[1].

1.2. POLYBROMINATED DIPHENYL ETHERS (PBDES)

Fire kills yearly more than 3500 people in Europe, more than 2000 in Japan and more than 4000 people in the USA[107]. Moreover, fire causes property damage in excess of US$10.7 billion in the USA alone[108]. Therefore, the use of flame retardants corresponds to a strong reduction in fire incident and thus saves lives. There are four different groups of chemicals used as flame retardants, inorganic, polyhalogenated hydrocarbon, organophosphorus and nitrogen-based flame retardants.

Table 1 | **Composition of commercial BDE mixtures.**

Mixture	Commercial product	Ingredients
pentaBDE	DE-71 (Great Lakes Chemicals) Bromkal 70-5DE (Chemische Fabrik Kalk)	0-1% triBDEs (17, 18, 33) 24-38% tetraBDEs (47, 49, 51, 66) 50-62% pentaBDEs (85, 99, 100, 102) 4-12% hexaBDEs (138, 139, 140, 153, 154) 0-0.3% heptaBDEs (183, 184)
octaBDE	DE-79 (Great Lakes Chemicals) Bromkal 79-8DE (Chemische Fabrik Kalk)	0.5% pentaBDEs 12% hexaBDEs (138, 144, 153, 154) 45% heptaBDEs (171, 180, 183) 33% octaBDEs (196, 197, 201, 203) 10% nonaBDEs (206, 207, 208) 0.7% decaBDE (209)
decaBDE	DE-83R (Great Lakes Chemicals) Bromkal 82-0DE (Chemische Fabrik Kalk)	0-1% octaBDEs (196, 197, 203) 0.3-10% nonaBDEs (206, 207, 208) 92-99% decaBDE (209)

Compositions of BDE mixtures are adapted from Germer[109].

The largest group is that of polyhalogenated hydrocarbons, with the subgroup of brominated flame retardants (BFRs). BFRs contain the group of polybrominated biphenyls (PBBs), which were banned in the 1970s[110], and others, such as tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD), which are still in use. A third major class is that of polybrominated diphenyl ethers (PBDEs), which are widely used in a variety of consumer products such as computers, electric components, television sets, textiles, carpets, polyurethane foam and cars. Commercially used PBDEs have been marketed as three mixtures consisting of diverse brominated congeners. These mixtures are known as pentabrominated BDE, octabrominated BDE and decabrominated BDE (Table 1). The amount of BFRs in the end

product is up to 30% of total weight, in individual cases even more. Whereas pentaBDE and octaBDE were banned in the European Union in 2004 and in several states in the USA, decaBDE was banned in the EU not until 2008 and is still produced in the USA. The use of decaBDE is not subject of any regulatory restriction in Asia. Nevertheless, despite banned production in the EU, PBDEs are still present in consumer goods produced elsewhere. Moreover, due to their persistent nature, they are still abundant in consumer products produced in the EU before June 2008.

1.2.1. CHEMICAL PROPERTIES OF PBDES

The general chemical structure of PBDEs consists of two benzene rings bound by an oxygen atom, with a diverse number of bromine atoms in ortho-, meta- and/or para-position. The empirical formula of all PBDEs is $C_{12}H_{(10-(m+n))}Br_{(m+n)}O$ (Figure 6).

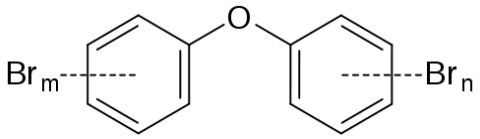

Figure 6 | **General structure of PBDEs (m + n = 1 - 10).** PBDEs consist of two benzene rings bound by an oxygen atom with a diverse number of bromine atoms.

They consist of 209 congeners, which are consecutively numbered by quantity and position of their bromine substitutes, starting with BDE-1, with a single bromine atom in the ortho-position, and ending with BDE-209 with 10 bromine atoms. In the event of fire, the relatively weak bromine carbon bonds are cleaved, and free bromine radicals form, which disrupt the pyrolytic chain reaction by scavenging oxygen radicals.

1.2.2. PBDES AS ENVIRONMENTAL POLLUTANTS

PBDEs are additive flame retardans, which are not chemically bond to the polymer matrix. Due to this lack of chemical bonds, these BFRs leak out of the matrix and are almost ubiquitously present in the environment. Thus, PBDEs have been found in the air, sediments, sludge, soil, house dust, food, birds and marine and terrestrial animals as well as in humans[111-120]. In contrast to PCBs, whose levels have been decreasing, PBDEs have been found in rapidly rising levels in the environment and in humans for the past three decades[112,121-123]. Lower brominated PBDE congeners show higher bioaccumulating and persisting properties than higher brominated compounds; their bioaccumulation factor lies over 5000[113]. In wild life as well as in human tissue, PBDE congeners BDE-28, -47, -99, -100, -153, -154 and -183 are particularly

observed[124]. The still widely used BDE-209 is also found in the environment[112,118], where it can be broken down to the lower brominated congeners[125,126].

Besides debromination, PBDEs are also amenable to other metabolic processes. Thus, phenolic metabolites have been recently found in blood samples from wild animals such as fish, birds and mammals[127-129], and hydroxylated PBDEs have also been identified in blood samples from mice and rats treated with their parent compounds[130,131]. Additionally, metabolites have been recently found in human blood samples as well[132].

Distribution and transport of PBDEs in the environment take place by particles and sediments, the exposition of animals by the food chain. The exposure and the body burden in animals correspond to their position in food chain, which is why predators are higher exposed than prey, and invertebrates are lower exposed than vertebrates[133].

1.2.3. HUMAN EXPOSURE AND BODY BURDEN

The main exposition pathways for humans are the food chain and the gastrointestinal tract. Among food, fish has the highest content of PBDEs, followed by meat and dairy products[119,134-136]. Other sources of PBDE exposure can be found in the occupational setting and in the indoor environment. For example, several studies indicated that house dust is a major source of exposure to PBDEs[135,137-139]; especially for toddlers, dust has been estimated to account for 80 – 93% of PBDE exposure[138]. Particularly alarming are the high levels of PBDEs in human tissue in North America as they are one to two orders of magnitude higher than those found in Europe and Japan[140-142]. In the human body, PBDEs are present in blood, liver, adipose tissue, placenta and in very high amounts in breast milk[143].

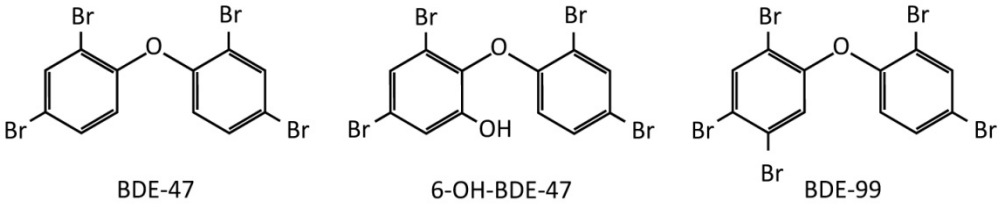

Figure 7 | **The PBDE congeners BDE-47, 6-OH-BDE-47 and BDE-99.** Chemical structures of 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47), its hydroxylated metabolite 6-hydroxy-2,2´,4,4´-tetrabromodiphenyl ether (6-OH-BDE-47) and 2,2´,4,4´,5-pentabromodiphenyl ether (BDE-99).

The highest amount of PBDE was detected in a study from Gill *et al.* with 956 ng/g lipid in a sample of human breast milk[114]. Given the high levels found in breast milk, it has been concluded that a breastfed infant would be exposed to approximately 306 ng/kg body weight (bw)/day[119]. Five tetra-, penta- and hexaBDE congeners (BDE-47, -99, -100, -153, -154) predominate in

human tissues and usually constitute for 90% of total body burden[118], although this does not correspond to their amount in commercial PBDE mixtures[144]. To facilitate PBDE hazard assessment for humans, two of these congeners, the tetra-brominated BDE-47, its hydroxylated metabolite 6-OH-BDE-47 and the penta-brominated BDE-99, are employed in this work (Figure 7)[145].

PBDEs are able to cross the placenta, so that similar concentrations can be found in maternal and fetal blood[145]. PBDE concentrations e. g. in human fetal blood reach up to 460 ng/g lipid[146]. Thus, exposure to PBDEs already starts *in utero* during development and continues after birth through breast-feeding and ingestion of house dust. Therefore, the highest PBDE levels are found in infants and toddlers[145]. In contrast to that, PCB concentrations increase with age[147].

1.2.4. DNT OF PBDEs

The greatest concern of PBDEs for potential adverse health effects relates to their developmental neurotoxicity[86,87,118,133,148]. Such concern is motivated by a number of studies that showed behavioral abnormalities after PBDE exposure during development in rodents[149-158]. E. g. mice were exposed directly to different PBDEs on postnatal day (PND) 3 or 10, and behavioral syndromes were monitored up to 6 months after PBDE administration. All PBDEs tested in this set-up (BDE-47, -99, -153, -183, -203, -206, -209) caused long-lasting changes in behavior. The most prominent effect was decreased habituation, although some other syndromes like e. g. anxiety-like behavior were observed as well. Other research groups used altered study designs, especially different exposure protocols and different species, like different rat (*Rattus norvegicus*) and mouse (*Mus musculus*) strains or killifish (*Fundulus heteroclitus*). In these studies, it was irrelevant whether animals were exposed prenatally, postnatally or perinatally. BDE-47 caused hyperactivity and an increase in locomotor activity in wistar rats after a single treatment on GD6[159,160]. Hyperactivity as well as decreased thigmotaxis were also observed in CD-1 Swiss mice after continuous treatment with BDE-99 from GD6 to PND21[161,162]. Additionally, the decabrominated BDE-209 led to hyperactivity in C57/BL6/J mice exposed from PND2 to 15[163]. The BDE mixture DE-71 caused hyperactivity in killifish after prenatal exposure[164], and perinatal treatment of Long-Evans rats with BDE-99 led to increased sweet preference in males[165] and decreased sexual behavior in females[166]. Moreover, postnatal exposure of rodent pups to DE-71 as well as to BDE-99 caused impaired learning and memory[155,167]. Two epidemiological studies have recently shown a correlation between elevated PBDE exposure during development and lowered IQ and hyperactivity in children[86,87].

Toxicokinetic studies demonstrated that lower brominated PBDEs were usually well-absorbed[168-170], whereas in case of decaBDE, only 10% of the given dose were taken up[171]. After absorption, PBDEs accumulate in several tissues, including the brain, and highest concentrations were measured in adipose tissue[124]. The half-life of tetra- and hexa-BDEs is in the range of 20 to 120 days in rodents, with increase proportional to the degree of bromination[124,172]. However, species as well as gender differences in the rate of excretion have been shown[168,170]. Once absorbed, PBDEs are metabolized to mono- and dihydroxylated metabolites[124,127,130,131,169,173], while higher brominated PBDEs are also metabolized to lower brominated congeners[171,174]. Additionally, high doses of PBDE metabolites are found in human blood samples. In maternal blood samples, approximately 17% of the found PBDEs are metabolites, whereas in fetal blood samples, 45% of all PBDEs are metabolized[132]. This is of concern insofar as hydroxylation of PBDEs is suspected to cause an increase in toxicity[175].

1.2.5. MECHANISMS OF PBDE-INDUCED DNT

Although various studies have shown the developmentally neurotoxic properties of PBDEs, the underlying mechanism still remains unclear. Two general, and not mutually exclusive, possible mechanisms are emerging: The systemic affection of thyroid hormone signalling and a direct effect of PBDEs on the developing brain.

Thyroid hormones are known to play a key role in brain development[25], and several studies have found that PBDEs interfere with the thyroid system during development. Zhou *et al.* first reported that weaning rats exposed to DE-71 or DE-79 had decreased levels of T_4[176]. These findings were confirmed by several other studies in mice, rats and ranch mink (*Mustela vison*), using different PBDEs[163,177-182]. These studies also disclose alterations in several other components of the TH system (e. g. thyrotropin (TSH), transthyretin (TTR) and T_3). Behavioral studies in hypothyroid animals during development have evidenced decreases in learning and habituation, anxiety-like behavior and an increase in locomotor activity[183]. Developmental exposure to PBDEs caused all of these effects as well, which suggests indeed that disturbances of the TH system may contribute to their developmental neurotoxicity. Whether such endocrine disrupting properties of PBDEs are caused by direct interaction with cellular TH signalling or indirectly by interference with systemic TH levels has been unknown so far.

Several other environmental chemicals can disrupt thyroid function and thus lead to a decrease in THs. Among these are propylthiouracil (PTU), which inhibits thyroid peroxidase, perchlorate, which interferes with iodine uptake into the thyroid, and PCBs[145]. PCBs may act by inducing T_4 metabolism[184] and interfere with cellular TH signalling in hNPCs[82]. Furthermore,

bisphenol A (BPA) inhibits the differentiation of oligodendrocyte precursor cells (OPCs) induced by exposure to T_3 *in vitro*[185].

There are only a few studies that have investigated biochemical changes in brains occurring in animals after exposure to PBDEs. Viberg and colleagues found a decreased number of cholinergic nicotinic receptors in the hippocampus of mice following postnatal exposure to BDE-99 or -153[152,155]. In contrast to that, a study performed in ranch mink (*Mustela vison*) did not find any changes in nicotinic receptor levels in the cerebral cortex following chronic developmental exposure to DE-71[186]. Other studies of Viberg *et al.* detected a decrease in the levels of brain derived neurotrophic factor (BDNF), growth associated protein 43 (GAP-43, neuromodulin) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in mice after exposure to BDE-209, -206 or -203[157,158]; and a decrease of CaMKII was also found in mice exposed to BDE-47 in a study by Dingemans *et al.*[175]. Additionally, a reduction in hippocampal long-term potentiation and post-tetanic potentiation as well as a decrease in postsynaptic proteins involved in glutamate receptor signalling (e. g. GluR subunits NR2B and GluR1) were observed in this study. According to these findings, two proteomic analyses by Alm *et al.* revealed changes in the levels of a number of proteins in hippocampus, striatum and cortex of BDE-99 exposed mice, like GAP-43 and BDNF[187,188], which are both closely related to neural development and plasticity[189,190]. A number of studies examined potential effects and mechanisms of PBDEs *in vitro*, which ranged from cell death over changes in protein expression to alterations in signal transduction. PBDE-induced cell death in rodent and human cells was observed in several studies using different congeners and mixtures[191-196]. The cause of cytotoxicity in all these different cell models was induction of apoptosis. Most other *in vitro* studies showed effects of PBDEs on signal transduction in human and rodent cells. BDE-47 as well as BDE-99 caused an increase in intracellular Ca^{2+} [175,197,198]. The mixture DE-71 was reported to lead to an inhibition of vesicular dopamine uptake in rat brain synaptosomes[199], whereas BDE-99 caused an increase of cGMP, calmodulin and soluble guanylate cyclase in rat cerebellar granule neurons (CGN)[200]. Kodavanti *et al.* reported an increase of 3H-phorbol ester (PDBu) binding in these cells after exposure to several PBDE congeners[201].

Structural similarities between PBDEs and halogenated dibenzodioxins and dibenzofurans give rise to concern about dioxin-like toxicity of PBDEs. Dioxins bind and thereby activate the aryl hydrocarbon receptor (AhR)[202], which results in developmental defects[203]. The AhR is a member of the basic helix-loop-helix/Per-Arnt-Sim-(bHLH/PAS-)transcription factor family. After ligand binding, the AhR translocates from the cytoplasm to the nucleus and heterodimerizes with the AhR nuclear translocator (Arnt). The AhR/Arnt complex recognizes specific DNA sequences, known as dioxin response elements (DRE) or xenobiotic response

elements (XRE), with promoters of regulated target genes, like the xenobiotic metabolizing enzyme cytochrome P450 (CYP) 1A1 (Figure 8).

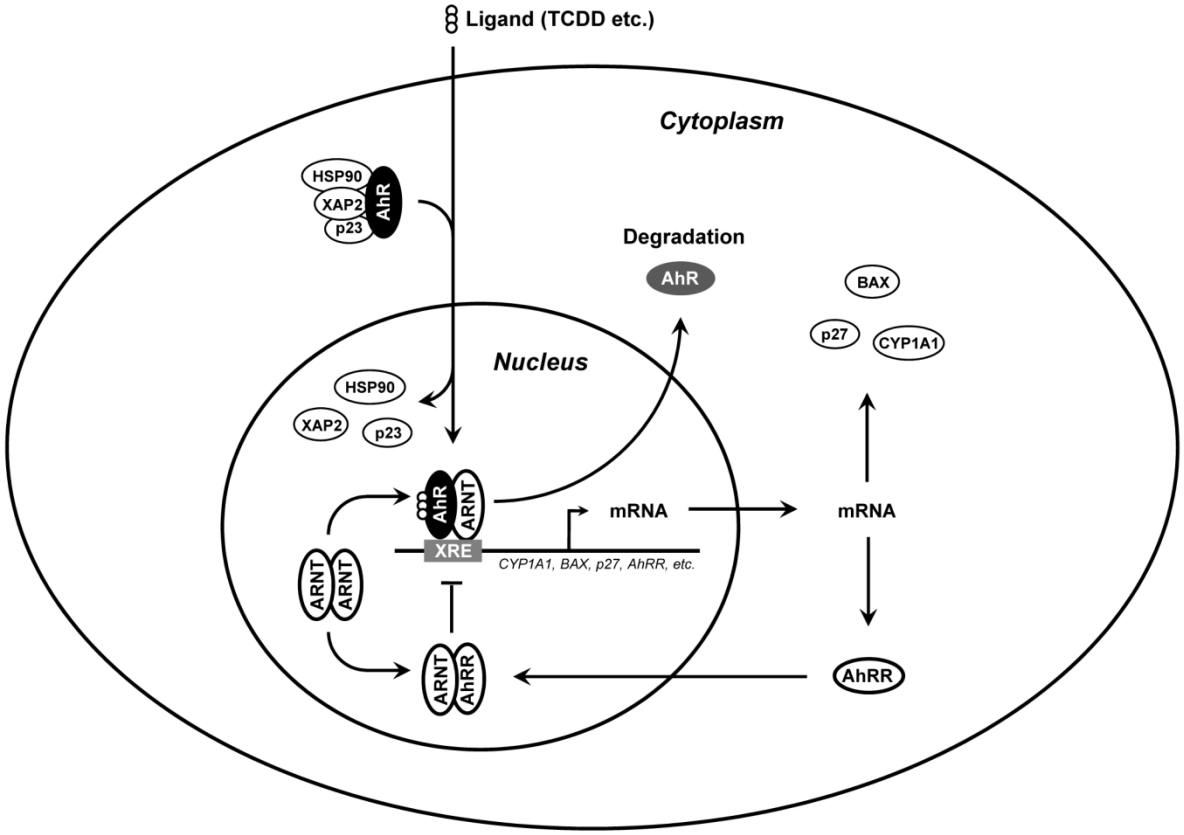

Figure 8 | **The AhR signal transduction.** The AhR translocates after ligand binding into the nucleus and heterodimerizes with Arnt. The AhR/Arnt complex binds to the XRE and induces transcription of specific target genes (adapted from Mimura and Fujii-Kuriyama)[202].

Activation of the AhR by PBDEs was suggested, and several studies showed interactions of PBDEs and the AhR signal pathway *in vitro*[204-206] as well as *in vivo*[207,208]. Yet, others found no activation of AhR target genes[209]. A recent study by Wahl *et al.* showed that low grade purified BDE-47 contaminated with furan triggered AhR activation and toxicity, while highly purified BDE-47 did not[210]. This might explain the discrepancies observed in the interactions of PBDEs and AhR signalling.

However, classical AhR ligands like dioxins cause morphological abnormalities of brains and deficits in behavior in invertebrates and vertebrates[211-213]. Therefore, it has been proposed that AhR activation causes DNT.

1.3. AIM OF THIS STUDY

Although animal studies are the gold standard in toxicology, they have limitations; and extrapolation of the data to human responses is often unsatisfactory. Thus, human *in vitro* test systems are needed to achieve better predictability of toxic responses to humans. Moreover, species differences of biological processes on the molecular level can only be investigated *in vitro*. Therefore, the following aims were pursued:

1. To establish a human and murine neurosphere culture as a cell system approach for DNT and to investigate their cell biological characteristics – with special regard to species-specific differences
2. To examine the influence of halogenated (PBDE) and polycyclic (PAH) aromatic hydrocarbons on DNT specific endpoints of hNPCs and msNPCs like proliferation, migration and differentiation
3. To elucidate the underlying mechanisms of PBDE-induced DNT in hNPCs and msNPCs

2. PUBLICATIONS

2.1. PUBLICATIONS AS FIRST AUTHOR

2.1.1. POLYBROMINATED DIPHENYL ETHERS INDUCE DEVELOPMENTAL NEUROTOXICITY IN A HUMAN *IN VITRO* MODEL: EVIDENCE FOR ENDOCRINE DISRUPTION

Timm Schreiber, Kathrin Gassmann, Christine Götz, Ulrike Hübenthal, Michaela Moors, Guido Krause, Hans Merk, Ngoc-Ha Nguyen, Thomas Scanlan, Josef Abel, Christine Rose, Ellen Fritsche

[*Environ Health Perspect* **118(4)**:572-578]

Despite extensive information on human exposure and body burden, there is no information on possible neurodevelopmental adverse effects in humans from PBDE exposure. Therefore, any potential risk for adverse nervous system effects in humans has to be extrapolated from animal studies[145]. To facilitate PBDE hazard assessment for humans, the impact of PBDEs on human neurodevelopment *in vitro* and the mechanisms underlying these changes were investigated in this study.

For these analyses, two of the most prominent congeners found in human tissues, the tetra-brominated BDE-47 and the penta-brominated BDE-99, were used. The effects of these PBDEs on the developmental neurotoxicity (DNT)-specific endpoints proliferation, migration and differentiation as well as on cell viability were investigated in a human model, which mimics brain development *in vitro*[80,82,214,215]. Furthermore, competition-studies with the thyroid hormone receptor (THR) agonist triiodothyronine (T_3) and antagonist NH-3 were performed to investigate the involvement of thyroid hormone (TH) disruption in the observed effects of PBDEs.

PBDEs (0.1 – 10 μM) were not cytotoxic for hNPCs over a period of 2 weeks. Furthermore, proliferation was not affected as well. In contrast to that, both investigated PBDE congeners significantly inhibit migration and differentiation of hNPCs in a concentration-dependent manner. Consequences of PBDE exposure for cell migration have not been investigated so far in any cell type, neither *in vitro* nor *in vivo*. Thus, this is the first report dealing with these chemicals and their ability to interfere with human progenitor cell motility. Similar to PBDE effects on neural migration, consequences of PBDE exposure for neural differentiation have not been studied so far. Therefore, this is the first report showing that this group of flame retardants can directly interfere with birth of neurons and oligodendrocytes in this human *in vitro* model.

Whether PBDEs disturb brain development by interfering with migration and differentiation not only *in vitro* but also *in vivo* needs to be investigated by appropriate animal experiments.

Competition-studies revealed that PBDE actions on migration and differentiation were completely antagonized by co-treatment of neurospheres with T_3, and simultaneous administration of PBDE and the THR antagonist NH-3 did not cause an additive effect. Thus, the two PBDE congeners BDE-47 and -99 directly disturb migration and differentiation of hNPCs *in vitro* by endocrine disruption of cellular TH signalling.

To test if long-term exposure (1 week) to PBDEs also influences calcium signalling and thus contributes to the developmentally neurotoxic effects of PBDEs in hNPC, calcium influx was measured in PBDE treated hNPCs stimulated with ATP or ACh. Neither BDE-47 nor -99 influenced the response of the cells towards these stimuli, indicating that they do not alter expression of proteins involved in Ca^{2+}-influx. Whether PBDEs interfere with downstream targets of calcium signalling in hNPCs like CaMKII or calcineurin has to be further elucidated.

In summary, BDE-47 and -99 disturb neural migration and differentiation in a human *in vitro* model for brain development by disruption of cellular TH signalling. The estimated intake of an infant is up to 4.1 μg/kg/day[135]. Assuming an average molecular weight of 500 g/mol for PBDEs, this equals an exposure of 8 nM. Moreover, within this study, it was shown that there is a 60-fold accumulation in intracellular concentration of PBDEs. This is in concert with an *in vivo* study where up to a 150-fold increase in brain tissue was measured in mice after oral exposure[153]. Therefore, infant exposure could result in a brain concentration of 0.5 – 1.2 μM. Considering that 0.1 μM BDE-99 decreases neuronal differentiation by ~ 40%, current PBDE exposure levels might be of concern for human health. Assessing subtle changes in human IQ or behavior in epidemiological studies is not trivial and needs large numbers of study subjects. Such investigations are needed to reveal if PBDEs as a hazard identified in this study actually pose a risk for human brain development *in vivo*.

2.1.2. BDE-47 and its Hydroxylated Metabolite 6-OH-BDE-47 Modulate Calcium Homeostasis in Primary Human Neural Progenitor Cells

Kathrin Gassmann*, Guido Krause*, Milou Dingemans*, **Timm Schreiber***, Josef Abel, Åke Bergman, Hans Merk, Michaela Moors, Christine Rose, Remco Westerink, Ellen Fritsche

*contributed equally

[*Environ Health Perspect*, in review]

Previous data demonstrated that high and low doses of PBDEs administered during pregnancy alter neurobehaviour of the offspring, ranging from altered motor activity over changes in sweet preference to impairment of cognitive functions[150,153,167,216,217]. Calcium (Ca^{2+}) plays an important role in a number of physiological processes, including cell proliferation, differentiation and apoptosis. Additional research demonstrated disturbances in long-term potentiation (LTP) in brain slices as well as in Ca^{2+}-homeostasis in PC12 cells[175,218]. Human neural precursor cells (hNPCs) have recently become commercially available. In this context, an *in vitro* test system for developmental neurotoxicants based on a neurosphere culture from hNPCs has already been developed. To determine the usefulness of these cells for measuring calcium signalling, the electrophysiological phenotypes of hNPCs were characterized. Additionally, acute effects of 6-OH-BDE-47 and BDE-47 on Ca^{2+}-homeostasis were examined in hNPCs to investigate if the previously observed increased activity of 6-OH-BDE-47 compared to BDE-47 is also relevant in developing human brain cells.

Physiological stimulants, like ATP, ACh and glutamate, can affect Ca^{2+}-homeostasis in primary human neural progenitor cells as shown in this study. However, application of GABA did not induce an increase in $[Ca^{2+}]_i$ in any of the hNPCs, thus suggesting the absence of functional GABA receptors. Furthermore, the PBDE congener BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 disturbed Ca^{2+}-homeostasis in primary human neural progenitor cells, and their effects appeared stronger than in PC12 cells[198]. The hNPCs responded to lower concentrations and showed bigger reactions on the same amounts of PBDE. In PC12 cells, an initial transient and a late persistent increase in $[Ca^{2+}]_i$ during exposure to 6-OH-BDE-47 could be observed, a reaction that was also seen in hNPCs. Additionally, a shift of the baseline was observed that did not occur in the PC12 cells. Possible origins for increase in $[Ca^{2+}]_i$ are influx of extracellular Ca^{2+} or release from intracellular Ca^{2+}-stores, such as ER, mitochondria and the

nucleus. To explore these possibilities, the imaging experiments were performed under calcium free conditions and in combination with the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-inhibitor Thapsigargin (TG) and the mitochondrial uncoupler FCCP.

In case of BDE-47, the removal of Ca^{2+} from external saline resulted in a dramatic decrease in the number of responding cells, which leads to the suggestion that the increase is caused by influx of extracellular Ca^{2+}. The remaining peaks were completely absent in presence of TG. In case of the hydroxylated metabolite 6-OH-BDE-47, the removal of Ca^{2+} from external saline caused a decrease in the amplitude of the initial increase in $[Ca^{2+}]_i$ and the complete loss of the late persistent increase. The shift of baseline caused by exposure to 20 μM 6-OH-BDE-47 still remained after depletion of ER Ca^{2+}-stores. As these currents did not occur in cells treated with TG and FCCP after application of 6-OH-BDE-47, it was concluded that they originated from mitochondrial Ca^{2+}-stores. However, in approximately 90% of the cells, an increase in $[Ca^{2+}]_i$ remained even after depletion of ER and mitochondrial Ca^{2+}-stores. The origin of these currents has to be elucidated in future. The combined data indicate that the initial transient increase depends on intracellular Ca^{2+}-release from the ER, whereas the shift of baseline is mainly due to Ca^{2+}-release from mitochondria. The late persistent increase in $[Ca^{2+}]_i$ seems to be caused by influx of extracellular Ca^{2+}, since it is absent under Ca^{2+}-free conditions. The underlying mechanism of the PBDE-induced disruption of Ca^{2+}-homeostasis has to be further investigated.

In summary, exposure to BDE-47 or 6-OH-BDE-47 causes an increase in $[Ca^{2+}]_i$ in hNPCs. This increase, mainly released from ER and mitochondria, occurs from concentrations ≥2 μM (BDE-47) and 0.2 μM (6-OH-BDE-47). This reveals that 6-OH-BDE-47 has a potency at least one order of magnitude higher than the parent compound BDE-47. Human exposure to hydroxylated metabolites of PBDEs results from uptake from natural sources and from internal oxidative metabolism[124]. Whereas an association between delayed human neurodevelopmental and prenatal exposure to PCBs was reported in cohort studies[99], epidemiologic evidence for a similar association of PBDEs is still missing. However, the strong Ca^{2+}-homeostasis-disrupting effect of these hydroxylated metabolites is a critical factor that should be taken into account for human PBDE risk assessment.

2.1.3. NEURAL DEVELOPMENT IN MICE AND MEN: SAME BUT DIFFERENT?

Timm Schreiber, Kim Quasthoff, Thomas Rockel, Christine Götz, Ulrike Hübenthal, Giersiefer S, Hans Merk, Ngoc-Ha Nguyen, Thomas Scanlan, Josef Abel, Ellen Fritsche

[In preparation]

Animal studies are the gold standard in toxicology and safety testing. However, due to their unsatisfactory extrapolation to humans, their use is limited. Therefore, smart human *in vitro* test systems are needed to confirm applicability of animal testings for humans. Within the last years, our group established a human neurosphere-based *in vitro* system that is apt of modeling basal processes of brain development like proliferation, migration and differentiation in the culture dish. For a direct comparison of species under the same experimental conditions, murine neurosphere cultures of two developmental stages (E16 and PND3) were set up. Murine NPCs proliferated faster than their human counterparts, although the maximum size of the spheres remained smaller than the one of human spheres. Additionally, murine NPCs migrated and differentiated faster than human cells, whereas human NPCs built more neurons and showed a longer lifespan in culture than murine ones. Moreover, migration seemed to be higher structured and was guided by radial glia only in human cultures.

Besides these general characteristics, the effects of polybrominated diphenyl ethers (PBDEs), which are known developmental neurotoxicants[86,87,145], on murine vs. human neural development were investigated. In contrast to the profound effects in hNPCs[219], PBDEs affected msNPC development to a much lesser extent and caused no inhibition of msNPC migration or neurogenesis. However, independent of developmental stage (E16 or PND3), they inhibited oligodendrocyte differentiation in a concentration-dependent manner. PBDE effects in human and mouse NPCs were rescued by co-administration of thyroid hormone (T_3), thus indicating that DNT of these compounds is mediated by endocrine disruption of cellular TH signalling. In order to determine the reason for the species discrepancy in PBDE effects, we treated spheres with T_3 or the THR antagonist NH-3. For THR activation, mouse NPC migration and differentiation were accelerated less by TH than human NPCs. On the other hand, THR antagonism by NH-3 mimicked PBDE effects, which were again greater in human compared to mouse NPCs. We suggest that this is due to different THR expression as murine spheres expressed little THRα_1 with neglectable amounts of THRβ, whereas human cells expressed higher amounts of THR α_1, β_1 and β_2. This hypothesis is supported by the observations that

THRβ induces neural differentiation[220,221], whereas THRα is more related to proliferation[222]. In summary, BDE-47 and -99 disturb neural development in human and murine neural progenitor cells by disruption of cellular TH signalling. Species-specific discrepancies in effects of PBDEs are propably due to different expression patters of thyroid hormone receptors. Accordingly, human neural progenitor cells show an enhanced sensitivity towards PBDE-induced neurotoxicity in comparison to their murine counterparts.

2.2. PUBLICATIONS AS CO-AUTHOR

2.2.1. HUMAN NEUROSPHERES AS THREE-DIMENSIONAL CELLULAR SYSTEM FOR DEVELOPMENTAL NEUROTOXIC TESTING

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[*Environ Health Perspect* **117(7)**:1131-1138]

Developmental neurotoxicity (DNT) of environmental chemicals is a serious threat to human health. In humans, it can result in learning deficits and mental retardation as well as epilepsy or schizophrenia. Current DNT testing guidelines propose investigations in rodents, which require large numbers of animals. The goal of this study was to establish a three-dimensional test system for DNT screening based on human fetal cells. Assays suited for detecting disturbances in basic processes of brain development were established by employing human neural progenitor cells (hNPCs), which grow as neurospheres. Furthermore, effects of mercury and oxidative stress on these cells were assessed.

It was shown that human neurospheres imitate proliferation, differentiation and migration *in vitro*. Whereas the hNPCs proliferated over time in culture after stimulation with EGF and FGF, they settled down under growth factor withdrawal and in presence of a poly-D-lysine/laminin matrix, and cells started to migrate radially out of the spheres. During migration, hNPCs differentiated, and $GFAP^+$, $O4^+$ and β(III)-$tubulin^+$ glial- and neuron-like cells were found. Exposure to the pro-apoptotic agent Staurosporine further suggests that they possess functional apoptosis machinery. Furthermore, hNPCs undergo caspase-independent apoptosis when exposed to high amounts of oxidative stress. The developmental neurotoxicants MeHgCl and $HgCl_2$ decreased migration distance in a dose-dependent manner. Additionally, the number of neuronal-like cells in differentiated hNPCs decreased with increasing concentrations of the mercury compounds.

In summary, human neurospheres are apt to mimic basic processes of brain development, and these processes can be modulated by developmental neurotoxicants. Thus, they are a 3D cell system that is a promising tool for DNT testing.

The author of this dissertation established the immunocytochemical stainings with the O4 antibody as a marker for oligodendrocytes and investigated the development and maturation of oligodendrocytes over time. Furthermore, the author performed several of the migration and differentiation analyses with MeHgCl and $HgCl_2$.

2.2.2. Species-Specific Differential AhR-Expression Protects Human Neural Progenitor Cells against Developmental Neurotoxicity of PAHs

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[*Environ Health Perspect*, in revision]

Persistent organic pollutants (POPs) pose a risk of causing adverse effects on human health and the environment. The main substance classes are polycyclic aromatic hydrocarbons (PAHs), like 3-methylcholantrene (3-MC) and benzo(a)pyrene (B(a)P), dioxins, like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and PCBs. *In utero* exposure to PAH through maternal cigarette smoking, PCBs and/or dioxins results in cognitive deficits in children[1,223-230]. These deficits are thought to involve the POP-activated aryl hydrocarbon receptor (AhR). AhR ligands cause morphological abnormalities of brains or deficits in cognition and behavior in several species[211-213]. If AhR activation is the underlying mechanism for reported DNT effects after POP exposure in humans is not known. Therefore, the effects of PAHs on murine and human neural development were investigated.

Although there was no disparity in human neurospheres between controls and spheres exposed to 3-MC, TCDD or MNF, there was a significant inhibition of msNPC proliferation after MNF exposure. These results were confirmed by FACS analyses for DNA content using propidium iodine-staining. Subsequently, the influence of POPs on migration was examined; independent of exposures, all human neurospheres adhered to the matrix, and cells migrated out of the sphere in the same manner. In contrast to the results in hNPCs, AhR activation by 3-MC and B(a)P reduced msNPC migration distance. Because of these observed differences between species, AhR and Arnt mRNA expression was determined by real-time RT-PCR analyses. Comparison of gene expression between human and mouse NPCs showed that genes of AhR signalling and AhR gene battery were generally expressed in higher copy numbers in msNPCs than in hNPCs. Moreover, AhR mRNA copies were close to detection limit, and AhR protein was absent in hNPCs.

In summary, we showed that, in contrast to mouse, human neurospheres are protected against PAH-induced DNT due to absence of AhR. An accumulating body of evidence indicates that AhR signalling in humans is less functional than in most laboratory animals. This should be taken into account for human risk assessment of TCDD and related xenobiotics.

The author of this dissertation established the murine neurosphere culture and verified the experimental condition for analyses of these spheres.

3. DISCUSSION

3.1. NEUROSPHERES AS A MODEL FOR DNT

3.1.1. HUMAN NEUROSPHERES

In humans, DNT leads to learning deficits and mental retardation[231,232]. To investigate the DNT potential of chemicals, a three-dimensional human neurosphere system was established and characterized. This *in vitro* test system mirrors the basic processes of brain development, namely proliferation, migration, differentiation and apoptosis.

After stimulation with EGF and FGF, hNPCs proliferated over time in culture, and increase in sphere size was dependent on EGF concentration in media. Cultivation without growth factors as negative control did not change size. Additionally, FACS analyses of propidium iodine-stained neurosphere single-cell suspensions revealed that approximately 2.7% of the population showed a typical cell cycle distribution for proliferating cells[214]. This corresponds to findings of Reynolds and Rietze, who showed by a single-cell clonogenic assay that 2.4% of neurosphere cells were capable of proliferation[233]. Moreover, indirubin, a G_2/M blocking agent, increased the number of proliferating cells, whereas withdrawal of growth factors resulted in G_1 arrest[214]. After mitogen withdrawal, the spheres attached to a poly-D-lysine/laminin matrix, and cells started to migrate out of the spheres. Real-time phase-contrast microscopy revealed that cells migrated radially as well as tangentially and connected and disconnected to the surrounding cells[214]. The cues for this behavior *in vitro* are so far unknown. *In vivo* directed migration is guided by chemical gradients and direct cell-cell interactions. However, the distance that hNPCs migrated over time was highly robust and reproducible, and they also differentiated into the three major cell types of the brain, neurons, astrocytes and oligodendrocytes. Moreover, cells lost nestin expression over time. After 2 days of differentiation, a ratio of approximately 10% neuronal and 90% glial cells was observed[214], which mimics the physiologic distribution of cells in human brain[235].

To establish an *in vitro* test system that identifies DNT potential of chemicals, the known developmental neurotoxicant mercury (Hg) was used[1]. The treated neurospheres mimicked the effects of Hg *in vitro* that were identified in human brain[236]. Organic (MeHgCl) and inorganic ($HgCl_2$) mercury decreased migration distance of hNPCs[214]. During development, mercury poisoning caused microencephaly and brain disorganization by disturbances in cell proliferation and migration[231,235]. Moreover, Hg exposure caused an increased glia/neuron ration in the neurospheres[214]. These effects were also observed in postmortem brains, indicated by a decreased number of neurons[95,236]. In contrast to Hg, cAMP, a well-described compound for inducing neuronal differentiation[237], caused an increase in migration distance and number of neurons in

differentiated hNPCs[214,238]. Additionally, stimulation with T_3 caused a significant increase in number of oligodendrocytes[82]. These data demonstrate the dynamic abilities of the cell system.

Oxidative stress induces apoptosis in many different cell types and thus results in developmental brain pathology or neurodegenerative diseases[50]. Therefore, ROS-induced programmed cell death was examined in hNPCs. While Staurosporine induced caspase-dependent apoptosis in human NPCs, H_2O_2 caused caspase-independent apoptosis revealed by $TUNEL^+$ cells without caspase-3/-7 activation[214]. This corresponds to findings by Dare *et al.* that H_2O_2 leads to caspase-independent apoptosis in primary rat CGN[239]. Additionally, comparison between hNPCs and the human neuroblastoma cell line SH-SY5Y showed that hNPCs were less sensitive towards oxidative stress[214], which is in concert with the knowledge that primary cells are less sensitive to various stresses than cancer cells[240,241].

In summary, human neurospheres mimicked basic processes of brain development, like proliferation, migration, differentiation and apoptosis. These processes were highly robust and reproducible and can thus be quantified *in vitro*. Moreover, the *in vivo* effects of the developmental neurotoxicant Hg were mirrored *in vitro*, and the methods applied are suitable for medium-throughput screenings. Thus, human neurospheres offer a method for DNT hazard identification. However, neurospheres do not simulate higher brain structures and functional networking, which is why their applicability is limited.

3.1.2. SPECIES-SPECIFIC DIFFERENCES

For detection of disturbances of brain development by chemicals, animal studies are the gold standard in toxicology. However, the predictability of animal experiments for human health effects is often unsatisfactory due to species differences. It has been estimated that up to 40% of potential drugs fail during early clinical trials because of unsuitable pharmacodynamic features[242,243]. Additionally, an international validation study compared results from toxicity studies in animals with information from poisoning centers and showed a coefficient of correlation of 0.56 between the LD_{50} in rats and lethal concentrations of the same chemicals in human blood[243]. Therefore, the European REACH legislation demands that alternative human *in vitro* models are needed to identify chemicals that are hazardous to human health. As a response, human cell culture models have been developed that are suitable to identify these chemicals. One problem of these human-based *in vitro* models is the interpretation of data. When *in vitro* results differ from animal data, it is important to distinguish whether there are species or *in vitro – in vivo* differences. One way to address this problem is a comparison of the same *in vitro* system from animals and humans. Therefore, we did not only establish a human test system for DNT testing

but added a murine neurosphere culture, which than allows extrapolation for humans via comparison of murine *in vitro* to *in vivo* results from animal testings. This will decrease the uncertainty associated with animal to human extrapolation.

Although the basic processes of neural development were the same in murine and human spheres, they also showed differences. After NPC preparation (mouse) or defrosting (human), neurospheres were formed within several days, which proliferated after stimulation with EGF and FGF over time. Murine neurospheres (E16 and PND3) proliferated significantly faster than their human counterparts, although the maximal size of the spheres remained smaller than the one of human spheres. Moreover, murine NPCs migrated and differentiated faster than human cells. In contrast, human spheres built more neurons and showed a longer lifespan than murine ones. Additionally, migration seemed to be higher structured and guided by radial glia (RG) only in human cultures. Latest research revealed a new population of radial glia-like cells in the outer subventricular zone (OSVZ) of the developing cortex that is unique for humans[244]. These cells were termed OSVZ radial glia-like cells (oRGs), and they may distinguish cortical growth in humans from that in other species. Further characterization of RG in human neurospheres should shed more light on species-specific differences in brain development.

Summarizing one can say that murine neural progenitor cells develop faster than their human counterparts and mimic species-specific differences *in vitro*. This is the first report that compared murine and human brain development under identical experimental conditions.

3.2. POLYCYCLIC AROMATIC HYDROCARBONS

Some of the uncertainties associated with animal to human extrapolation concern persistent organic pollutants (POPs). POPs bioaccumulate through the food chain and are of concern due to their lipophilicity; they cross the human placenta and possibly affect human neural development. The majority of POPs are known AhR ligands and activators of AhR signalling. Therefore, it has been proposed that AhR activation causes developmental neurotoxicity (DNT). A relationship between AhR activation and morphological abnormalities of brains or deficits in cognition and/or behavior was shown in several species[211-213,245]. If AhR activation is the underlying mechanism for the reported DNT effects after POP exposure in humans is not known.

We showed that msNPC proliferation was completely blocked by AhR antagonists, whereas proliferation of hNPCs was unaffected[246]. Nevertheless, spheres generated from AhR knockout mice proliferated at the same speed than their wildtype counterparts, thus indicating that the AhR is not perquisite for NPC proliferation. This is in contrast to findings in human liver and

neuroblastoma cells, where exogenous AhR activation inhibits cell proliferation[247,248]. On the contrary, AhR activation causes increased proliferation in human MCF breast cancer cell line[249]. In the murine hepatoma cell line 1c1c7, AhR activation inhibits proliferation[250]. However, mouse embryonic fibroblasts from AhR knockout mice grow more slowly than wild-type cells[251]. Thus, AhR effects on proliferation are cell type- and species-dependent, and cell type specificity is not consistent throughout species.

Similar results were obtained for NPC migration; whereas AhR stimulation caused an inhibition of migration in murine cells, human cells remained unaffected. A number of studies showed that modulation of cell migration by AhR ligands differs between cell types and species[252-255]. To explore the underlying mechanisms for the observed species-specific differences, the expression of genes was investigated that belong to the AhR machinery as well as genes that are AhR-regulated. Human and mouse NPCs expressed AhR, Arnt, AhRR, CYP1B1 and c-Myc, whereas quantifiable amounts of cyp1a1 were only found in msNPCs. Interestingly, it appeared that copy numbers of AhR and Arnt in human cells were close to detection limit and 8- to 100-fold lower than in msNPCs. Moreover, induction of cyp1a1 and 1b1 by AhR ligands was only seen in murine cells[246]. These findings are in concert with a study by Abbott *et al.*, where similar differences between mice and humans were reported[256].

Thus, it was shown that human neurospheres are protected against PAH-induced DNT due to absence of the AhR, which should be taken into account for risk assessment of POPs.

3.3. POLYBROMINATED DIPHENYL ETHERS

3.3.1. EFFECTS OF PBDES ON NEURAL DEVELOPMENT

Human exposure to brominated flame retardants is of concern because PBDEs impair neurodevelopment in animals and humans[86,87,257]. However, the mechanisms by which PBDEs interfere with human brain development are inscrutable. To shed light upon the consequences that PBDE exposure causes to developing brain cells, their effects on the development of hNPCs and msNPCs were studied *in vitro*.

PBDEs (0.1 – 10 μM) were not cytotoxic for proliferating or differentiating hNPCs over a period of 2 weeks as demonstrated by different independent methods[219]. This is in agreement with findings in msNPCs (E16 and PND3), where PBDEs caused no cytotoxicity over a period of 4 days[260], and to findings of other groups in rodent and human cells. The technical PBDE mixture DE-71 caused cell death in rat cerebellar granule cells only in concentrations >20 μM[192], while BDE-47 induced cytotoxicity in rat hippocampal neurons at a concentration of 41.2 μM[195].

Furthermore, BDE-99 induced cell death in human astrocytoma cells only in concentrations >25 μM[191]. In contrast to these findings, BDE-47 was reported to be cytotoxic for human neuroblastoma cells in concentrations >5 μM[196]. Moreover, the higher brominated BDE-209 (>10 μM) was toxic to human hepatoma cells HepG2[193]. Reason for cytotoxicity in all these different cell models was induction of apoptosis. Giordano *et al.* demonstrated that DE-71 exposure induces oxidative stress[258]. That this production of reactive oxygen species (ROS) was responsible for DE-71-induced apoptosis of rat cerebellar granule neurons (CGN) became obvious by showing that intracellular glutathione (GSH) content was the most important determinant of CGN susceptibility to DE-71 neurotoxicity. Transgenic rat neuron-astrocyte co-cultures with proficient vs. deficient GSH synthesis supported these findings as astrocytes rich in GSH protected neurons against DE-71-induced neurotoxicity, while astrocytes with poor GSH content did not[259]. Protection of neurons against PBDE-induced cytotoxicity by presence of astrocytes is thus the probable reason for hNPC insensitivity towards PBDE-dependent cell death. Therefore, such a human co-culture system like differentiated neurospheres, where the three major cell types of the brain are present in 'physiological' ratios, seems to be a superior *in vitro* method for assessing hazards of chemicals to humans compared to simple monolayer cell lines.

Human or murine neurosphere proliferation assessed by monitoring of sphere diameter[214] was also not affected by BDE-47 or -99[219,260]. This is in agreement with results from a T-screen assay[259], a functional assay based on T_3-dependent cell proliferation of the rat pituitary tumor cell line GH3, where no effects of these two congeners on proliferation were observed either[262]. In contrast, one of the hydroxylated BDE-47 metabolites, 2-OH-BDE-47 (5 – 10 μM), inhibited proliferation of the H295R adrenocortical carcinoma cell line[263]. However, it has to be considered that this concentration of reactive metabolite causing an antiproliferative effect in these cells is high, and the authors did not investigate effects of the parent compound. Inhibition of proliferation by BDE-47 was also seen in 5L rat hepatoma cells. The authors demonstrated that, rather than BDE-47 itself, AhR activation by the BDE-47 contaminant 1,2,3,7,8-pentabromo-dibenzofuran was responsible for the effects on proliferation[210]. This was confirmed by the observation that highly purified BDE-47 does not stimulate the AhR[209]. Therefore, we ensured that the PBDEs used in this study were contaminant-free. Increased proliferation was observed in DE-71-treated MCF-7 breast cancer cells. This stimulation was estrogen receptor-dependent, and thus, DE-71 acts as an endocrine disruptor in this estrogen receptor-positive cell line[264]. Hence, interaction of PBDEs with cell proliferation seems to be congener- and cell type-specific. So far, all data available on this topic have been obtained in tumor cells. This is the first work

employing normal human and murine stem/progenitor cells for determining effects of PBDEs on cell proliferation.

In contrast to proliferation, both investigated PBDE congeners inhibited migration and differentiation of hNPCs significantly in a concentration-dependent manner[219]. Consequences of PBDE exposure for cell migration have been investigated neither *in vitro* nor *in vivo* so far, so this is the first report showing that these chemicals have the ability to interfere with human progenitor cell motility. However, a recent proteomics study by Alm *et al.* suggests that PBDE exposure might cause disturbances in cell motility also *in vivo*[188]. In this work, a single dose of BDE-99 given on mouse PND 10 caused changes in brain protein expression after 24 hrs, and one third of those proteins was related to the cytoskeleton, including actin. The importance of the actin cytoskeleton for neuronal migration has been reviewed extensively[265], leaving room for the speculation that PBDEs might interfere with migration through alteration of cytoskeleton-related protein expression. In contrast to the findings in hNPCs, PBDEs caused no inhibition of msNPCs migration[260]. This negative finding is astonishing as the proteomic study by Alm *et al.*, which was also done in mice, clearly shows dysregulation of protein expression participating in migration. However, PBDE effects on migration *in vivo* were not analyzed in this study. Moreover, our mouse *in vitro* data stem from E16 and PND3 mice. Thus, *in vivo* migration studies and *in vitro* investigations in NPCs generated from PND10 mice have to be performed to finally elucidate this matter.

Similar to PBDE effects on neural migration, consequences of PBDE exposure for neural differentiation have not been studied so far *in vitro* or *in vivo*. Therefore, this is the first report showing that this group of flame retardants can directly interfere with birth of neurons and oligodendrocytes in this human *in vitro* model. Neurogenesis was the most sensitive endpoint with a LOAEL for BDE-99 of 0.1 μM and a NOAEL of 10 nM. In contrast, msNPC neuronal differentiation was not affected by PBDEs up to a concentration of 10 μM. Contrarily, oligodendrogenesis was affected in both human and murine neurospheres. In hNPCs, oligodendrocyte differentiation was inhibited significantly at concentrations starting from 1 μM BDE-47 or -99, whereas in msNPCs, a significant inhibition was observed only after exposure to 10 μM BDE-99[260]. Thus, human NPCs seem to be more sensitive towards PBDE-induced disturbances of cell differentiation than their murine counterparts. There are two more studies identifying mechanisms of species-specific differences towards neurotoxic substances *in vitro*. One describes species-dependent modulation of copper-mediated neurotoxicity by amyloid precursor protein (APP) in *Caenorhabditis elegans*[266]. Cu toxicity induced by APP was dependent on conservation of histidine residues at positions corresponding to 147 and 151 of human APP. In the second one, we showed that in contrast to mouse, human neurospheres were protected

against PAH-induced DNT due to absence of AhR. These data indicate that *in vitro* studies identifying molecular mechanisms of species-differences in susectibility towards neurotoxicants might be valuable tools for risk assessment.

Whereas these effects were seen at micromolar concentrations of PBDEs, a study by Mundy *et al.* indicated that concentrations present in medium underestimate tissue concentrations by up to two orders of magnitude[267]. Therefore, we measured intracellular PBDE concentration employing ^{14}C-BDE-47. After 2 and 7 days of differentiation, PBDE accumulation in human NPCs was approximately 45- and 60-fold respectively. BDE-47 accumulation in murine NPCs was in a similar range of approximately 35-fold after 2 days of differentiation. These data reflect PBDE accumulation in PND10 and 19 mouse brains after 1 and 7 days *in vivo* (20- to 150-fold), which was calculated from a study of Viberg *et al.*[153]. Other lipophilic substances, like methylmercury and PCBs, support the accumulation factors of PBDEs *in vitro* and *in vivo*; as in three-dimensional cellular systems (e. g. brain slices), accumulation of these compounds is comparable to accumulation *in vivo*[268].

In the past, it was hypothesized that the developing mouse brain is especially vulnerable during a defined period of development, the so-called brain growth spurt (BGS). The BGS occurs in the neaonate, spanning the first 3 – 4 weeks, and is characterized by axonal and dendritic outgrowth[269]. The enhanced sensitivity during this period is reflected by a study of Xing *et al.*, who showed that postnatal mice (exposure from mother milk during lactation) were more susceptible to PBDE-induced changes in synaptic plasticity than embryonic mice (exposure during pregnancy)[270]. We did not find increased susceptibility towards PBDEs in earlier neurosphere development (E16 vs. PND3) *in vitro*. This might be due to two independent mechanisms. We investigated the effects of PBDEs on cell migration and differentiation, whereas Xing and colleagues showed that PBDEs inhibited the long-term potentiation (LTP) of excitatory postsynaptic potential (EPSP) in adult mice exposed during development. If PBDEs also affect EPSP in differentiated neurospheres needs to be investigated in future.

3.3.2. Disturbances of Calcium Homeostasis

Disruption of calcium balance might contribute to PBDE-induced disturbances of NPC development as Ca^{2+}-signalling is a key player in developmental processes[41,271], and it is known that PBDEs disturb calcium homeostasis *in vivo* and *in vitro*[145]. Additional research demonstrated disruption of long-term potentiation (LTP) in brain slices caused by BDE-47[198,218]. Furthermore, it was shown in a recent study that the congener BDE-99 caused an increase in $[Ca^{2+}]_i$ in PC12 cells[197]. Moreover, the effects on calcium homeostasis were stronger for the hydroxylated

metabolite 6-OH-BDE-47 than for the parent compound BDE-47[198]. The maintenance of the intracellular Ca^{2+}-homeostasis is crucial for normal cell functions. Thus, disruption of Ca^{2+}-homeostatic processes may induce production of reactive oxygen species (ROS) and affect neurotransmitter release, activation of phosphokinases, phosphatases and phospholipases, protease activity, apoptotic processes and other Ca^{2+}-dependent enzyme activities such as nitric oxide synthase (NOS). Moreover, Ca^{2+} determines LTP and synaptic plasticity and is hence involved in learning and memory[272]. Therefore, within this study, we tested if BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 disturb intracellular Ca^{2+}-homeostasis of NPCs as one mechanism causing DNT in hNPCs.

$[Ca^{2+}]_i$ of NPCs was modulated by ATP, ACh and glutamate. Thereby, ATP and ACh evoked Ca^{2+}-transients independent of differentiation times (12, 24 or 48 hrs in culture) and application form (bath or puff application). However, glutamate caused increases in $[Ca^{2+}]_i$ at early differentiation time points (12 hours) in a significantly smaller number of responding cells than at later time points (24 – 48 hours)[273]. In contrast to this, cells did not respond to high extracellular potassium. These results indicate the presence of functional receptors for ATP, ACh and glutamate, while voltage-gated Ca^{2+}-channels were either not expressed or not functional. Additionally, the most important inhibitory transmitter in the adult CNS, GABA, did not induce an increase in $[Ca^{2+}]_i$, thus suggesting the absence of functional GABA receptors. These findings show in summary that hNPCs provide a suitable model to investigate the effects of some, but not all chemicals on $[Ca^{2+}]_i$[272]. Lack of responses towards potassium and GABA might be due to relatively little maturation of the human cultures as modifications of ligand-induced Ca^{2+}-responses by age in neural cell models were already described[274-276].

Exposure to the PBDE congener BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 resulted in a disturbed Ca^{2+}-homeostasis in primary human neural progenitor cells. 6-OH-BDE-47 caused disturbances of $[Ca^{2+}]_i$ at a lower concentration than the parent compound, as it was also seen in the rat pheochromocytoma cell line PC12[198]. However, in hNPCs, the effects of BDE-47 and its hydroxylated metabolite appeared stronger than in PC12 cells. Acute exposure of hNPCs to concentrations as low as 2 μM BDE-47 or 0.2 μM 6-OH-BDE-47 resulted in a significant increase in $[Ca^{2+}]_I$, whereas in PC12 cells, rather high concentrations of 20 μM BDE-47 and 1 μM 6-OH-BDE-47 were needed to evoke similar increases in $[Ca^{2+}]_i$[175,198]. In both cell models, 6-OH-BDE-47 provoked an initial transient and a late persistent increase in $[Ca^{2+}]_i$. Additionally, hNPCs responded with a shift of baseline[273]. Recent studies demonstrated that high concentrations of different PBDE congeners and structurally related non-planar PCBs increased $[Ca^{2+}]_i$ in cultured neuronal cells and brain preparations from rodents[277]. Rosin and Martin were the first to show that mixtures of PCBs alter the uptake processes of Ca^{2+} in cell preparations[277].

Moreover, PCBs and PBDEs induce entry of Ca^{2+} into different neural cell types[278-282] and disrupt Ca^{2+}-homeostasis in rodent microsomes isolated from different brain regions[283]. PBDEs also affect Ca^{2+}-homeostasis in the human neuroblastoma cell line SH-SY5Y[284]. In comparison to PCB/PBDE effects in all these different models, hNPCs seem to be the most sensitive cells with regard to disturbances of $[Ca^{2+}]_i$ homeostasis.

Possible origins for increase in $[Ca^{2+}]_i$ are influx of extracellular Ca^{2+} by voltage-gated Ca^{2+}-channels (VGCCs)[282] or store-operated Ca^{2+}-entry (SOCE) channels[285] or release from intracellular Ca^{2+}-stores, such as endoplasmic reticulum (ER), mitochondria and the nucleus. Furthermore, the loss of membrane integrity can result in an unspecific increase in $[Ca^{2+}]_i$, which was overruled in this case because no increase in LDH-activity in the media was measured. In case of BDE-47, removal of external Ca^{2+} caused a significant decrease in number of responding cells. On the contrary, for 6-OH-BDE-47, removal of extracellular Ca^{2+} resulted in a decrease in the amplitude of the initial increase in $[Ca^{2+}]_i$ and the complete loss of the late persistent increase. Therefore, it can be concluded that these increases originate completely (late increase) or partly (initial increase) from influx of extracellular Ca^{2+}. Generally, the PCB- and PBDE-induced effects on Ca^{2+}-homeostasis have been attributed to influx of extracellular Ca^{2+}. PCB-induced influx of extracellular Ca^{2+} requires L-type VGCCs as well as activation of excitatory glutamate and GABA receptors[281,282]. As hNPCs do not possess functioning VGCCs and GABA receptors, this mechanism can not be involved in the PBDE-induced increase in $[Ca^{2+}]_i$. Although it is possible that SOCE channels are involved, the specific channels mediating the PBDE-induced increase in $[Ca^{2+}]_i$ remain to be identified.

The remaining BDE-47-induced peaks in Ca^{2+}-free conditions were completely absent in presence of Thapsigargin (TG), an inhibitor of ER Ca^{2+}-channels. This is in agreement with the findings in PC12 cells[198], where the initial increase is also absent after TG treatment. Activation of inositol 1,4,5-triphosphate receptor (IP_3R) and/or ryanodine receptor (RyR) is involved in the principle mechanisms affecting release of Ca^{2+} from ER stores. A PCB-induced sensitization of the ryanodine receptor complex in rat brain was shown in several studies[276,287,288]. To investigate if Ca^{2+}-efflux from the ER is triggered by activation of the RyR in hNPCs, neurospheres were stimulated with the direct RyR agonist 4-chloro-m-cresol (CMC) and the receptor sensitizer caffeine. Puff application of 500 µM CMC or 20 mM caffeine did not elicit any increases in $[Ca^{2+}]_i$ in neurospheres after different differentiation times (data not shown). Thus, the RyR seemed not to be involved in PBDE-induced Ca^{2+}-release. PCBs also induce a release of Ca^{2+} from inositol triphosphate (IP_3)-sensitive Ca^{2+}-stores from ER[289], but the involvement of the IP_3R was not investigated in this work and has to be elucidated in future.

After depletion of ER Ca^{2+}-stores, the shift of baseline caused by exposure to 20 μM 6-OH-BDE-47 still remained in hNPCs. As these currents disappeared in cells co-treated with TG and the mitochondrial uncoupler FCCP, we concluded that they originate from mitochondrial Ca^{2+}-stores. However, in approximately 90% of the cells, an increase in $[Ca^{2+}]_i$ remained even after depletion of endoplasmic and mitochondrial Ca^{2+}-stores[273]. Furthermore, in PC12 cells treated with TG and FCCP, no more increase in $[Ca^{2+}]_i$ occurred after treatment with 6-OH-BDE-47[41,197]. The underlying mechanism of these currents has to be elucidated in future. Possible mechanisms are inhibition of endoplasmic and mitochondrial Ca^{2+}-ATPases, mobilization of Ca^{2+} from ER through interactions with IP_3R and sigma receptor or disruption of plasma, endoplasmic and mitochondrial membranes.

To test if long-term exposure (1 week) to PBDEs also influences calcium signalling and thus contributes to the developmentally neurotoxic effects of PBDEs in hNPC, calcium influx in PBDE treated hNPCs stimulated with ATP or ACh was measured. Neither BDE-47 nor -99 influenced the response of the cells towards these stimuli, thus indicating that they do not alter expression of proteins involved in Ca^{2+}-influx[219]. Recently, it was shown that BDE-203 and BDE-206 increased CaMKII expression in mouse hippocampus[158]. If PBDEs interfere with downstream targets of calcium signalling in hNPCs like CaMKII or calcineurin has to be further investigated.

The combined data indicate that the initial transient increase depends on intracellular Ca^{2+}-release from the ER, whereas the shift of baseline is mainly due to Ca^{2+}-release from mitochondria. The late persistent increase in $[Ca^{2+}]_i$ seems to be caused by influx of extracellular Ca^{2+}, since it is absent under Ca^{2+}-free conditions.

3.3.3. ENDOCRINE DISRUPTION

During normal development, TH guides neural migration and maturation of neural and glial cells[290,291]. Therefore, hypothyroidism during development causes a large number of neuroanatomical and behavioral effects[292-294]. Due to similar neurobehavioral alterations observed after PBDE exposure in rodents, endocrine disruption of the TH system by PBDEs has been studied intensively. Hypothyroidism of dams and/or offspring was found in a variety of different studies after pre- or postnatal PBDE exposure[145]. However, behavioral toxicity of BDE-47 has been observed without alterations in serum T_4 and T_3 levels as well, thus suggesting that PBDEs cause toxicity by a mechanism beyond changes in body TH homeostasis[295,296]. These findings might be explained by the data generated in this work, as the two PBDE congeners BDE-47 and -99 directly disturbed migration and differentiation of NPCs *in vitro* by endocrine disruption of

cellular TH signalling. This was confirmed by two observations: (i) PBDE actions were completely antagonized by co-treatment of neurospheres with T_3, and (ii) simultaneous administration of PBDE and the THR antagonist NH-3 did not cause an additive effect[218]. However, reporter gene analyses in THRα and β overexpressing Chinese hamster ovary cells (CHO) revealed that both PBDE congeners did not act as agonists or antagonists of THRα or β[297]. One possible reason for discrepancies seen between this work and results in that overexpression system might be that PBDEs act via disturbance of recruitment of THR co-factors. In primary cells, receptors, co-factors and responsive elements are present at a fine-tuned equilibrium, which is not the case in transfected cells, which overexpress only certain players of the machinery.

That nuclear hormone receptor co-factors might be crucial in endocrine disruption by polyhalogenated aromatic compounds was already discussed in a previous work of our group where it was found that the non-coplanar PCB118 induced oligodendrocyte differentiation in hNPCs[82]. This *in vitro* work represented the *in vivo* finding that Aroclor treatment led to an increased expression of TH-dependent genes such as RC3/neurogranin and myelin basic protein (MBP) in fetal rat brains[298]. An equivalent rodent study employing PBDEs has not been performed yet. Only in fish, the fathead minnow (*Pimephales promelas*), TH disruption on the basis of THR-dependent gene expression was observed after BDE-47 exposure[299].

Interference of PBDEs with the TH-regulated basic transcription element-binding protein (BTEB) was suggested as a possible mode of action of PBDE toxicity. However, a study by Denver *et al.* showed that T_3 upregulates BTEB in primary neurons and astrocytes, but not in neonatal oligodendrocytes[300]. If BTEB is involved in oligodendrocyte precursor cell (OPC) performance has not been studied so far. Another possible candidate for a PBDE target molecule causing the effects seen in neurosphere development is brain-derived neurotrophic factor (BDNF), whose protein levels were altered after PBDE exposure in developing mouse brains[156]. BDNF belongs to the TH regulated genes[301,302], increases proliferation of rodent OPCs[303] and is related to migration and differentiation of oligodendrocytes and neurons as well[190,304,305]. If BDNF is involved in disturbances of migration and differentiation of neurospheres has to be elucidated.

Moreover, BDE-99 increases protein expression of GAP-43 in developing rat brains[188]. GAP-43 is expressed in OPCs, and its expression is rapidly downregulated during maturation of oligodendrocytes[306]. It can be speculated that the increase in GAP-43 in rodent brains after PBDE treatment might be related to a delay in oligodendrocyte differentiation and therefore corresponds to the findings in murine neurospheres. Whether GAP-43 also determines migration and/or differentiation of NPCs is not known.

Human NPCs showed an enhanced sensitivity towards neurotoxic effects of PBDEs compared to murine cells. As these effects are supposed to be mediated through disruption of TH signalling, we investigated the effects of thyroid hormone receptor agonism by T_3 and antagonism by NH-3 on development of human and murine NPCs. Like the PBDEs, THR modification caused stronger effects in human than in murine neurospheres. Thus, this is the first report showing that human and murine NPCs show different sensitivity towards THR modulation[260]. Because it is well described that humans are born with a more matured thyroid system[39], the neurosphere assay reflects these species-specific developmental differences seen *in vivo* in the culture dish. To explore these species-specific differences in TH signalling, the expressions of THRs in neurospheres were investigated. Murine spheres expressed little THRα_1 and hardly any THRβ, whereas human cells expressed comparatively high amounts of THR α_1, β_1 and β_2. Although NH-3 binds to THRα as well as to THRβ, it has a higher affinity to THRβ[307]. As inhibition of THRα leads to decreased proliferation of avian neurogenic precursors[222] (but PBDEs did not interfere with NPC proliferation), and as the induction of THRβ induces neural differentiation[220,221] (and PBDEs disturbed neural differentiation of NPCs), it is likely that PBDEs interfere with THRβ signalling of human NPCs. This is supported by the observation that mouse NPCs hardly express any THRβ, and neurogenesis is not impaired by PBDEs in this system. As murine oligodendrogenesis is affected by PBDEs, it is possible that PBDEs also interfere with the THRα_1, which is known to be expressed in proliferating OPCs[308]. It can be assumed that disturbances of TH signalling through THRα might specifically lead to inhibition of OPC proliferation and therefore result in a lower number of $O4^+$ cells. Due to the low number of oligodendrocytes in neurospheres (approximately 5% of all cells), this is not measurable in our proliferation assay. The precise mechanism of PBDE interference with oligodendrogenesis has to be determined prospectively.

Thus, human NPCs are more receptive to PBDE-induced DNT due to their matured thyroid system.

3.3.4. Perspective on Risk Assessment of PBDEs

In summary, BDE-47 and -99 disturb neural migration and differentiation of hNPCs by endocrine disruption of cellular TH signalling *in vitro*. For human neurospheres, neurogenesis was the most sensitive endpoint with a LOAEL of 0.1 µM and a NOAEL of 10 nM. The NOAEL for murine neurospheres was actually 10 µM with no LOAEL determined. For murine NPCs, oligodendrogenesis was the most sensitive endpoint with a NOAEL of 1 µM and a LOAEL of 10 µM. In contrast to that, the NOAEL for oligodendrogenesis in human spheres was 0.1 µM,

and the LOAEL was 1 μM. Additionally, exposure to BDE-47 or 6-OH-BDE-47 caused an increase in $[Ca^{2+}]_i$ in hNPCs. This increase, mainly due to release of calcium from ER and mitochondria, occured from concentrations ≥2 μM (BDE-47) and 0.2 μM (6-OH-BDE-47). This reveals that 6-OH-BDE-47 has a potency at least one order of magnitude higher than the parent compound BDE-47. Human exposure to hydroxylated metabolites of PBDEs results from uptake from natural sources as well as from internal oxidative metabolism[170]. Furthermore, a recent study by Qiu *et al.* showed that approximately 45% of detectable PBDEs are present as metabolites in human fetal blood. In maternal blood, PBDE metabolites represent only 20% of all PBDEs[174]. The contribution of hydroxylated PBDE metabolites to DNT is so far unknown, and their potential to alter neurobehavior has to be investigated in future. However, their ability to interfere with $[Ca^{2+}]_i$-homeostasis is cause for concern.

For PBDEs, neurobehavioral disturbances in murine pups were already observed after administration of a single oral dose in the range of μg/kg body weight (bw). In a study by Kuriyama *et al.*, a single oral dose of 0.7 mg/kg bw led to increased motor activity[158], and application of 0.6 mg/kg bw caused hyperactivity in newborn mice[160]. Disturbances of learning and memory could be observed after a single oral dose of 0.9 mg/kg bw[158]. Estimated PBDE exposure of an infant from North America through breast-feeding is about 0.3 μg/kg bw/day, with a range of 0.003 - 4.1 μg/kg bw/day[135]. Therefore, the estimated median intake of an infant is 2000 times lower than the concentrations leading to neurobehavioral changes in mice, whereas the maximal intake is only 150 times lower. Regarding the NOAEL of the animal testings (0.45 mg/kg bw)[153] and a safety factor of 10 for interspecies extrapolations and 10 for intraspecies differences, a tolerable daily intake (TDI) of 4.5 μg/kg bw/day can be derived, which lies well above the maximum intake of an infant and does not lead to concern.

However, within this study, we showed that human neural progenitor cells are much more sensitive to PBDEs than their murine counterparts due to differences in thyroid hormone receptor expression. The NOAEL for effects of BDE-99 on neurogenesis was 1000 times lower in human cells than in murine cells. Moreover, assuming an average molecular weight for PBDEs of 500 g/mol and an intake of 4000 ng/kg, this equals an exposure of 8 nM. Taken into account that there is a 60-fold accumulation in tissue concentration of PBDEs in this human *in vitro* system and an up to 150-fold increase in brain tissue in mice after oral exposure *in vivo*[153], infant exposure could result in a brain concentration of 0.5 – 1.2 μM. These calculations were supported by the study of Dingemans *et al.*, who estimated that concentrations in the brain could reach a peak value of 1.2 μM[198], based on data from a toxicokinetic study of ^{14}C-BDE-47 in neonatal mice[309]. Considering that 0.1 μM BDE-99 (approx. 6 μM tissue concentration) decreases neuronal

differentiation by 40%, and that a NOAEL of 10 nM (approx. 0.6 μM tissue concentration) was identified, current PBDE exposure levels are likely to be of concern for human health.

This concern seems to be supported by two recent epidemiological studies[86,87]. The first one by Roze *et al.* showed that maternal blood concentrations of 0.2 – 1.6 ng/g lipid correlated with poor neuropsychological functioning of Dutch school children. These children elicited poor fine motor skills and attention deficits dependent on PBDE blood levels. Taken a blood lipid content of 2 mg/ml into account, this equals a blood concentration of 1 – 7 pM. Considering that cord blood concentrations can exceed maternal blood concentrations by a factor of 4^{132}, and that brain PBDE concentrations can exceed blood levels by a factor of 10^{309}, this results in a brain concentration of approx. 0.04 – 0.28 nM. The second study by Herbstman *et al.* demonstrated an inverse association between elevated cord blood levels of PBDEs (median 1.4 – 11.2 ng/g lipid, approx. 0.06 – 0.5 nM brain concentration) and adverse neurodevelopmental test scores for mental and psychomotor development and lowered IQ[87]. Therefore, a reevaluation of PBDE risk assessment seems indicated.

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ABBREVIATIONS

3-MC	3-methylcholantrene
cAMP	cyclic AMP
AhR	aryl hydrodrocarbon receptor
APP	amyloid precursor protein
Arnt	aryl hydrocarbon receptor nuclear translocator
B(a)P	benzo(a)pyrene
BDNF	brain derived neurotrophic factor
BFR	brominated flame retardants
BGS	brain growth spurt
BPA	bisphenol A
BrdU	Bromdesoxyuridin
BTEB	basic transcription element-binding protein
bw	body weight
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CGN	cerebellar granule neurons
CHO	Chinese hamster ovary cells
CMC	4-chloro-m-cresol
CNS	central nervous system
CYP	cytochrom P450
DNT	developmental neurotoxicity
DDT	dichlorodiphenyltrichloroethane
DRE	dioxin response element
ECVAM	European Centre for the Validation of Alternative Methods
ED	embryonic day
EGF	epidermal growth factor
EPA	Environmental Protection Agency
EPSP	excitatory postsynaptic potential
ER	endoplasmatic reticulum
ESAC	ECVAM Scientific Advisory Committee
EST	embryonic stem cell test
FACS	fluorescence-activated cell sorting
FAS	fetal alcohol syndrome
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

FGF	fibroblast growth factor
FSS	fetal solvent syndrome
GAP-43	growth associated protein 43
GD	gestational day
GFAP	glial fibrillary acidic protein
GluR	glutamate receptor
GSH	glutathione
GW	gestational week
HBCD	hexabromocyclododecane
hNPC	human neural progenitor cell
IP_3	inositol 1,4,5-triphosphate
IP_3R	inositol 1,4,5-triphosphate receptor
LD	letal dose
LOAEL	low observed adverse effect level
LTP	long-term potentiation
MBP	myelin basic protein
MNF	3´methoxy-4´nitroflavone
MOS	margin of safety
msNPC	mouse neural progenitor cell
NEC	neural epithelial cell
NH-3	{4-[4-Hydroxy-3-isopropyl-5-(4-nitrophenylethynyl)-benzyl]-3,5-dimethyl}-acetic Acid
NOAEL	no observed adverse effect level
NOS	nitric oxide synthase
NSC	neural stem cell
OPC	oligodendrocyte precursor cell
oRG	outer subventricular zone radial glia
OSVZ	outer subventricular zone
PAH	polycyclic aromatic hydrocarbons
PMA	para-Methoxyamphetamine
PBB	polybrominated biphenyl
PBDE	polybrominated diphenly ether
PCB	polychlorinated bisphenyl
PDBu	3H-phorbol ester
PND	postnatal day

POP	persistent organic pollutant
PTU	propylthiouracil
RG	radial glia
ROS	reactive oxygen species
RXR	retinoic X receptor
RyR	ryanodine receptors
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SOCE	store-operated Ca^{2+}-entry
TBBPA	tetrabromobisphenol A
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TG	Thapsigargin
TH	thyroid hormone
THR	thyroid hormone receptor
TRE	thyroid hormone response element
TSH	thyrotropin
TTR	transthyretin
VGCC	Voltage-gated Ca^{2+}-channel
WEC	whole embryo culture
XRE	xenobiotic response elements

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since 04/2007	PhD work at the Institut für umweltmedizinische Forschung gGmbH at the Heinrich-Heine University Düsseldorf

DECLARATION

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbständig verfasst und aussschließlich die von mir angegebenen Hilfsmittel verwendet habe.

Timm Schreiber

Düsseldorf, den

5. MANUSCRIPTS

PUBLICATION LIST

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Gassmann K*, Krause G*, Dingemans ML*, **Schreiber T***, Abel J, Bergman A, Rose CR, Westerink RH & Fritsche E. (In review) BDE-47 and its hydroxylated metabolite 6-OH-BDE47 modulate calcium homeostasis in primary human neural progenitor cells. *Environ Health Perspect.*

*contributed equally

Schreiber T, Quasthoff KN, Rockel TD, Götz C, Hübenthal U, Giersiefer S, Merk HF, Nguyen NH, Scanlan TS, Abel J & Fritsche E. (In preparation) Neural Development of Mice and Men: Same but Different?

Moors M, Rockel TD, Abel J, Cline JE, Gassmann K, **Schreiber T**, Schuwald J & Fritsche E. (2009) Human neurospheres as three-dimensional cellular systems for developmental neurotoxicity testing. *Environ Health Perspect* **117**:1131-1138.

Gassmann K, Abel J, Bothe H, Haarmann-Stemmann T, Merk HF, Quasthoff KN, **Schreiber T** & Fritsche E. (In revision) Species-specific differential AhR-expression protects human neural progenitor cells against developmental neurotoxicity of PAHs. *Environ Health Perspect.*

Polybrominated Diphenyl Ethers Induce Developmental Neurotoxicity in a Human in Vitro Model: Evidence for Endocrine Disruption

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[*Environ Health Perspect* **118(4)**:572-578]

Polybrominated Diphenyl Ethers Induce Developmental Neurotoxicity in a Human *in Vitro* Model: Evidence for Endocrine Disruption

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Background: Polybrominated diphenyl ethers (PBDEs) are persistent and bioaccumulative flame retardants, which are found in rising concentrations in human tissues. They are of concern for human health because animal studies have shown that they possess the potential to be developmentally neurotoxic.

Objective: Because there is little knowledge of the effects of PBDEs on human brain cells, we investigated their toxic potential for human neural development *in vitro*. Moreover, we studied the involvement of thyroid hormone (TH) disruption in the effects caused by PBDEs.

Methods: We used the two PBDE congeners BDE-47 and BDE-99 (0.1–10 μM), which are most prominent in human tissues. As a model of neural development, we employed primary fetal human neural progenitor cells (hNPCs), which are cultured as neurospheres and mimic basic processes of brain development *in vitro*: proliferation, migration, and differentiation.

Results: PBDEs do not disturb hNPC proliferation but decrease migration distance of hNPCs. Moreover, they cause a reduction of differentiation into neurons and oligodendrocytes. Simultaneous exposure with the TH receptor (THR) agonist triiodothyronine rescues these effects on migration and differentiation, whereas the THR antagonist NH-3 does not exert an additive effect.

Conclusion: PBDEs disturb development of hNPCs *in vitro* via endocrine disruption of cellular TH signaling at concentrations that might be of relevance for human exposure.

Key words: brain, development, human, *in vitro*, neural, neural progenitor cells, neurosphere, PBDE, toxicology. *Environ Health Perspect* 118:572–578 (2010). doi:10.1289/ehp.0901435 [Online 7 December 2009]

Polybrominated diphenyl ethers (PBDEs) are persistent and bioaccumulative flame retardants that are of concern because they are ubiquitous, are potentially toxic, and have been found at rapidly rising levels in humans during the past few decades [reviewed by Costa et al. (2008)]. PBDEs are widely used by industry as flame retardants in, for example, textiles, electrics, plastics, and furniture. Over time, PBDEs diffuse out of the matrix and bioaccumulate in the environment (Schecter et al. 2006). Furthermore, these chemicals are primarily indoor pollutants and are found at high levels in household dust and other home and workplace environmental samples (Schecter et al. 2005a; Stapleton et al. 2005). The abundance in household dust especially causes high exposure of toddlers and children (Fischer et al. 2006). PBDEs also accumulate in the human body; very high levels have recently been found in milk (Kalantzi et al. 2004; Schecter et al. 2003, 2005b), blood including fetal blood (Mazdai et al. 2003; Morland et al. 2005; Schecter et al. 2005b; Sjodin et al. 2004), placenta (Doucet et al. 2009), and adipose tissue (Johnson-Restrepo et al. 2005; She et al. 2002). Although levels of dioxins, dibenzofurans, and polychlorinated biphenyls (PCBs) have been declining in human tissues, PBDE levels have increased substantially during the past two decades (Schecter et al. 2005b; Sjodin et al. 2004).

The high levels of PBDEs in the human population, especially in infants and toddlers, are of vast concern because these compounds are chemically similar to PCBs and have been shown to be developmentally neurotoxic in rodents. Various PBDE congeners cause behavioral alterations, such as hyperactivity, and disrupt performance in learning and memory tests in perinatally exposed mice and rats [reviewed by Costa et al. (2008)].

Despite extensive information on human exposure and body burden, there is no information on possible neurodevelopmental adverse effects in humans from PBDE exposure. Therefore, any potential risk for adverse nervous system effects in humans has to be extrapolated from animal studies (Costa et al. 2008). To facilitate PBDE hazard assessment for humans, we investigated the impact of PBDEs on human neurodevelopment *in vitro* and studied the mechanisms underlying these changes. For these analyses, we used two of the most prominent congeners found in human tissues, the tetrabrominated congener BDE-47 and the pentabrominated congener BDE-99 [reviewed by Costa et al. (2008)]. We investigated the effects of these PBDEs on the developmental neurotoxicity (DNT)–specific end points proliferation, migration, and differentiation, as well as cell viability, in a human model that mimics brain development *in vitro* (Fritsche et al. 2005; Moors et al. 2007, 2009, 2010). Furthermore, we performed competition studies with the thyroid hormone (TH) receptor (THR) agonist triiodothyronine (T_3) and the THR antagonist NH-3, or {4-[4-Hydroxy-3-isopropyl-5-(4-nitrophenylethynyl)-benzyl]-3,5-dimethyl}-acetic acid, to investigate the involvement of TH disruption in the observed effects by PBDEs.

Materials and Methods

Chemicals. BDE-47 and BDE-99 were a kind gift from U. Strähle from the Karlsruhe Institute of Technology and were diluted in dimethyl sulfoxide (DMSO) at stock concentrations of 1, 10, and 100 mM (purity of both PBDEs were 98.88%). ^{14}C-BDE-47 (55 μCi/mg, stated purity > 96%) was a kind gift from K. Crofton from the U.S. Environmental Protection Agency and was diluted in toluene at a stock concentration of 131 mM. T_3 (Sigma-Aldrich, Munich, Germany) and NH-3 (Nguyen et al. 2002) were diluted in ethanol (300 mM) and DMSO (1 mM), respectively.

Cell culture. Normal human neural progenitor cells (hNPCs; Lonza Verviers SPRL, Verviers, Belgium) generated from gestational week 16 were cultured as free-floating neurospheres in proliferation medium [Dulbecco's modified Eagle medium and Hams F12 (3:1)

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supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/mL epidermal growth factor (EGF; Biosource, Karlsruhe, Germany), 20 ng/mL recombinant human fibroblast growth factor (rhFGF; R&D Systems, Wiesbaden-Nordenstadt, Germany), and penicillin and streptomycin (1:100 vol/vol; Invitrogen) at 37°C with 7.5% CO_2 as previously described (Moors et al. 2007, 2009). Differentiation was initiated by growth factor withdrawal in differentiation medium [Dulbecco's modified Eagle medium and Hams F12 (3:1) supplemented with N2 (Invitrogen)] and plating onto a poly-D-lysine/laminin matrix.

Chemical exposure. For viability, migration, and differentiation analyses, neurospheres were preincubated for 1 week with PBDEs (0.1, 1, or 10 µM) in proliferation medium; afterward, differentiation was initiated and spheres differentiated with the same concentrations of PBDEs in differentiation medium for 48 hr (migration measurements) or 7 days (differentiation analyses). This treatment scheme is supposed to imitate exposure of fetal cells during expansion and differentiation *in vivo*. For proliferation analyses, neurospheres were treated for 2 weeks with PBDEs (0.1, 1, or 10 µM) in proliferation medium. For cotreatment with T_3 or NH-3, spheres were incubated for 48 hr (migration) or 7 days (differentiation) with the indicated concentrations after differentiation was initiated.

Viability assay. Cell viability was measured using the alamarBlue assay (CellTiter-Blue assay, Promega, Madison, WI, USA), which measures mitochondrial reductase activity, and the CytoTox-ONE assay (Promega), which determines lactate dehydrogenase release, according to the manufacturer's description. In addition, to determine whether PBDEs cause cell death in differentiating cells in the migration area, cells were incubated for 5 min with 0.1% trypan blue (Sigma-Aldrich). Afterward, dead cells were counted under a light-field microscope (Olympus, Hamburg, Germany).

Proliferation analyses. For proliferation analyses, spheres were cultured in proliferation medium with or without 20 ng/mL EGF/rhFGF as previously described (Moors et al. 2009). After 0 and 14 days, sphere size was determined by software analyses (MetaMorph, version 7.1; Universal Imaging Corp., West Chester, PA, USA).

Migration assay. For analyses of hNPC migration, the distance from the edge of the sphere to the farthest migrated cells was measured 48 hr after initiation of differentiation at four defined positions per sphere (Moors et al. 2007).

Immunocytochemistry. After differentiating for 7 days, the cells were fixed in 2% paraformaldehyde for 30 min and stored in phosphate-buffered saline (PBS) at 4°C until immunostaining was performed. For antibodies against intracellular epitopes, the fixed slides were washed twice for 5 min each in PBS containing 0.1% Triton X-100 (PBS-T). After that, slides were incubated with primary antibodies for 30 min at 37°C in PBS-T containing 10% goat serum. After three additional washes with PBS, the cells were incubated for 30 min with appropriate fluorochrome-labeled secondary antibodies in PBS containing 0.1 µg/mL Hoechst 33258 to label cell nuclei, followed by three washes with PBS for 10 min each. After brief drying, slides were mounted with Vectashield mounting medium (Linaris, Wertheim, Germany). For antibodies against cell-surface epitopes, the same protocol was used with the exception that PBS-T was replaced by PBS in all steps. The primary antibodies were mouse monoclonal IgG anti-β(III)tubulin (1:100; Sigma-Aldrich) and mouse monoclonal IgM anti-O4 (1:50; Millipore, Billerica, MA, USA). The appropriate secondary antibodies were coupled with Alexa Fluor 488 or rhodamine red (1:100; Jackson ImmunoResearch, Dianova GmbH, Hamburg, Germany). For analyses, slides were examined with a fluorescence microscope (Olympus), and photomicrographs were taken at the edge of the sphere with a ColorViewXS digital camera (Olympus). Stained cells were counted manually in relation to the total number of nuclei in the field.

RNA preparation and reverse-transcriptase polymerase chain reaction. Total RNA was prepared from five differentiated neurospheres either untreated or treated with 10 µM PBDE using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA). Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed as previously described (Fritsche et al. 2007). Primer sequences for nestin are CAGCTGGCGCACCTCAAGATG (forward) and AGGGAAGTTGGGCTCA GGACT (reverse). Primer sequences for β-actin are CCCCAGGCACCAGGGCGTGAT (forward) and GGTCATCTTCTCGCGGTT GGCCTTGGGGT (reverse).

Calcium imaging. We treated neurospheres for 1 week with 10 µM BDE-47 or BDE-99. Subsequently, differentiation was induced under ongoing PBDE exposure. After 24 hr, ratiometric calcium imaging was performed using a wide-field epifluorescence system (TILL Photonics, Martinsried, Germany) attached to an upright microscope (Axioskop; Zeiss, Oberkochen, Germany) with a 40× water-immersion objective (0.8 numerical aperture; Olympus Europe, Hamburg, Germany). Excitation was generated by a monochromator; emission was detected by a CCD camera (imago super-VGA; TILL). Cells were passively loaded by addition of the calcium indicator dye fura-2 acetoxymethyl ester (15 µM; Teflabs, Invitrogen) for 90 min. Fura-2 fluorescence was alternately excited at the isosbestic point (357 nm) and at the calcium-sensitive wavelength (380 nm), and the ratio of fluorescence emission (F_{357}/F_{380}) in regions of interest positioned around cell somata was calculated. Adenosine triphosphate (ATP) and acetylcholine (ACh) were puff-applied using a Picospritzer II (General Valve/Parker Hanifin, Flein/Heilbronn, Germany) coupled to standard micropipettes with a tip diameter of around 1.5 µm (Hilgenberg, Waldkappel, Germany) placed at a distance of approximately 10–20 µm above the cell layer. Data analysis was performed using TILLVision and IgorPro software (Wavemetrics, Lake Oswego, OR, USA). To determine effects on $[Ca^{2+}]_i$, we used the normalized F_{357}/F_{380} ratio for puff application. Any change in the normalized ratios to ≥ 1.2 was considered an increase and was used for further data analysis.

^{14}C-BDE-47 accumulation. After mitogen withdrawal neurospheres were allowed to attach to culture dish for 4 hr; afterward, cells were exposed to 1 µM ^{14}C-BDE-47. The cells were incubated for 7 days at 37°C, and half of the medium was changed every 2 days. At the end of the incubation period, the medium was removed, and cells were washed once with 500 µL PBS. Cells were lysed in 100 µL lysis buffer (Stratagene). ^{14}C-BDE-47 concentrations were determined by liquid scintillation counting in residual medium and cell lysates in Roti-Szint (Carl Roth, Karlsruhe, Germany). Intracellular ^{14}C-BDE-47 concentrations were calculated after background subtraction (same treatment without spheres) by a standard concentration curve and normalized to sphere volumes. Percent nonspecific binding to the culture dish was determined by subtracting intracellular and medium ^{14}C-BDE-47 from total ^{14}C-BDE-47 added to the cultures.

Statistics. For multifactor analyses analysis of variance in combination with the Bonferroni post hoc test was used. Student's *t*-test was used for two group comparison. The significance value was set at $p \leq 0.05$.

Results

Effects of PBDEs on hNPC viability. To determine viability of cells, we preincubated spheres for 7 days with different concentrations of BDE-47 or BDE-99 under proliferating conditions followed by further differentiation in presence of PBDEs for 7 days or for 14 days as proliferating spheres. Neither mitochondrial activity nor release of lactate dehydrogenase changed significantly compared with the DMSO controls. Additionally, visual inspection of migration areas after staining with 0.1% trypan blue indicated that in all samples the number of dead or damaged cells was < 1% (data not shown). Thus, BDE-47 or BDE-99 did not cause cytotoxicity of

hNPC [see Supplemental Material, Figure 1 (doi:10.1289/ehp.0901435)].

Effects of PBDEs on hNPC proliferation. For assessment of hNPC proliferation, we cultured neurospheres with and without PBDEs (0.1–1 μM) for 2 weeks. We determined increases in cell number by measuring sphere diameter (Moors et al. 2009). In contrast to the negative control without mitogens, PBDEs did not impair sphere growth over time [see Supplemental Material, Figure 2 (doi:10.1289/ehp.0901435)].

Effects of PBDEs on hNPC migration. To measure migration, we determined the distance between the sphere edge and the farthest migrated cells 48 hr after plating. The solvent controls migrated 942.1 ± 42.9 μm within 48 hr, whereas BDE-47–treated hNPCs migrated 838.9 ± 11.6 μm (89.0 ± 1.2% of control; 0.1 μM), 755.3 ± 20.7 μm (80.2 ± 2.2% of control; 1 μM), and 660.7 ± 21.4 μm (70.1 ± 2.3% of control; 10 μM) and BDE-99 exposed cells migrated 781.9 ± 14.8 μm (83.0 ± 1.6% of control; 0.1 μM), 674.5 ± 24.4 μm (71.6 ± 2.6% of control; 1 μM), and 609.6 ± 33.6 μm (64.7 ± 3.6% of control; 10 μM). These data show that PBDEs reduce hNPC migration in a concentration-dependent manner (Figure 1).

Effects of PBDEs on hNPC differentiation. To investigate the influence of PBDEs on differentiation of hNPCs, we preincubated spheres with PBDEs for 7 days under proliferating conditions. After 7 additional days of differentiation under PBDE exposure, we performed immunocytochemical staining for β(III)tubulin (neurons) and O4 (oligodendrocytes; Figure 2). The number of control and PBDE-exposed nuclei in the differentiation zones, visualized with Hoechst, did not differ [see Supplemental Material, Figure 3 (doi:10.1289/ehp.0901435)]. Manual counting of immunopositive cells under blinded conditions revealed that 26.6 ± 3.2% of the control cells were positive for β(III)tubulin, whereas PBDE exposure reduced immunopositive cells to 21.6 ± 1.8% (0.1 μM), 16.8 ± 2.1% (1 μM), and 13.3 ± 3.0% (10 μM) for BDE-47 and to 16.3 ± 1.5% (0.1 μM), 13.4 ± 0.5% (1 μM), and 8.3 ± 2.0% (10 μM) for BDE-99. The effects of PBDE exposure on oligodendrogenesis were stronger than on neurogenesis. Although 5.7 ± 0.8% of all differentiated cells were immunoreactive for O4 after 7 days of differentiation in the control cultures, in the BDE-47–exposed groups only 4.5 ± 0.5% (0.1 μM), 3.9 ± 0.6% (1 μM), and 2.8 ± 0.6% (10 μM) stained O4 positive, and in the BDE-99–treated cells 4.5 ± 0.6% (0.1 μM), 2.7 ± 0.6% (1 μM), and 0.4 ± 0.4% (10 μM). Thus, PBDEs inhibit neural differentiation of hNPCs in a concentration-dependent manner.

To determine whether PBDEs lead to cell type–specific inhibition of migration, causing cells to remain within the sphere, or whether PBDEs lead to an actual delay in differentiation, we performed real-time RT-PCR analyses of the entire spheres with nestin, a marker of undifferentiated progenitor cells. Control spheres displayed only weak expression of nestin after 7 days of differentiation. In contrast, BDE-47 and BDE-99 increased nestin expression 4- and 5-fold, showing that PBDEs delay differentiation of hNPCs (Figure 3B).

PBDEs interfere with THR signal transduction. To study involvement of endocrine disruption in the observed PBDE effects, we employed the THR agonist T_3 and the antagonist NH-3. After initiating differentiation, we exposed the spheres to 10 μM BDE-47 or BDE-99 with or without 3 nM T_3 or 1 μM NH-3. Stimulation with T_3 alone increased migration distance significantly to 1181.6 ± 7.4 μm (121.1 ± 0.6% of control) compared with 975.6 ± 7.4 μm in the controls (Figure 3A). In contrast, NH-3 inhibited migration of hNPCs to 735.2 ± 7.2 μm, (78.2 ± 1.8% of controls). Coadministration of BDE-47 or BDE-99 and T_3 rescued the inhibitory effects of PBDEs completely, the cells migrated over a distance of 1107.4 ± 8.4 μm (113.5 ± 1.2% of control; BDE-47) and 1109.2 ± 18.6 μm (113.8 ± 2.6% of control; BDE-99). In contrast, cotreatment of PBDEs with NH-3 did not have an additive effect, indicating that these substances inhibit migration through an identical mechanism.

To address the question of whether endocrine disruption of the TH system is also responsible for PBDE-induced changes in differentiation, we analyzed nestin expression 7 days after cotreatment with PBDEs and T_3 or NH-3. T_3 rescued the PBDE-induced cellular increase in nestin expression, whereas NH-3 showed no additive effects in combination with PBDEs (Figure 3B). These results support the notion that PBDEs delay neural differentiation by interfering with cellular THR signaling.

PBDEs do not influence calcium signaling. We treated neurospheres for 1 week with 10 μM BDE-47 or -99. Subsequently, we induced differentiation under ongoing PBDE exposure. After 24 hr, we stimulated neurospheres with 1 mM ATP or 500 μM ACh to induce calcium signaling. ATP caused a $[Ca^{2+}]_i$ increase in 94.7 ± 4.1% of all cells with a maximal amplitude of 1.5 ± 0.1, whereas ACh caused a $[Ca^{2+}]_i$ elevation in 29.2 ± 3.6% of all cells with an amplitude of 1.32 ± 0.0. However, BDE-47 and BDE-99 did not change number of responding cells or amplitude of response (Figure 4).

PBDEs accumulate in hNPCs. Exposure of hNPCs to 1 μM ^{14}C-BDE-47 for 7 days resulted in a cellular concentration of 61.16 ± 6.34 μM, which equals an accumulation factor of 60. Only 2% of ^{14}C-BDE-47 remained in the media, indicating that 91% of ^{14}C-BDE-47 was bound to the culture dish [see Supplemental Material, Figure 4 (doi:10.1289/ehp.0901435)].

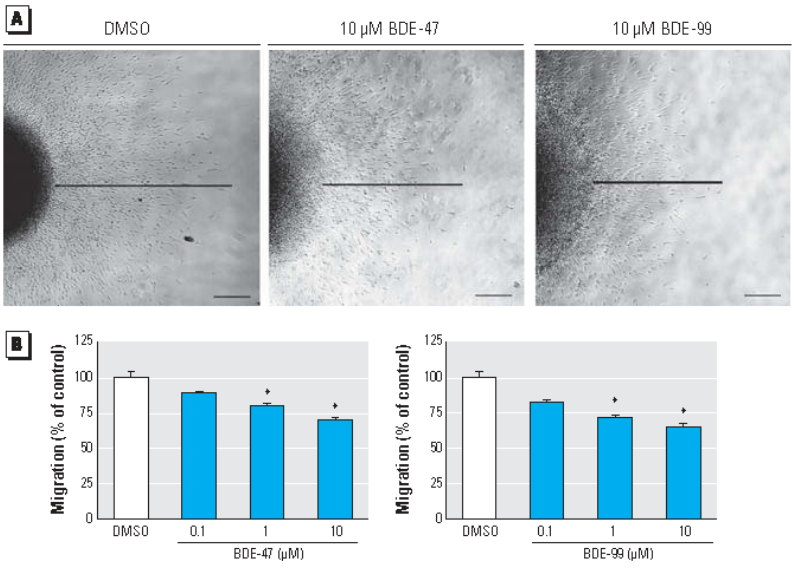

Figure 1. PBDEs inhibit migration of neural progenitor cells: phase-contrast images (*A*) and quantification (*B*) of cell migration. Migration distance was measured at four defined spots from the edge of the sphere to the farthest migrated cell after 48 hr. All data are mean ± SE of three independent experiments (five spheres/experiment). Bars, 200 μm.
*$p \leq 0.05$.

Discussion

Human exposure to brominated flame retardants is of concern because PBDEs impair neurodevelopment in animals, rising concentrations of these compounds are found in human tissues, and nothing is known about their developmentally neurotoxic effects in humans (Costa et al. 2008). Moreover, the mechanisms by which PBDEs interfere with brain development are not known. To shed light onto the effects caused by PBDE exposure in human developing brain cells, we studied their effects on the development of hNPCs *in vitro*. These cells are primary human fetal neuroprogenitors and grow as three-dimensional, complex cellular systems, called neurospheres, in culture. Recently, we have established several end points for DNT testing in such neurospheres—proliferation, migration, differentiation, and apoptosis—by employing end-point–specific controls (Moors et al. 2007, 2009). We showed that this neurosphere system is able to reveal exogenously induced disturbances in these basic processes of brain development. In the present study, we applied this novel testing method to unravel the DNT potential of PBDEs for such developing human cells.

PBDEs (0.1–10 μM) are not cytotoxic for proliferating or differentiating hNPCs over a period of 2 weeks, as we demonstrated by different, independent methods [see Supplemental Material, Figures 1 and 2 (doi:10.1289/ehp.0901435)]. In contrast to our findings, the technical PBDE mixture DE-71 (> 20 μM) caused cell death in rat cerebellar granule cells (Reistad et al. 2006), and BDE-99 (> 25 μM) induced cytotoxicity in human astrocytoma cells (Madia et al. 2004). BDE-47 was reported to cause cell death in hippocampal neurons (41.2 μM), human neuroblastoma cells (> 5 μM), and human fetal liver hematopoietic cells (> 50 μM) (He et al. 2008a, 2008b; Shao et al. 2008). Moreover, the higher brominated congener BDE-209 (> 10 μM) was toxic to human HepG2 hepatoma cells (Hu et al. 2007). The cause of cytotoxicity in all these different cell models was induction of apoptosis. Giordano et al. (2008) demonstrated that DE-71 exposure induces oxidative stress. This production of reactive oxygen species is responsible for DE-71–induced apoptosis of rat cerebellar granule neurons (CGN), as indicated by intracellular glutathione (GSH) content, which is a most important determinant of CGN susceptibility to DE-71 neurotoxicity. Moreover, transgenic rat neuron/astrocyte cocultures with proficient versus deficient GSH synthesis support these findings: Astrocytes rich in GSH protected neurons against DE-71–induced neurotoxicity, whereas astrocytes poor in GSH content did not (Giordano et al. 2009). Protection of neurons against PBDE-induced cytotoxicity by the presence of astrocytes is thus the probable reason for differentiated hNPC insensitivity toward PBDE-dependent

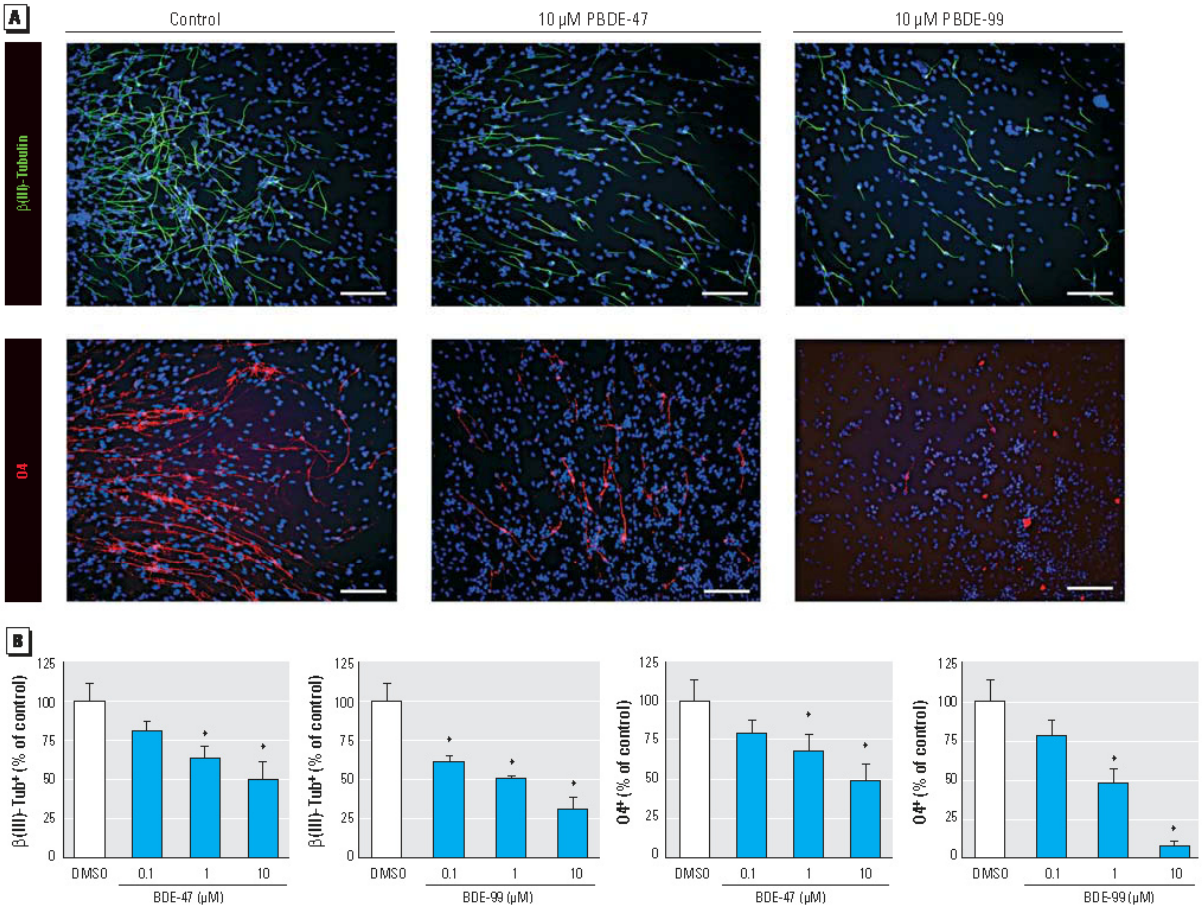

Figure 2. PBDEs inhibit differentiation of hNPCs. (*A*) Representative photomicrographs of hNPCs after 7 days of differentiation. Cells were stained with antibodies against β(III)tubulin [β(III)Tub+] for neurons and O4+ for oligodendrocytes. Cell nuclei were counterstained with Hoechst. Bars, 50 μm. (*B*) Quantification of immunostaining after PBDE treatment. All data are mean ± SE of three independent experiments (five spheres/experiment).
*$p \leq 0.05$.

cell death, whereas proliferating precursors are known to possess better defense mechanisms than do postmitotic neural cells (Madhavan et al. 2006). Moreover, such human coculture systems, such as differentiated neurospheres, where the three major cell types of the brain are present in "physiological" ratios, seem to be superior *in vitro* methods for assessing hazards of chemicals to humans compared with simple monolayer cell lines.

Neurosphere proliferation assessed by monitoring of sphere diameter (Moors et al. 2009) was also not affected by presence of BDE-47 or -99 [see Supplemental Material, Figure 2 (doi:10.1289/ehp.0901435)]. This is in agreement with results from a T-Screen assay (Gutleb et al. 2005), a functional assay based on T_3-dependent cell proliferation of the rat GH3 pituitary tumor cell line, where also no effects of these two congeners on proliferation were observed (Hamers et al. 2006). In contrast, one of the hydroxylated BDE-47 metabolites, 2-OH-BDE-47 (5–10 µM), inhibited proliferation of the H295R adrenocortical carcinoma cell line (Song et al. 2009). However, this concentration of reactive metabolite causing an antiproliferative effect in these cells is high, and the researchers did not investigate effects of the parent compound. Inhibition of proliferation by BDE-47 was also seen in 5L rat hepatoma cells (Wahl et al. 2008). However, the researchers demonstrated that arylhydrocarbon receptor (AhR) activation by the BDE-47 contaminant 1,2,3,7,8-pentabromodibenzofuran was responsible for the effects on proliferation, rather than BDE-47 itself. Highly purified BDE-47 does not stimulate the AhR (Peters et al. 2004). This supports our data because PBDEs used in the present study are contaminant-free. Increased proliferation was observed in DE-71–treated MCF-7 breast cancer cells. This stimulation was estrogen receptor dependent, so DE-71 acts as an endocrine disruptor in this estrogen receptor–positive cell line (Mercado-Feliciano and Bigsby 2008). Hence, interaction of PBDEs with cell proliferation seems to be congener and cell type specific. So far, all data available on this topic have been obtained in tumor cells. This is, to our knowledge, the first report employing normal human cells or human stem/progenitor cells for determining effects of PBDEs on cell proliferation.

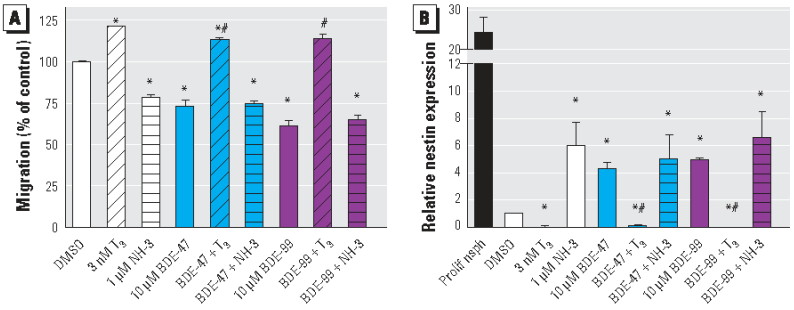

Figure 3. PBDEs disrupt cellular TH signaling. (*A*) hNPC migrated for 48 hr in the presence of the indicated substances, and migration distance was quantified. (*B*) hNPCs differentiated for 7 days in the presence of the indicated substances. Proliferating neurospheres (Prolif nsph) were used as positive control. Real-time PCR analyses for nestin were quantified with a product-specific copy number standard and normalized for β-actin expression. All data (% DMSO control) are shown as mean ± SE of three independent experiments (five spheres/experiment).
*$p \le 0.05$ versus control; #$p \le 0.05$ versus respective PBDE treatment.

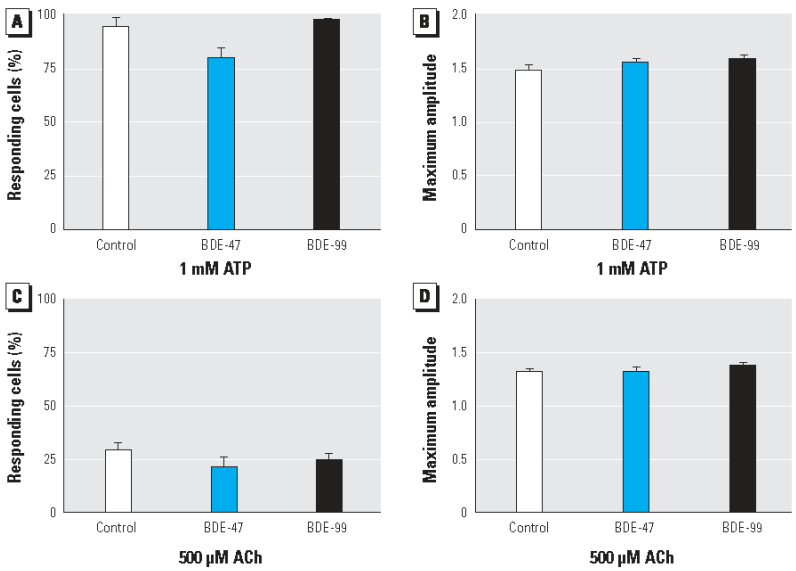

Figure 4. Long-term PBDE exposure does not interfere with calcium signaling. Neurospheres were incubated with 10 µM BDE-47 or BDE-99 for 7 days under proliferating conditions and for an additional day during differentiation. Afterward, hNPCs were loaded with the fura-2 dye and puff-exposed to 1 mM ATP (*A*,*B*) or 500 µM ACh (*C*,*D*). After excitation, the ratio of fluorescence emission (F_{357}/F_{380}) in regions of interest positioned around cell somata was calculated. Any change in normalized ratios (F_{357}/F_{380}) ≥ 1.2 was considered as an increase and used for further analysis. All data are mean ± SE of three independent experiments (five spheres/experiment).

Similar to PBDE effects on neural migration, to our knowledge, consequences of PBDE exposure for neural differentiation have not been studied so far. Therefore, this is the first report showing that this group of flame retardants can directly interfere with birth of neurons and oligodendrocytes in this human *in vitro* model. Interference of BDE-47 with neuronal differentiation was also suggested in the fathead minnow because this congener reduced basic transcription element–binding protein (BTEB) expression in their brains (Lema et al. 2008). BTEB is known to be involved in neural differentiation in rodents (Denver et al. 1999). Moreover, BDE-99 altered protein expression of growth-associated protein-43 and brain-derived neurotrophic

factor in developing rodent brains (Alm et al. 2008). Both proteins are closely related to neural development and plasticity [reviewed by Strittmatter et al. (1992) and Binder and Scharfman (2004)]. Whether PBDEs disturb brain development by interfering with migration and differentiation not only *in vitro* but also *in vivo* needs to be investigated by appropriate animal experiments. However, because our study was performed in normal cells of human origin, possible species differences should first be addressed by migration and differentiation analyses in rodent neurospheres. Moreover, the developmental stage of cells needs to be considered for rodent *in vitro* and *in vivo* analyses: hNPC generated from gestational week 16 correspond approximately to embryonic day 16 to postnatal day 3 of mouse development.

During normal development, neural migration and maturation of neural and glial cells are guided by TH (Alvarez-Dolado et al. 1999; Wong and Leung 2001). Therefore, hypothyroidism during development causes a large number of neuroanatomical and behavioral effects (Haddow et al. 1999; Schalock et al. 1977; Zoeller and Crofton 2005). Because of similar neurobehavioral alterations observed after PBDE exposure in rodents, endocrine disruption of the TH system by PBDEs has been studied intensively. Hypothyroidism of dams and/or offspring was found in a variety of different studies after pre- or postnatal exposure [reviewed by Costa et al. (2008)]. This reduction in serum thyroxine (T_4) or T_3 levels is thought to be caused by induction of the phase II enzyme UDP-glucuronosyl transferase, causing accelerated TH metabolism (Zhou et al. 2002), and by preventing TH from binding to its plasma transport protein transthyretin (Meerts et al. 2000). However, behavioral toxicity of BDE-47 without alterations in serum T_4 and T_3 levels was also observed, suggesting that PBDEs cause toxicity by a mechanism beyond changes in body TH homeostasis (Gee and Moser 2008; Gee et al. 2008). These studies might be explained by the data generated in our experiments because the two PBDE congeners BDE-47 and -99 directly disturb migration and delay differentiation of hNPCs *in vitro* by endocrine disruption of cellular TH signaling. We confirmed this by two observations: *a*) PBDE actions are completely antagonized by cotreatment of neurospheres with T_3 and *b*) simultaneous administration of PBDE and the THR antagonist NH-3 did not cause an additive effect. Although NH-3 binds to THRα as well as THRβ, it has a higher affinity to THRβ (Nguyen et al. 2002). Because inhibition of THRα leads to decreased proliferation of avian neurogenic precursors (Lezoualc'h et al. 1995; but PBDEs do not interfere with hNPC proliferation), and the induction of THRβ induces neural differentiation (Jones et al. 2003; Lebel et al. 1994; and PBDEs disturb neural differentiation of hNPCs), it is likely that PBDEs interfere with THRβ signaling of hNPCs. However, reporter gene analyses in THRα and β overexpressing Chinese hamster ovary cells revealed that neither PBDE congener acted as agonist or antagonist of THRα or THRβ. One possible reason for discrepancies between our study and results in the overexpression system might be that PBDEs act via disturbance of recruitment of THR cofactors. In primary cells, receptors, cofactors, and responsive elements are present at a fine-tuned equilibrium, which is not the case in transfected cells, which overexpress only certain elements of the cellular machinery. That nuclear hormone receptor cofactors might be crucial in endocrine disruption by polyhalogenated aromatic compounds is discussed in our previous work (Fritsche et al. 2005), in which we found that the noncoplanar PCB-118 induced oligodendrocyte differentiation in hNPCs. That *in vitro* work represented the finding *in vivo* that Aroclor treatment led to an increased expression of TH-dependent genes such as RC3/neurogranin and myelin basic protein in fetal rat brains (Zoeller et al. 2000). To our knowledge, an equivalent rodent study has not yet been performed with PBDEs. Only in the fathead minnow, TH disruption on the basis of THR-dependent gene expression was observed after BDE-47 exposure (Lema et al. 2008). Thus, this is the first work showing that PBDEs can directly interfere with cellular TH signaling in human neural cells.

Besides endocrine disruption, we also investigated whether PBDEs disturb calcium homeostasis. Calcium signaling is a key player in developmental processes (Ciccolini et al. 2003; Greer and Greenberg 2008), and BDE-47 disturbs calcium homeostasis in rat PC12 pheochromocytoma cells after 20 min of exposure (Dingemans et al. 2008). BDE-47 exerts similar short-term effects on hNPCs (Gassmann K, Krause G, Dingemans M, Schreiber T, Abel J, Bergman A, et al., unpublished observations). To test whether long-term exposure (1 week) to PBDEs also influences calcium signaling and thus contributes to the developmentally neurotoxic effects of PBDEs in hNPC, we measured calcium signaling in PBDE treated hNPCs stimulated with ATP or ACh. Neither BDE-47 nor BDE-99 influenced the response of the cells toward these stimuli, indicating that they do not alter expression of proteins involved in Ca^{2+} flux. Recently, Viberg (2009) showed that BDE-203 and BDE-206 increase Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) expression in mouse hippocampus. Whether PBDEs interfere with downstream targets of calcium signaling in hNPCs, such as CaMKII or calcineurin, has to be further elucidated.

In summary, BDE-47 and BDE-99 disturb neural migration and differentiation in a human *in vitro* model for brain development by disruption of cellular TH signaling. The question is now how the lowest observed effect levels from this study relate to actual human PBDE exposure. Therefore, we measured intracellular PBDE concentrations by employing ^{14}C-BDE-47. Because medium concentration-dependent intracellular PBDE accumulation *in vitro* follows a linear kinetic (Mundy et al. 2004) and neurosphere material is limited, we measured only 1 μM ^{14}C-BDE-47 medium concentration. After 7 days of differentiation, PBDE accumulation is approximately 60-fold. These data support other *in vitro* data where PBDEs accumulate up to 100-fold in neuronal cells (Mundy et al. 2004). It also reflects PBDE accumulation in postnatal day 10 and 19 mouse brains after 7 days *in vivo* (20- to 140-fold) that we calculated from a study of Viberg et al. (2003). PBDE exposure of human infants through breast milk is up to 4,000 ng/kg/day (Jones-Otazo et al. 2005). Assuming an average molecular weight of 500 g/mol for PBDEs, this equals an exposure of 8 nM. Taking into account that there is a 60-fold increase in tissue concentration of PBDEs in our human *in vitro* system and up to a 140-fold increase in brain tissue in mice after oral exposure *in vivo* (Viberg et al. 2003), infant exposure could result in a brain concentration of 0.5–1.1 μM. Considering that 0.1 μM BDE-99 (~ 6 μM tissue concentration) decreases neuronal differentiation by approximately 40% (Figure 2), current PBDE exposure levels are likely to be of concern for human health.

Assessing subtle changes in human IQ or behavior in epidemiologic studies is not trivial and needs large numbers of study subjects. Such investigations are needed to reveal whether PBDEs as a hazard identified in this study actually pose a risk for human brain development *in vivo*.

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BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 modulate calcium homeostasis in primary human neural progenitor cells

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BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 modulate calcium homeostasis in primary human neural progenitor cells

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Short running title

(6-OH-)BDE-47 modulates Ca^{2+} homeostasis in hNPCs

Key words

Polybrominated diphenyl ether, calcium, human, brain, neural, brominated flame retardant, neurotoxicity, intracellular calcium stores, neurosphere

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Abbreviations

CMC: 4-chloro-m-cresol

ER: Endoplasmatic reticulum

FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

hNPC: Human neural progenitor cell

LDH: Lactate-dehydrogenase

LTP: Long-term potentiation

PCB: Polychlorinated biphenyl

PBDE: Polybrominated diphenyl ether

SERCA: Sarcoplasmatic/endoplasmatic Ca^{2+}-ATPase

SOCE: store-operated Ca^{2+} entry

TG: Thapsigargin

VGCC: voltage-gated Ca^{2+} channel

Outline of manuscript section headers

Abstract

Background: Polybrominated Diphenyl Ethers (PBDEs) are bioaccumulating flame retardants, which are found in rising concentrations in human tissue. Animal studies have raised concern for their developmental neurotoxic potential. One suggested mechanism of how PBDEs exert developmental neurotoxicity (DNT) is by increasing the intracellular calcium concentration ($[Ca^{2+}]_i$). It is suggested that not only the parent compounds but also their oxidative metabolites contribute to their toxicity. However, acute PBDE effects on Ca^{2+} homeostasis of developing primary human brain cells are so far unknown.

Objective: Therefore, human neural progenitor cells (hNPCs) were exposed to 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47) and its hydroxylated metabolite 6-hydroxy-2,2´,4,4´-tetrabromodiphenyl ether (6-OH-BDE-47) to determine possible effects on $[Ca^{2+}]_i$.

Methods: Membrane potential and voltage-gated ion currents were measured using whole-cell patch-clamp in differentiated hNPCs. $[Ca^{2+}]_i$ concentrations were determined by imaging with the fluorescent dye Fura-2.

Results: Acute exposure of hNPCs to BDE-47 (≥2 µM) or 6-OH-BDE-47 (≥0.2 µM) resulted in a significant increase in $[Ca^{2+}]_i$. 6-OH-BDE-47 (20 µM) caused an initial transient peak, followed by a persistent increase in baseline that turned into a second strong increase in $[Ca^{2+}]_i$ after ~10 min. Pharmacological manipulation revealed that the initial transient Ca^{2+} increase originated from endoplasmatic reticulum Ca^{2+} stores, whereas the second, late increase was mainly due to influx of extracellular Ca^{2+}. The persistent increase in baseline appeared due to Ca^{2+} release from mitochondria.

Conclusion: These findings indicate that Ca^{2+} homeostasis of developing human brain cells is highly sensitive towards PBDE exposure and supports the concern that PBDEs are developmentally neurotoxic to humans.

Introduction

Polybrominated diphenyl ethers (PBDEs) are nowadays ubiquitously present in the environment, in animals and in humans. Among those, 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47) is the predominant congener in human blood samples (Hites 2004). *In vivo*, BDE-47 is converted to hydroxylated metabolites in mice and rats (Marsh et al. 2006; Staskal et al. 2006b) and high amounts of hydroxylated metabolites are found in blood of humans (Athanasiadou et al. 2008), including fetal blood (Qiu et al. 2009). The current greatest concern for potential adverse health effects of PBDEs relates to their developmental neurotoxicity (DNT). Previous data demonstrated that high and low doses of PBDEs administered during pregnancy or postnatally during the brain growth spurt alter neurobehaviour of offspring in rodents ranging from altered motor activity over changes in sweet preference to impairment of cognitive functions (rev. in Costa and Giordano 2007, Fonnum and Mariussen 2009). Recently, two epidemiological studies observed that prenatal exposure towards PBDEs also affect neurological outcome in children (Roze et al. 2009; Herbstman et al. 2009). One of the proposed mechanisms how PBDEs exert DNT is disruption of calcium (Ca^{2+}) homeostasis as shown in rat pheochromocytoma (PC12) cells, human neuroblastoma (SH-SY5Y) cells and in isolated rat brain organelles *in vitro* (Dingemans et al. 2008; Kodavanti and Ward 2005; Yu et al. 2008). Thereby, hydroxylated metabolites of PBDEs are more potent than their parent compounds (Dingemans et al. 2008), suggesting that bioactivation by oxidative metabolism might add to the neurotoxic potential of PBDEs. Ca^{2+}, an early-response second messenger, plays a key role in a number of physiological processes including cell proliferation, differentiation and apoptosis as well as a wide variety of specific neuronal processes including dendritic spine growth, synaptic plasticity and neurotransmission (Berridge et al. 2000; Ciccolini et al. 2003). Ca^{2+} influx as well as Ca^{2+} release from intracellular stores, such as endoplasmic reticulum (ER) and mitochondria, define the magnitude, time course, and spatial spread of the Ca^{2+} signal (Berridge et al. 2000; Delmas and Brown 2002).

Recently, we showed that PBDEs are endocrine disruptors of thyroid hormone signaling in normal human neural progenitor cells (hNPCs) causing disturbances in neural migration and differentiation (Schreiber et al. 2010). This cell culture model mimics basic processes of brain development *in vitro*: proliferation, migration, differentiation and apoptosis (Fritsche et al. 2005; Moors et al. 2007; Moors et al. 2009; Moors et al. 2010). In the present study we extend this knowledge by showing that short-term PBDE exposure affects intracellular Ca^{2+} signaling in hNPCs.

Materials and Methods

Chemicals. BDE-47 and 6-OH-BDE-47 were synthesized and purified (~99% purity) at Stockholm University as described by Marsh et al. (1999). All other chemicals used (unless otherwise noted) were purchased from Sigma–Aldrich (Taufstein, Germany) and were of the highest purity available.

Cell culture. The Normal Human Neural Progenitor cells (hNPCs, Lonza, Verviers SPRL, Belgium) were prepared from a single donor. They were cultured in proliferation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/ml EGF (Biosource, Karlsruhe, Germany) and 20 ng/ml rhFGF (R&D Systems, Wiesbaden-Nordenstadt, Germany) in a humidified 92.5% air/7.5% CO_2 incubator at 37 °C in suspension culture (Moors et al. 2007; Moors et al. 2009). For Ca^{2+} imaging and patch clamp experiments, hNPCs were differentiated by growth factor withdrawal in differentiation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with N2 (Invitrogen)) and plating onto poly-D-lysine/laminin coated cover glasses (Menzel GmbH & Co KG, Braunschweig, Germany; MatTek, Ashland MA, USA).

Electrophysiology. Experiments were performed at room temperature. During experiments, differentiated hNPCs were constantly perfused with artificial saline containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 20 glucose, bubbled with 95% O_2 / 5% CO_2 resulting in a pH of 7.4.

Somatic whole-cell patch-clamp recordings were carried out using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA, USA) coupled to a personal computer via a digidata 1322A interface (Molecular Devices). Data were acquired at 1 to 10 kHz using PClamp 8.2 software (Molecular Devices). Electrodes were pulled from borosilicate glass (Hilgenberg, Waldkappel, Germany) and filled with internal saline containing (in mM): 125 K-Acetate, 10 KCl, 10 HEPES, 4 NaCl, 0.5 EGTA, 1 $MgCl_2$, pH 7.2 (KOH). Electrode resistance was 3-4 MΩ. The performed voltage-step protocol induced large capacitive as well as passive currents and leak subtraction (P/4) was performed to uncover voltage-gated currents activated by membrane depolarization. Data analysis was performed with Clampfit 8 and IgorPro Software.

Calcium imaging. Ratiometric calcium imaging was performed as described previously (Dingemans et al. 2008). Briefly, differentiated hNPCs were loaded with 5-15 µM Fura-2 AM (Molecular Probes, Invitrogen) in saline for 20 min at room temperature. Subsequently, the cells

were washed with external saline and placed on the stage of a microscope equipped with a TILL Photonics Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany). Fluorescence was recorded every 0.5-6 sec at 510 nm (excitation wavelengths: 340 or 357 and 380 nm) with a CCD camera (TILL Photonics GmbH) and the fluorescence ratio (F340/F380 or F357/380) was calculated. Data collection and digital camera and polychromator control were performed by imaging software (TILLvisION, version 4.01). Changes in the fluorescence ratio were analyzed using custom-made Excel macros (Microsoft Corp., Redmond, WA, USA) or Igor Pro Software (Wavemetrics, Lake Oswego, OR, USA).

Following 5 min baseline recording, cells were exposed to 0.2-20 µM BDE-47 or 0.02-20 µM 6-OH-BDE-47 for 20 min. Maximum and minimum ratios were determined after 25 min recording by addition of ionomycin (5 µM) and ethylenediamine tetraacetic acid (EDTA, 17 mM), respectively, as a control for experimental conditions.

For the experiments under Ca^{2+}-free conditions, cells were washed after Fura-2 loading with Ca^{2+}-free external saline (containing 10 µM EDTA) just before the imaging experiments. In specific experiments, where intracellular Ca^{2+} stores were emptied by 10 min pre-incubation with 1 µM thapsigargin (TG) and 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), cells were exposed to 20 µM BDE-47 or 6-OH-BDE-47 for 15 min.

Receptor agonists were either bath-applied or puff-applied using a Picospritzer II (General Valve/Parker Hanifin, Flein/Heilbronn, Germany) coupled to standard micropipettes with a tip diameter of around 1.5 µm (Hilgenberg, Waldkappel, Germany) placed at a distance of approximately 10–20 µm above the cell layer. Exposure to receptor antagonists was performed by bath application.

LDH assay. Cell viability was measured using a lactate-dehydrogenase (LDH) assay (CytoTox-One, Promega) as previously described (Moors et al. 2009). Briefly, supernatants from cells that were exposed to 0.02-20 µM BDE-47 and 6-OH-BDE-47 for 20 minutes were collected prior to detection of fluorescence (Ex 540 nm/Em 590 nm). Complete lysis of cells served as a positive control.

Data analysis and statistics. To determine effects on $[Ca^{2+}]_i$, the fluorescence ratio of each cell under baseline conditions was normalized. An increase in the normalized fluorescence ratio of more than 1.2 ($\geq$ 1.02 in case of glutamate application) was considered as an increase in $[Ca^{2+}]_i$ and was used for further data analysis. All data are presented as mean ± SE from the number of cells indicated. Statistical analyses were performed using GraphPad Prism. We compared

continuous data using paired Student's *t*-test and categorical data were compared using Fisher´s exact and chi-square tests. For multifactor analysis ANOVA in combination with the Bonferroni *post hoc* test was used. A p-value < 0.05 was considered statistically significant.

Results

Electrophysiology. Membrane potential and characteristics of voltage-gated ion channels were determined in differentiated hNPCs using whole-cell patch-clamp measurements. Cells had a mean membrane potential of -42.4 ± 10.7 mV, a membrane resistance of 1.0 ±0.6 GΩ, and a membrane capacitance of 6.4 ± 3.8 pF (n = 11). To reveal the functional expression of voltage-gated ion channels, cells were held in the voltage-clamp mode at -80 mV and then subjected to a rectangular voltage step protocol from -100 to +50 mV in 10 mV increments. In some experiments, cells were additionally hyperpolarized to -110 mV before the voltage-step protocol to remove inactivation of voltage-gated sodium channels. The voltage-step protocol revealed the presence of outward currents (n = 10/11 cells; Suppl. Fig. 1). In two of these cells, we detected small fast inward currents. In none of the cells tested, injection of depolarizing currents were able to induce action potentials in the current clamp mode.

General characterization of calcium signaling in hNPCs. Calcium imaging experiments were performed with differentiated hNPCs. Cells did not show spontaneous calcium transients after different differentiation times ranging from 12 hours to 96 hours (Figure 1). To probe for the presence of voltage-gated ion channels, we applied saline containing 80 mM potassium by puff application for 2.5 s to the cells. However, only a minority of cells (3 of 125 cells, differentiated for 48 and 96 hours) responded to the application of potassium with a calcium increase (data not shown). In contrast to this, virtually all cells responded to puff application of ATP (0.5 or 1 mM for 1 s; Figure 1). After puff application of acetylcholine (0.5 mM for 1 or 2 s) about 40% reacted (Fig. 1). The calcium increase induced by acetylcholine was largely blocked by atropine (48/53 cells; not shown), indicating that it was due to the activation of muscarinic receptors on the cells. The percentage of cells responding to ATP and acetylcholine as well as the amplitude of the induced calcium transients was independent of the differentiation time (either 12, 24 or 48 hrs in culture) and the application form (bath application or puff-application), although the bath application yielded in slightly higher amplitudes (Fig. 1B).

Application of glutamate (1 mM; 1-2.5 s) induced calcium increases in the majority of cells (Fig. 1). The amplitudes of the glutamate-induced calcium transients were, however, quite small as

compared to those induced by ATP or acetylcholine and at early differentiation time points (12 hours) the number of responding cells was significantly smaller than at later timepoints (Fig 1 B). Therefore, for all additional experiments a differentiation time of 24 or 48 hours was chosen. In none of the cells studied (n=60), application of GABA (1 mM, 2.5 s) induced an increase in intracellular calcium (Fig 1 A).

BDE-47 as well as 6-OH-BDE-47 cause an increase in $[Ca^{2+}]_i$ in human neural progenitor cells. The vehicle alone (0.1% DMSO) slightly increased $[Ca^{2+}]_i$ in 27% of the cells (Figure 2 & 3). BDE-47 (2 and 20 µM) or 6-OH-BDE-47 (0.2, 2 and 20 µM) initiated a $[Ca^{2+}]_i$ increase in 42 and 83% or 45, 54 and 100% of responding cells, respectively (Figure 2 and 3). The amplitude of the solvent control increases in $[Ca^{2+}]_i$ amounted to 1.16 ± 0.01, whereas the amplitude was concentration-dependently increased by BDE-47, amounting to 1.24 ± 0.03 and 2.11 ± 0.15, respectively. Application of 6-OH-BDE-47 (0.2, 2 and 20 µM) also resulted in a concentration-dependent increase in $[Ca^{2+}]_i$ amplitude to 1.30 ± 0.05, 1.44 ± 0.08 and 5.74 ± 0.45. Notably, 6-OH-BDE-47 was effective already at lower concentrations than BDE-47 and the increase in amplitude was larger (Figure 2 & 3). There was also a difference in response time: while the increase in $[Ca^{2+}]_i$ occurred 3 to 5 minutes after BDE-47 (20 µM) application with multiple peaks, $[Ca^{2+}]_i$ elevation took place immediately and persistently after 6-OH-BDE-47 at concentrations ≥ 0.2 µM. Only 20 µM 6-OH-BDE-47 induced a transient initial $[Ca^{2+}]_i$ increase within the first 5 min after application followed by a late (> 10 min) $[Ca^{2+}]_i$ increase accompanied by a general elevation of the baseline. Some single cells showed a very strong $[Ca^{2+}]_i$ increase without recovery (Figure 2).

Increases in $[Ca^{2+}]_i$ originate from extracellular and intracellular stores. After removal of extracellular Ca^{2+} no increase in $[Ca^{2+}]_i$, was observed after application of the solvent control DMSO (data not shown), while BDE-47 (20 µM) initiated only a few fast transient $[Ca^{2+}]_i$ increases (Figure 4B) though much less compared to BDE-47 exposure in Ca^{2+}-containing medium. Additionally, the number of responsive cells decreased from 83% to 26% and the amplitude of the $[Ca^{2+}]_i$ increase was significantly smaller (1.16 ± 0.07, Figure 4B, C). In contrast, 6-OH-BDE-47 exposure under Ca^{2+}-free conditions did not change the number of responding cells. The initial transient and the shift of baseline in $[Ca^{2+}]_i$ were still present, though the late increase was absent. The average and the maximal amplitude of $[Ca^{2+}]_i$ was significantly lower in Ca^{2+}-free medium (1.48 ± 0.02 and 2.58 ± 0.11, respectively, Figure 4A, C, D). These

data indicate that the parent compounds and the hydroxylated metabolites increase $[Ca^{2+}]_i$ in hNPCs through different mechanisms.

To identify the intracellular stores responsible for the observed increase in $[Ca^{2+}]_i$, we depleted ER and mitochondrial Ca^{2+} stores with TG and FCCP, respectively, in Ca^{2+}-free medium. TG and TG/FCCP pretreatment transiently increased $[Ca^{2+}]_i$, but after several minutes the baseline stabilized. Exposure of hNPCs to 20 µM BDE-47 in Ca^{2+}-free medium in the presence of TG reduced the number of responding cells to 0%. Exposure to 20 µM 6-OH-BDE-47 in Ca^{2+}-free medium in the presence of TG reduced the number of responding cells to 84%. Additionally, the average and maximum amplitude of the response decreased further to 1.13 ± 0.01 or 1.41 ± 0.02 in comparison to 6-OH-BDE-47 exposure only in Ca^{2+}-free medium (Figure 4C, D). However, the 6-OH-BDE-47-induced shift of the baseline still remained in presence of TG, but appeared with a delay of several minutes. Interestingly, depletion of ER and mitochondrial Ca^{2+} stores with both TG and FCCP under Ca^{2+}-free conditions eliminated this baseline shift. However, a single transient increase after 5 to 10 min after application remained in 89 % of all cells (Figure 4C, D).

BDE-47 and 6-OH-BDE-47 are not cytotoxic to hNPCs. Exposure of hNPCs with up to 20 µM BDE-47 or 6-OH-BDE-47 for 20 min did not result in significant LDH release compared to solvent control (Supplementary Fig. 2), indicating that the observed effects on Ca^{2+} homeostasis were not caused by cytotoxicity.

Discussion

Prenatal exposure towards PBDEs affects neurological outcome in children (Roze et al. 2009; Herbstman et al. 2009) as was previously observed in rodents (rev. in Fonnum and Mariussen 2009). Disturbances in long term-potentiation (LTP) possibly by altered calcium homeostasis might thereby be one of the underlying mechanisms (Dingemans et al. 2008; Kodavanti and Ward 2005; Xing et al. 2009). We have already shown that a one week exposure with BDE-47 and BDE-99 did not alter hNPC calcium responses towards ATP, acetylcholine (ACh) and glutamate (Schreiber et al. 2010). However, the direct, short-term influences of PBDEs on $[Ca^{2+}]_i$ had so far not been investigated in primary human neural cells. Therefore, the aim of the present study was to determine the acute effects of BDE-47 and the hydroxylated metabolite 6-OH-BDE-47 on $[Ca^{2+}]_i$ homeostasis of hNPCs.

First, we analyzed several basic electrophysiological properties of hNPCs by patch-clamp experiments and measured $[Ca^{2+}]_i$ after application of different neurotransmitters. The data of the

patch-clamp experiments concerning the membrane characteristics were in the same ranges as those reported earlier from cells derived from neurospheres (Piper et al. 2000). Similar to the results obtained by Piper et al., the voltage-step protocol revealed the presence of outward currents, most likely representing a mixture of several potassium currents. The neurotransmitters ATP, ACh and glutamate evoke Ca^{2+} transients in hNPCs. The percentage of cells responding to ATP and acetylcholine as well as the amplitude of the induced calcium transients presented here, were independent of the differentiation duration (either 12, 24 or 48 hrs in culture) and application form (bath application or puff-application). In case of glutamate, the number of responding cells was significantly smaller at short (12 hours) vs long differentiation durations (24-48 hours). It is not unlikely that this observation reflects a developmental regulation in Ca^{2+} signaling because modifications of ligand-induced Ca^{2+} responses by age *in vitro* are already described in other neural cell models (Gafni et al. 2004; He and McCarthy 1994; Takeda et al. 1995) and it is known that glutamatergic and GABAergic neurotransmitter receptors are differentially expressed during prenatal development also *in vivo* (Lujan et al. 2005). In contrast, cells did not respond to high extracellular potassium. These results indicate the presence of functional receptors to ATP, ACh, and glutamate, while voltage-gated Ca^{2+} channels (VGCCs) are either not expressed or not functional.

While it is the most important inhibitory transmitter in the adult CNS, GABA acts depolarizing in neurons of the developing hippocampus and other brain regions (Ben-Ari et al. 2007). The opening of $GABA_A$ receptor channels causes outward movement of chloride at this early stage of development. The resulting depolarization then opens VGCC and results in intracellular calcium transients. The same is true for astrocytes both in early postnatal development as well as in mature brain (Bernstein et al. 1996; Meier et al. 2008). However, in none of the cells grown from the neurosphere, application of GABA induced an increase in $[Ca^{2+}]_i$, suggesting the absence of functional GABA receptors. Taken together, these findings show that hNPCs provide a suitable model to investigate the effects of chemicals on $[Ca^{2+}]_i$.

Exposure to BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 caused Ca^{2+} transients in hNPCs. Thereby, 6-OH-BDE-47 was more potent at lower concentrations than the parent compound. This is in agreement with results in the rat pheochromocytoma cell line PC12 (Dingemans et al. 2008). However, the effects in hNPCs appear to be stronger than in PC12 cells. Already 2 µM BDE-47 or 0.2 µM 6-OH-BDE-47 significantly increases $[Ca^{2+}]_i$ in hNPCs

whereas in PC12 cells rather high concentrations (20 µM BDE-47 and 1 µM 6-OH-BDE-47) were needed to evoke similar responses (Dingemans et al. 2007; Dingemans et al 2008). In both cell models, 6-OH-BDE-47 instigated an initial transient and a late persistent increase in $[Ca^{2+}]_i$, whereas only in human neurospheres a shift of baseline was observed. Effects of PBDEs on Ca^{2+} homeostasis were also seen in SH-SY5Y cells after 1.5 – 24 hours (25.6 µM of the pentabrominated mixture DE-71; Yu et al. 2008) and in microsomes and mitochondria isolated from different rat brain regions after 20 min (ca. 7–20 µM DE-71; Kodavanti and Ward 2005). Although these measurements were carried out by endpoint determination and not in a time kinetic like in the present study, one may assume that Ca^{2+} homeostasis of primary human neural cells is more easily disturbed by PBDEs than immortalized cell lines or cell organelles from rodent brains.

Not only PBDEs, but also the known developmentally neurotoxic polychlorinated biphenyls (PCBs) disrupt Ca^{2+} homeostasis in cultured neuronal cells and brain preparations (comprehensively reviewed in Mariussen and Fonnum 2009). This interference with Ca^{2+} signaling is responsible for PCB-induced oxidative stress and apoptosis *in vitro*. Due to structural similarities between PCBs and PBDEs, disruption of $[Ca^{2+}]_i$ balance might also contribute to PBDE-induced DNT and the involved target structures might be similar. Possible origins for increases in $[Ca^{2+}]_i$ are influx of extracellular Ca^{2+} by VGCCs (Catterall 2000), store-operated Ca^{2+} entry (SOCE) channels (Parekh and Putney, Jr. 2005) or intracellular Ca^{2+} stores, such as ER, mitochondria or the nucleus. Furthermore, loss of membrane integrity can result in an unspecific increase in $[Ca^{2+}]_i$. We ruled out this last possibility in the present study because LDH-activity in the media did not increase upon PBDE treatment. On the contrary, the removal of Ca^{2+} from external saline results in a dramatic decrease in number of cells responding to BDE-47. For the hydroxylated metabolite 6-OH-BDE-47, removal of extracellular Ca^{2+} causes a decrease in the amplitude of the initial increase in $[Ca^{2+}]_i$ and the complete loss of the late persistent increase indicating that these increases originate partly (initial increase) or completely (late increase) from influx of extracellular Ca^{2+}. Inglefield and Shafer (2000b) showed that PCB-induced calcium oscillations in cultures of newborn rodent cortical neurons are generated via extracellular Ca^{2+} influx through L-type VGCCs. Moreover, the observed PCB-induced Ca^{2+} oscillations were attributed to increased excitatory synaptic activity of excitatory glutamate and GABA receptors (Inglefield and Shafer 2000a). As hNPCs do not possess functioning VGCCs or GABA receptors

(Fig. 1), these proteins cannot be involved in the increase in $[Ca^{2+}]_i$ after PBDE exposure of hNPCs. Though it is possible that SOCE channels are involved, it remains to be elucidated which specific channels mediate the PBDE-induced increase in $[Ca^{2+}]_i$.

We demonstrate that the residual BDE-47-induced $[Ca^{2+}]_i$ influx was completely inhibited when ER Ca^{2+} transport was blocked (Fig. 4) supporting the findings in PC12 cells that parts of the increase in $[Ca^{2+}]_i$ after BDE-47 exposure is due to ER Ca^{2+} release (Dingemans et al. 2008). The mechanisms affecting release of Ca^{2+} from ER stores involve activation of ryanodine (RyR) and/or inositol 1,4,5-triphosphate receptors (IP_3R). Wong et al. (1997) reported that *ortho*-PCBs induce a ryanodine-sensitive Ca^{2+} mobilization in membrane preparations from rat brain. Thereby, the *ortho*-PCB (PCB 95) interacts with FK506 binding protein (FKBP12) and ryanodine receptor complex (RyR) and sensitizes RyR-mediated Ca^{2+} release (Gafni et al. 2004; Wong et al. 2001). Microarray expression analysis revealed the presence of brain–specific RyR-3 mRNA in hNPCs (Moors & Fritsche, unpublished observation). As neurosphere stimulation with the direct RyR agonist 4-chloro-m-cresol (CMC, 500 µM) or the receptor sensitizer caffeine (20 mM) did not elicit an increases in $[Ca^{2+}]_i$ (data not shown), the RyR seems not to be involved in PBDE-induced Ca^{2+} release. If IP_3R is involved in PBDE-induced ER Ca^{2+} release - as was shown for PCBs earlier (Inglefield et al. 2001; Kang et al. 2004) - is subject of future investigations.

After depletion of extracellular Ca^{2+} and ER Ca^{2+} stores 20 µM 6-OH-BDE-47 still produced a shift of baseline (Fig. 4). Because this shift disappears after co-administration of the mitochondrial uncoupler FCCP, we suggest that these increases in $[Ca^{2+}]_i$ originate from mitochondrial Ca^{2+} stores. However, in approximately 90% of the cells a small 6-OH-BDE-47-induced increase in $[Ca^{2+}]_i$ remains even after depletion of ER and mitochondrial Ca^{2+} stores exclusively in hNPCs (Ciccolini et al. 2003; Dingemans et al. 2007). The origin of this increase has to be further elucidated.

In summary, exposure to BDE-47 (2 µM) or 6-OH-BDE-47 (0.2 µM) triggers an increase in $[Ca^{2+}]_i$ in hNPCs due to extracellular Ca^{2+} influx and release of Ca^{2+} from ER and mitochondria. Thereby, hNPC seem to be more sensitive to PBDE-induced disturbances of Ca^{2+} homeostasis than PC12 cells. Moreover, the metabolite 6-OH-BDE-47 is at least one order of magnitude more potent than the parent compound BDE-47. We calculated that infant exposure could result in a brain concentration of 0.5 – 1.1 µM PBDE (Schreiber et al. 2010). Considering that approximately 45% of detectable PBDEs are present as metabolites in human fetal blood (Qiu et

al. 2009), OH-PBDE-effects on hNPCs in our study are observed at relevant concentrations close to human exposure.

Thus, this study together with our previously published data that PBDEs disrupt cellular thyroid hormone signaling of hNPCs (Schreiber et al. 2010) provides two possible mechanistic explanations for the epidemiologic findings that PBDEs cause DNT in humans (Herbstman et al. 2009; Roze et al. 2009).

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Figure legends

Figure 1: Differentiated hNPCs express functional neurotransmitter receptors. (**A**) Representative traces of ratiometric calcium imaging of 24 h differentiated NPCs that were constantly perfused with artificial saline. Arrows indicate application of neurotransmitters for 1 s. In case of glutamate and GABA, the graph was smoothed by calculating the mean fluorescence of three timepoints. (**B**) Occurrence and amplitudes of ATP, ACh and glutamate-induced $[Ca^{2+}]_i$-increases on different differentiation time points under constant perfusion and after bath application (b.a.). Data are from 3-4 independent experiments (mean ± SE). Numbers above bars indicate the number of cells used for the data analysis. * Significances compared as indicated ($p < 0.05$).

Figure 2: BDE-47 and 6-OH-BDE-47 cause an increase in $[Ca^{2+}]_i$ in human neurospheres during acute exposure. Results are shown as representative traces of normalized F340/F380 ratio, from individual cells exposed to 0.2 to 20 µM BDE-47 (**A**) or 0.02 to 20 µM 6-OH-BDE-47 (**B**) for 20 min, applied at t = 5 min, as indicated by the bars on top of the recordings. Note the difference in scaling for 20 µM. (**C**) Representative recording with the characteristic initial transient increase, the late persistent increase and the shift of baseline in $[Ca^{2+}]_i$ used in this article.

Figure 3: Quantification of $[Ca^{2+}]_i$ disturbances and amplitude during exposure to BDE-47 or 6-OH-BDE-47. (A, B) Bars indicate the percentage of cells showing an increase in $[Ca^{2+}]_i$ within 20 min after application (threshold ≥ 1.2). (C, D) Bars indicate average and amplitudes of increase in $[Ca^{2+}]_i$ after application. Data are from 3-7 independent experiments (mean ± SE). Numbers above bars indicate the number of cells used for the data analysis. * Significances compared as indicated ($p < 0.05$).

Figure 4: Acute exposure to BDE-47 or 6-OH-BDE-47 leads to release of Ca^{2+} from intracellular stores in differentiated hNPCs. Results are shown as representative traces of $[Ca^{2+}]_i$ measurements of individual cells exposed to 20 µM 6-OH-BDE-47 (A) or BDE-47 (B) for 15 min, applied at t = 5 or 15 min. The diagrams show responding cells (C) and the amplitude (D). Data are from 3-7

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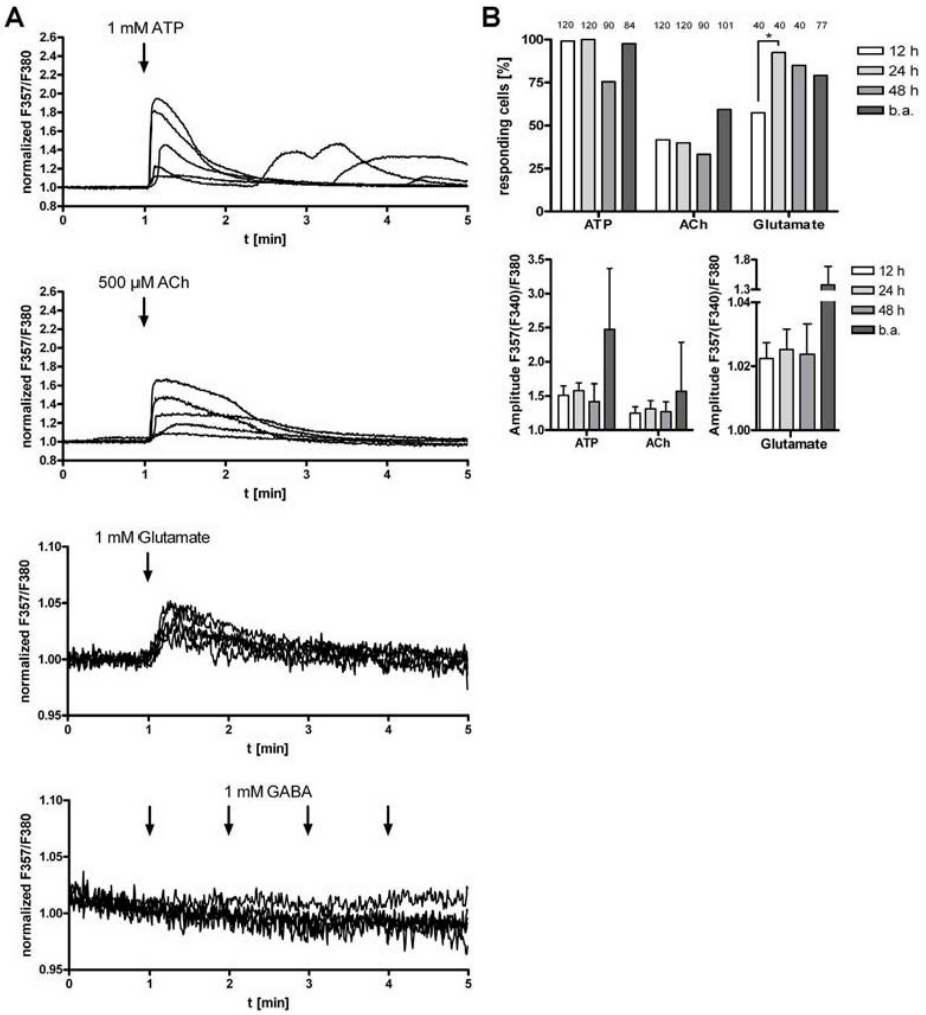

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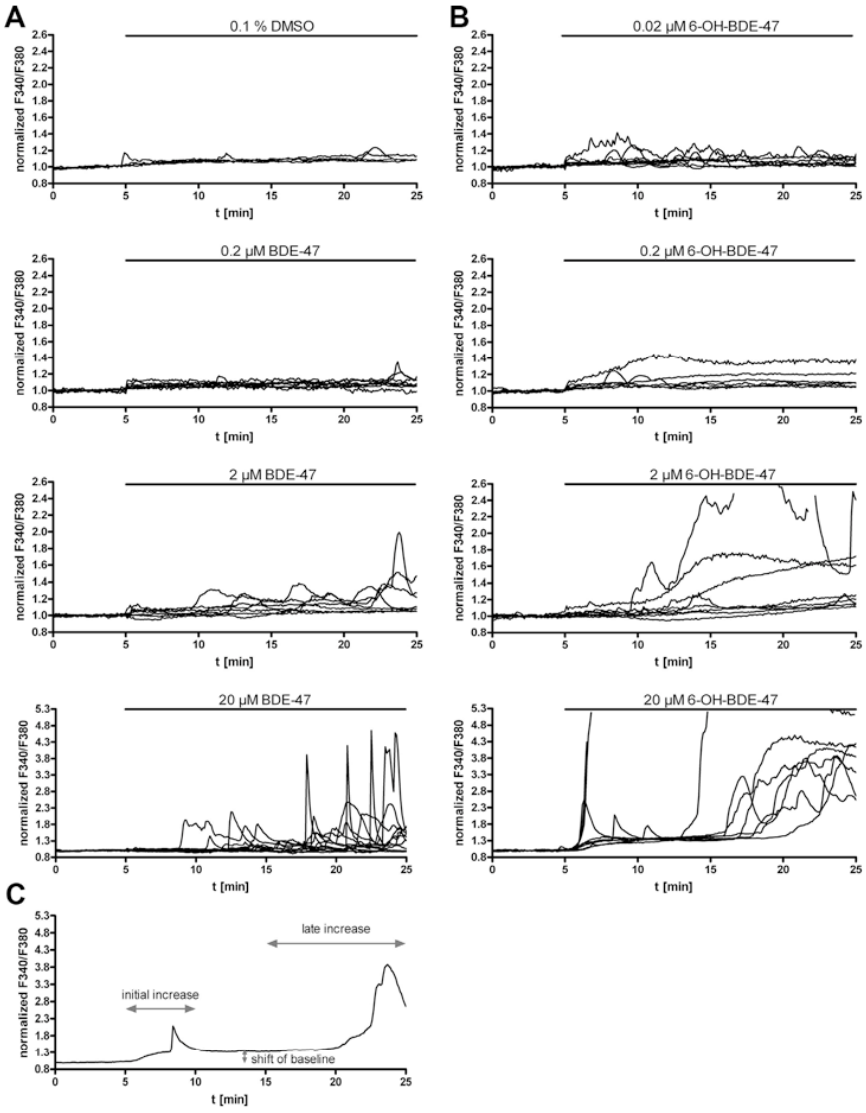

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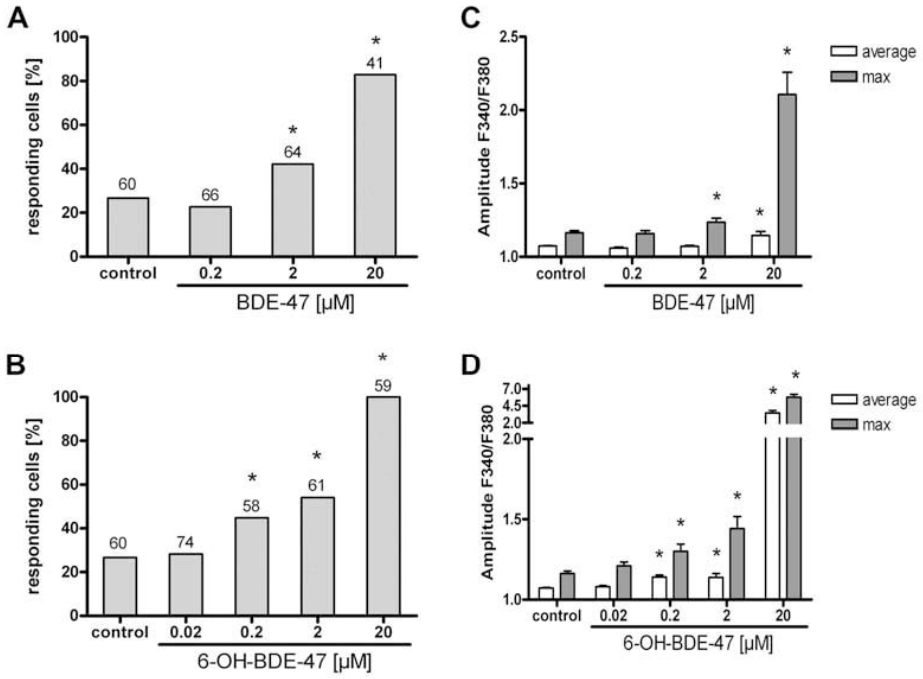

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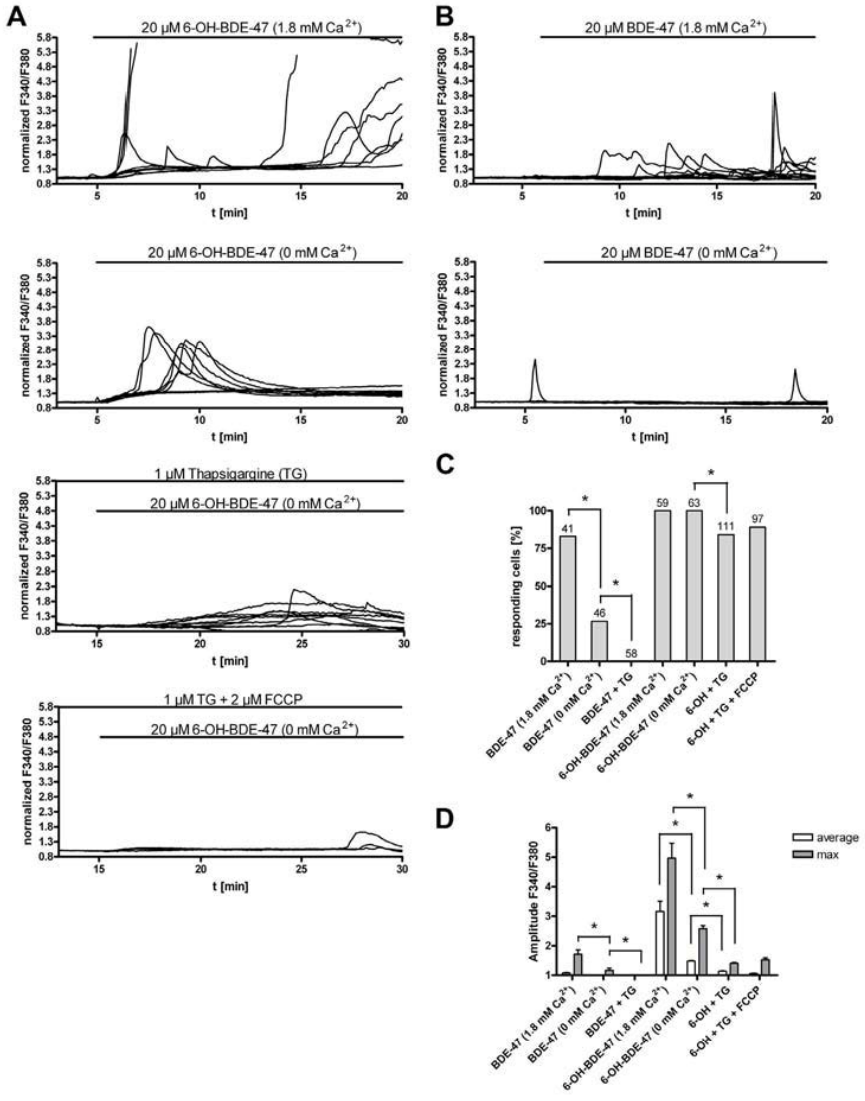

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197x252mm (300 x 300 DPI)

Supplemental Material

Supplementary Figure 1: Electrophysiological characterization. Leak-subtracted membrane currents in a voltage-clamped hNPC in response to a rectangular voltage step protocol from -100 to +50 mV in 10 mV increments, preceded by a hyperpolarization to -110 mV as indicated by the inset. Holding potential was -80 mV.

Supplementary Figure 2: PBDEs are not cytotoxic to hNPCs after 20 min. Quantification of cytotoxic effects of PBDEs measured by lactate dehydrogenase release. The LDH release was measured 20 min after exposure to the indicated concentrations and compared to the max LDH release after cell lysis. All data are mean ± SEM of three independent experiments. * Significances compared as indicated ($p < 0.05$).

Neural Development in Mice and Men: Same But Different?

Timm Schreiber, Kim Quasthoff, Thomas Rockel, Christine Götz, Ulrike Hübenthal,
Susanne Giersiefer, Hans Merk, Ngoc-Ha Nguyen, Thomas Scanlan,
Josef Abel, Ellen Fritsche

[In preparation]

Neural Development in Mice and Men: Same But Different?

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Abbreviations

BDNF: Brain-derived neurotrophic factor

CaMKII: Ca^{2+}/calmodulin dependent protein kinase

CGN: Cerebellar granule neurons

EGF: Epidermal growth factor

GSH: Glutathione PDL: Poly-D lysin

hNPC: human neural progenitor cell

msNPC: murine neural progenitor cell

Nsph: Neurosphere

PBDE: Polybrominated dephenyl ether

PCB: Polychlorinated biphenyls

rhFGF: recombinant human fibroblast growth factor

T_3: Triiodothyronine

TH: Thyroid hormone

THR: Thyroid hormone receptor

Abstract

Animal studies are the gold standard in toxicology, although they have limitations and extrapolation of the data to human responses is often unsatisfactory. Therefore, human *in vitro* models are needed to support data from animal testing. Within the last years, we established a human neurosphere-based *in vitro* system, which is apt of modelling basal processes of brain development like proliferation, migration and differentiation. Recently we also established a murine neurosphere culture for a direct comparison of species under the same experimental conditions. In this study we demonstrated that murine neural progenitor cells (NPCs) developed faster than their human counterparts and mimiced species-specific differences *in vitro*. Additionally, we investigated the effects of polybrominated diphenyl ethers (PBDEs), a known developmental neurotoxicant, on the neural development of mice *in vitro*. We showed that murine NPCs were less sensitive to DNT properties of PBDEs than their human counterparts, which was due to differences in cellular thyroid hormone signaling.

Introduction

For detection of disturbances of neuronal development by chemicals, animal studies are the gold standard in toxicology. However, the predictability of animal experiments for humans is often unsatisfactory due to species differences. It has been estimated that up to 40% of potential drugs failed during early clinical trials because of unsuitable pharmacokinetic features[1,2]. Additionally, an international validation study compared results from animal studies with information from poisoning centers and found a coefficient of correlation of 0.56 between the LD_{50} in rats and lethal concentrations of the same chemical in human blood[3]. Therefore, the European REACH legislation demands alternative human *in vitro* models to identify chemicals that are hazardous to human health. Others and we are developing human cell culture models that are suitable to identify hazardous chemicals. One problem of these human-based *in vitro* models is the interpretation of data. When *in vitro* results differ from animal data, it is important to distinguish if there are species or *in vitro* - *in vivo* differences. One way to address this problem is a comparison of the same *in vitro* system from animals and humans.

Within the last years, we established a human (GW 16) neurosphere-based *in vitro* system that is apt of modelling basal processes of brain development like proliferation, migration and differentiation[4,5,6]. Moreover, these processes can be modulated by developmental neurotoxicants. Thus, they are a three-dimensional cell system, which is a promising approach for DNT testing. Recently, we also established a mouse neurosphere culture (E16 and PND3) for a direct comparison of species under the same experimental conditions. In this study, we demonstrated that murine neural progenitor cells (NPCs) developed faster than their human counterparts and mimiced species-specific differences *in vitro*. Additionally, we investigated the effects of polybrominated diphenyl ethers (PBDEs), a known developmental neurotoxicant, on the neural development of mice *in vitro*. PBDEs are persistent and bioaccumulating brominated

flame-retardants that are known to alter neurobehaviour of the offspring when administered during pregnancy ranging from altered motor activity over changes in sweet preference to impairment of cognitive functions[7]. Moreover, PBDEs are found to cause disturbances of neural development in our human neurosphere-based *in vitro* system[8]. Here we showed that murine NPCs were less sensitive to DNT properties of PBDEs than their human counterparts, which was due to differences in cellular thyroid hormone signaling.

Material and Methods

Chemicals. BDE-47 and -99 were diluted in DMSO at stock concentrations of 1, 10 and 100 mM. T3 (Sigma-Aldrich, Munich, Germany) and NH-3[20] were diluted in ethanol (300 mM) and DMSO (1 mM), respectively.

Cell culture. Normal Human Neural Progenitor cells (hNPCs, Lonza Verviers SPRL, Belgium) generated from GW16 were cultured as free-floating neurospheres in proliferation medium as previously described[4,5,6]. Differentiation was initiated by growth factor withdrawal in differentiation medium and plating onto a poly-D-lysine/laminin matrix.

Murine progenitor cells were isolated from embryos of C57Bl/6 mice at day 16 (E16) of gestation or 3 days postnatal (PND3) and transferred to phosphate-buffered saline (PBS, Invitrogen GmbH, Karlsruhe, Germany). The age of the embryos was determined according to the staging criteria of Theiler, in which embryonic day 16 (E16) corresponds to Theiler stage 24[32]. Embryonic brains were dissected and transferred to Dulbebecco´s modified Eagle medium (DMEM, Invitrogen GmbH, Karlsruhe, Germany). The tissue was enzymatically digested with trypsin-EDTA (Invitrogen GmbH, Karlsruhe, Germany) for 20 to 30 minutes at 37°C to obtain single cell suspensions. After centrifugation for 5 minutes at 1000g, the pellets were resuspended in

proliferation medium. Afterwards, the murine spheres were cultivated under the same conditions as human spheres.

Viability assay. Cell viability was measured using the Alamar Blue Assay (CellTiter-Blue Assay, Promega, Madison, WI, USA) and the CytoTox-One Assay (Promega, Madison, WI, USA) according to the manufacturer's description.

Proliferation analyses. For proliferation analyses, spheres were cultured in proliferation medium with or without 20 ng/ml EGF/rhFGF as previously described[6]. After 0, 7 and 14 days, sphere size was determined by software analyses with the Metamorph analysis software package (Universal Imaging Corp., West Chester, PA, USA).

Migration assay. For analyses of msNPC migration, the distance from the edge of the sphere to the furthest migrated cells was measured after 24 and 48 hours[5]. Living cells migration analyses were performed as previously described[6].

Immunocytochemistry. After differentiating the cells were fixed in 2% paraformaldehyde for 30 min and stored in PBS at 4°C until immunostaining was performed as previously described[8]. For stainings with markers for radial glia, spheres were plated on a PDL/laminin matrix in proliferation medium for 5 hours. Afterwards spheres were fixed in 2% paraformaldehyde for 30 min and stained with primary antibodies (Nestin, 1:50; BD Science, Franklin Lakes, NJ, USA; GFAP, 1:100, Sigma; GLAST, 1:500, Chemicon, Temecula, CA, USA) at 4°C over night. Second antibody-staining was performed as previously described[8].

^{14}C-BDE-47 accumulation. PBDE accumulation was measured by liquid sintilation counting as previously described[8].

RNA preparation and reverse transcriptase-polymerase chain reaction. Total RNA was prepared from 5 differentiated neurospheres using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA). Real-time reverse-transcriptase-polymerase chain reaction (RT-PCR) was performed as previously described[33]. For primer sequences see supplemental material.

Statistics. For multifactor analyses ANOVA in combination with the Bonferroni post hoc test was used. Student's *t*-test was used for two group comparison. The significant value was set at $p \leq 0.05$.

Results

Murine NPCs proliferated faster than their human counterpart. We used the neurosphere assay[9] to investigate species-specific differences in development of neural progenitor cells (NPCs). We compared human NPCs (GW16) with their murine correlates (E16 and PND3) under exactly the same experimental conditions. After NPC preparation (mouse) or defrosting (human) neurospheres were formed within several days, and they proliferated after stimulation with EGF and FGF over time. Interestingly, murine NPCs proliferated significantly faster than their human counterparts up to a size of approximately 700 µm within 7 days. In contrast, human neurospheres grew constantly over the experimental time of 14 days and stopped to increase at a size of approx. 1200 µm (Figure 1).

Murine NPCs migrated faster than human cells and showed a different morphology of the migration area. After mitogen withdrawal, spheres of both species attached to the protein matrix, and cells started to migrate out of the spheres. Within 24 hours, murine cells traveled over a distance of 512.6 ± 17.4 (E16) and 604.2 ± 30.9 µm (PND3). In contrast, human cells migrated significantly slower and traveled over a distance of 337.9 ± 6.7 µm (Figure 2). This difference

remained after 48 hours. Although murine cells of E16 and PND3 migrated over a distance of 857.1 ± 27.0 and 848.7 ± 33.5 µm, human cells migrated only over a distance of 702.3 ± 14.2 µm. Moreover, human and murine cells showed different morphologies of the migration area. In human neurospheres, long processes that protrude out of the sphere followed by highly directed radial migrating cells characterized migration. This radial morphology of the migration area endured over the experimental time, and cells showed a radial glia like morphology. On the contrary, these processes were missing in murine neurospheres (E16 and PND3), and migration seemed to be more undirected [Supplemental Material, Movie 1].

Differentiation of murine and human NPCs. After mitogen withdrawal, cells migrated out of the sphere and differentiated to the three major cell types of the brain, neurons, astrocytes and oligodendrocytes (Figure 3). However, murine cells differentiated faster than human cells. Within 2 days of differentiation, morphologies of major cells were found, multipolar neurons, extensive astrocytes and membrane sheet-forming oligodendrocytes. In humans, extensive astrocytes and membrane sheet-forming oligodendrocytes could be found after 7 days of differentiation. Multipolar neurons were not found in human culture and neurons remained in a small bipolar shape even after 7 days. Interestingly, human oligodendrocyte precursor cells (OPCs) expressed the O4 epitope already in an early bipolar stage, whereas murine OPCs expressed O4 only in a later multipolar stage (data not shown). After 2 days of differentiation, 8.23 ± 0.54% (E16) and 11.06 ± 0.39% (PND3) of all cells in murine spheres were immunoreactive for β(III)tubulin. In human spheres 12.11 ± 2.42% of all cells were immunoreactive after 2 days and 29.41 ± 3.07% after 7 days (Figure 3b). Additionally, in murine spheres 5.56 ± 1.27% (E16) and 7.36 ± 0.49% (PND3) of all cells were positive for O4 after 2 days. Within that time only 0.17 ± 0.17% were immunoreactive in human spheres, after 7 days 5.69 ± 0.81% were positive. In murine spheres, cells started to form secondary cell aggregates after 4 days of differentiation that starts to detach

from the culture dish over time. Therefore, murine spheres were not analyzed after 7 days of differentiation.

To confirm that radial glial cells guided human migration, we plated neurospheres in proliferation medium, as EGF is an important factor for radial glia differentiation[10]. Within 5 hours the characteristic long processes were formed that were immunoreactive for nestin, GFAP and GLAST, common markers for radial glial cells (Figure 3c). Therefore, we state that radial glial cells guided human migration *in vitro*, in contrast to murine migration.

Effects of PBDEs on msNPCs development. To investigate viability after PBDE exposure, murine neurospheres (E16 and PND3) were pre-incubated under proliferative conditions for 2 days with concentrations from 0.1 to 10 µM BDE-47 or BDE-99, followed by differentiation in presences of PBDEs for 2 days. Neither mitochondrial activity nor release of lactate dehydrogenase changed significantly compared to the DMSO controls [see Supplemental Material, Figure 1]. Therefore, we concluded, that BDE-47 or BDE-99 did not cause cytotoxicity of msNPC. To determine msNPC proliferation, neurospheres were cultured with different concentrations of BDE-47 or BDE-99 (0.1 – 10 µM) for 7 days. Increase in cell number was determined by measuring sphere diameter[6]. Whereas spheres cultured without mitogens did not increase in diameter at all, PBDEs showed no effects on proliferation in comparison to solvent controls [Supplemental Material, Figure 2]. To measure migration, spheres were pre-treated with PBDEs under proliferative conditions; afterwards, differentiation was initiated in presence of PBDEs by mitogen withdrawal. The solvent control migrated over a distance of 817.4 ± 48.3 µm (E16) and 816.1 ± 37.4 µm (PND3) within 48 hours. However, treatment with different concentrations of BDE-47 or BDE-99 did not significantly affect migration distance (Figure 4a). For assessment on msNPC differentiation, spheres were pre-treated with PBDEs for 2 days under proliferating conditions. After 2 additional days of differentiation under PBDE exposure, we performed

immunocytochemical stainings for β(III)-tubulin (neurons, Figure 4b) and O4 (oligodendrocytes, Figure 4c). Manual counting of immunopositive cells revealed that 8.24 ± 0.54% (E16) and 10.80 ± 0.40% (PND3) of the control cells were positive for β(III)tubulin. Neither BDE-47 nor BDE-99 affected neurogenesis in msNPCs. In contrast to neurogenesis, exposure of msNPCs to PBDEs showed an inhibitory effect on oligodendrogenesis. While 5.56 ± 1.27% (E16) and 7.17 ± 0.56% (PND3) of all differentiated cells were immunoreactive for O4 after 2 days of differentiation in control cultures, there was a dose-dependent decrease in O4+ cells after PBDE exposure. Even so, only 10 µM BDE-99 decreased the number of oligodendrocytes significantly to 1.97 ± 0.50% (E16) and 2.73 ± 0.16% (PND3). Embryonic and postnatal murine NPCs showed the same sensitivity towards PBDE exposure; therefore we investigated the mechanism of PBDE toxicity only in neurospheres from embryonic mice.

In human neurospheres, the effects of PBDEs could be rescued with co-application of T38. To investigate if this was also true for murine neurospheres (E16), we analyzed the number of oligodendrocytes 2 days after differentiation had been initialized under exposure to 10 µM BDE-99 in combination with 3 nM T3. Whereas 5.67 ± 0.29% of all cells appeared immunoreactive for O4 in the solvent control (0.01% DMSO + 0.01% ethanol), after BDE-99 treatment only 2.26 ± 0.31% remained immunoreactive (Figure 5). Co-application of T3 rescued the effect of BDE-99 and increased the number of oligodendrocytes to 6.50 ± 0.32%.

Effects of PBDEs on hNPCs. Recently, we showed that PBDEs disturb human neural development *in vitro*[8]. According to the observations that mouse NPCs developed faster than their human counterparts, we changed the experimental procedure for msNPCs and shortened exposure times for proliferation to 7 days and for differentiation to 2 days pre-incubation and 2 days under mitogen withdrawal (instead of 14 days for proliferation and 7 + 7 days for differentiation). To exclude that those differences in the experimental procedure caused the

observed differences in response to PBDE exposure, we analyzed the effects of 10 µM BDE-99 on neurogenesis of hNPCs according to the exposition scheme for murine neurospheres. Although the number of neurons in the culture appeared to be significantly smaller within 2 days of differentiation (17.40 ± 1.16% vs. 27.05 ± 4.61% of all cells after 7 days), the effect of BDE-99 remained approximately the same [Supplemental Material, Figure 3a]. Additionally, we determined the NOAEL for the effects of BDE-99 on human neurogenesis, which was actually 0.01 µM [Supplemental Material, Figure 3b]. Moreover, in order to determine if accumulation of PBDEs was different between species, we performed accumulation studies with ^{14}C-BDE-47. Exposure to 1 µM ^{14}C-BDE-47 for 2 days resulted in an intracellular concentration of 34.5 ± 4.5 µM in murine cells and 45.5 ± 3.7 µM in human cells [Supplemental Material, Figure 4].

Cellular thyroid hormone signaling in murine and human NPCs. To study the involvement of cellular thyroid hormone signaling in the observed species-specific differences, we employed the THR agonist T_3 and the antagonist NH-3 and performed migration analyses. In both species, migration was increased after treatment with 3 nM T_3 to 129.8 ± 4.3% (mouse) and 121.1 ± 1.3% (human) of controls. However, exposure to 1 µM NH-3 resulted in an inhibition of migration only in human spheres (78.2 ± 3.8%), whereas murine spheres remained unaffected (Figure 6a). Moreover, we performed real-time RT-PCR analyses of the entire spheres for the marker of undifferentiated progenitor cells nestin. Stimulation with T_3 decreased expression of nestin significantly in hNPCs as well as in msNPCs in comparison to the controls (Figure 6b). In contrast, NH-3 inhibited differentiation of hNPCs and msNPCs, and led to an increase in nestin expression. However, the effects of T_3 and NH-3 appeared significantly weaker in msNPCs compared to hNPCs. Whereas T_3 caused a decrease in nestin expression to 5.3 ± 3.4% of control in human cells, it caused a decrease to 42.1 ± 11.8% of control in murine cells (Figure 6b). NH-3 increased the nestin expression to 289.9 ± 42.9% (human) and 177.4 ± 27.9% (mouse) of control.

In contrast to our findings in hNPCs, where both investigated PBDE congeners inhibited migration significantly in a concentration-dependent manner[8] (>1 μM), PBDEs caused no inhibition of msNPCs migration (Figure 1). This contradicts a proteomic study by Alm *et al.*, where a single oral dose of BDE-99 given on mouse PND10 caused changes in brain protein expression[12]. It was suggested that this might result in disturbances in cell motility, as one third of these changes in protein expression were related to the cytoskeleton including actin, importance of which for neural migration has been reviewed extensively[13]. However, disturbances of migration and resulting alterations in brain morphology were not analyzed in the study of Alm *et al.* In order to explore the observed differences in the response to the THR agonist T3 and the antagonist NH-3 in human and murine cells, we performed real-time RT-PCR analyses for the thyroid hormone receptor (THR) isoforms α1, β1 and β2. Therefore, we used murine and human neurospheres 24 hours after differentiation was initiated. In hNPCs we detected 64.99 ± 11.8 copies (per 1000 copies of β-actin) of THRα1, 0.73 ± 0.3 copies of THRβ1 and 1.09 ± 0.5 copies of THRβ2 (Figure 4b). In contrast, we detected a higher number of copies of THRα1 in msNPCs (0.285 ± 0.071) and a lower number of copies of THRβ1+2 (0.001 ± 0.0) in murine neurospheres (Figure 6c).

Discussion

Within this study, we showed that neurospheres were an excellent tool to study species-specific differences in neural development *in vitro*. Murine NPCs proliferated faster than their human counterparts, although the maximal size of the spheres remained smaller than of human spheres (Figure 1). Moreover, murine NPCs migrated and differentiated faster than human cells (Figure 2 & 3). In contrast, human spheres built more neurons and showed a longer lifespan than murine spheres. Moreover, migration seemed to be higher regulated and was guided by radial glia in

human cultures. Thus, we demonstrated that murine neural progenitor cells developed faster than their human counterparts and mimicked species-specific differences in vitro. This is, to our knowledge, the first report that compared murine and human neural development under the same experimental conditions.

Polybrominated diphenyl ethers (PBDEs) are of concern because rising concentrations of these compounds are found in human tissues, and they impair neurodevelopment in animals[7]. If developmental exposure of humans to PBDEs also causes behavioral abnormalities in children is not known yet. However, we showed that the two PBDE congeners BDE-47 and -99 disturbed neurodevelopment in vitro in primary human neural progenitor cells[8]. These data were supported by a cohort study that showed that children who had higher PBDE cord blood concentration (BDE-47, -99, and -100) scored lower on tests of mental and physical development[11].

PBDEs (0.1 – 10 μM) were not cytotoxic for msNPCs over a period of 4 days [Supplemental Material, Figure 1]. This is in agreement to our findings in hNPCs, were PBDEs caused no cytotoxicity over a period of 2 weeks[8], and to findings of other groups in rodent and human cells7. Neurosphere proliferation was also not affected by PBDE exposure [Supplemental Material, Figure 2]. This corresponds to our results in hNPCs[8].

Similar to species-specific PBDE effects on neural migration, we also observed differences in the effects on neurogenesis. Whereas neurogenesis in hNPCs was inhibited by PBDE exposure in a dose-dependent manner[8], we did not find any effects in PBDE exposed msNPCs (Figure 2). For human neurospheres, neurogenesis was the most sensitive endpoint with a LOAEL for BDE-99 of 0.1 μM. In this study, we determined the NOAEL for hNPCs with a concentration of 10 nM, whereas the NOAEL for msNPCs was actually 10 μM. Thus, the human cells were three orders of magnitude more sensitive than their murine counterparts. In contrast to the observations on neurogenesis, we found a concentration-depended inhibition of oligodendrocyte differentiation in

msNPCs (Figure 2). However, the observed effects appeared remarkably smaller than in hNPCs[8]. Whereas in hNPCs oligodendrogenesis is inhibited significantly at a concentration of 1 µM BDE-47 as well as BDE-99, in msNPCs a significant inhibition occurred only after exposure to 10 µM BDE-99 (Figure 2). Some other studies showed that species-specific differences towards neurotoxic substances existed. In a recent study, species-dependent modulation of copper-mediated neurotoxicity by amyloid precursor protein (APP) was observed[14].

In the past it was hypothesized that mice showed an enhanced vulnerability during a defined period of brain development, the so-called brain growth spurt (BGS). This was confirmed by a study of Xing et al., who showed that postnatal mice were more susceptible to PBDE-induced changes in synaptic plasticity than embryonic mice[15]. This contradicts the results of our study, where neurospheres of embryonic and postnatal mice showed the same sensitivity to PBDE-induced neurotoxicity. However, this might be due to two independent mechanisms.

In human neurospheres, the toxic effects of PBDEs were mediated by disruption of thyroid hormone signaling. Here, we investigated if the effects of PBDEs in murine spheres could be rescued with T_3 as well. Co-application of 3 nM T_3 completely rescued the effects of 10 µM BDE-99 in msNPCs (Figure 3). During the fetal and neonatal stages, TH deficiency delays oligodendrogenesis and leads to a permanently lower number of myelinated axons, affecting different steps of myelin sheath formation[16,17,18]. Thus, hypothyroidism during development causes a large number of neuroanatomical and behavioral effects[19,20,21]. Due to similar neurobehavioral alterations observed after PBDE exposure in rodents, endocrine disruption of the TH system by PBDEs has been studied intensively. Hypothyroidism of dams and offspring was found in a variety of different studies after pre- or postnatal exposure[7]. If TH supplementation also rescues the neurotoxic effects of PBDEs in vivo is not known yet and has to be investigated using appropriate animal testings.

The data generated in our experiments showed that the two PBDE congeners BDE-47 and BDE-99 directly disturbed migration and differentiation in hNPCs as well as oligodendrogenesis in msNPCs in vitro by endocrine disruption of cellular TH signaling. However, human cells showed an enhanced sensitivity towards neurotoxic effects of PBDEs. Therefore, we analyzed the effects of thyroid hormone receptor agonists (T_3) and antagonists (NH-3) on differentiation of human and murine NPCs. In both cases, reaction of murine cells appeared weaker than reaction of human cells (Figure 6). This is - to our knowledge - the first report showing that human and murine NPCs show different sensitivity to TH agonists and antagonists. However, it is known that humans are born with a full maturation of thyroid system, whereas rodents are born with a less developed thyroid system[22].

To explore these differences in TH signaling we investigated the expression of THR in the neurospheres. Whereas murine spheres expressed little THRα_1 and hardly any THRβ, human cells expressed higher amounts of THRα_1, β_1 and β_2 (Figure 4B). In our previous study, we stated that PBDEs interfered with THRβ signaling of hNPCs, because the THR antagonist NH-3 caused the same disturbances as the tested PBDEs. NH-3 binds to THRβ with a higher affinity then to THRα[23]. Additionally, it is known that an inhibition of THRα leads to decreased proliferation of avian neurogenic precursors[24], and the induction of THRβ induces neural differentiation[25,26]. MsNPCs hardly expressed any THRβ, although oligodendrogenesis was affected by PBDEs. Therefore, we suggested that PBDEs also interfered with the THRα_1, which is expressed in proliferating OPCs[27]. A disturbance of TH signaling through THRα_1 could lead to an inhibition of OPC proliferation, which would result in a lower number of $O4^+$ cells. That proliferation of neurospheres was not affected could be due to the low number of oligodendrocytes in neurospheres (approximately 5% of all cells). The precise mechanism hoe PBDEs interfere with oligodendrogenesis still has to be elucidated.

In summary, BDE-47 and -99 disturbed neural migration and differentiation in human NPCs by disruption of cellular TH signaling. In murine neural progenitor cells, only oligodendrogenesis was affected through the same mechanism. Neurobehavioral disturbances in rodent pups were already observed after administration of a single oral dose in the range of µg/kg body weight. In a study by Kuriyama et al., 0.7 mg/kg led to an increase in motor activity[28], and application of 0.6 mg/kg caused hyperactivity in newborn mice in another study[29]. Disturbances of learning and memory could be observed after a single oral dose of 0.9 mg/kg[30]. Estimated exposure of an infant through breast-feeding is about 0.3 µg/kg/day, with a range of 0.003 - 4.1 µg/kg/day[31]. Therefore, the estimated median intake of an infant is 2000 times lower than the concentrations leading to neurobehavioral changes in mice; the maximal intake is only 150 times lower. Within this study, we showed that human neural progenitor cells were much more sensitive to PBDEs than their murine counterparts due to differences in thyroid hormone system. The NOAEL for effects of BDE-99 on neurogenesis was 1000 times lower in human cells than in murine cells. Thus, PBDEs seemed to pose a serious risk for human health and might cause neurobehavioral disturbances in human children.

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Figures

Figure 1. Proliferation of human and murine neurospheres. (A) Photomicrographs of human and murine neurospheres in suspension cultures. (B) Assessment of proliferation by measuring sphere diameter over time. All data are mean ± SEM of three independent experiments. Scale bar: 100 µm. *, p-value < 0.05.

Figure 2. Migration of human and murine NPCs. (A) Photomicrographs of human and murine NPCs after 48h. Differentiation was initiated under withdrawal of growth factors and plating onto poly-D-lysine/laminin coated chamber slides. (B) Respective quantification of cells migration after 24 and 48 h. All data are mean ± SEM of three independent experiments. Scale bar: 50 µm. *, p-value < 0.05.

Figure 3. Differentiation of human and murine NPCs. (A) Representative immunostainings of cells in the migration area of human and murine neurospheres. Cells were differentiated for the indicated time points and stained with antibodies against β(III)-tubulin (neurons), GFAP (astrocytes), and O4 (oligodendrocytes). Cell nuclei were counterstained with Hoechst. (B) Cellular composition of the neurosphere migration area. The fraction of immunoreactive cells for β(III)-tubulin and O4 was determined relative to the total number of cell nuclei, expressed as percentage fraction. (C) Representative immunostainings of processes that protrude out of spheres after 5h of differentiation stained with antibodies against nestin, GFAP and GLAST. All data are mean ± SEM of three independent experiments. Scale bar: 100 µm. *, p-value < 0.05.

Figure 4. Effects of PBDEs on murine neural progenitor cells. Quantification of cell migration and of immunostainings after PBDE treatment. (A) Migration distance was measured at four defined spots from the edge of the sphere to the furthest migrated cell after 48 hours. Cells were

stained with antibodies against β(III)-tubulin for neurons (B) and O4 for oligodendrocytes (C). Cell nuclei were counterstained with Hoechst. All data are mean ± SEM of three independent experiments. *, p-value < 0.05.

Figure 5. PBDEs disrupt cellular thyroid hormone signalling in murine NPCs (E16). (A) Representative photomicrographs of msNPCs after 2 days of differentiation. Cells were stained with an antibody against O4 for oligodendrocytes. Cell nuclei were counterstained with Hoechst. (B) Quantification of immunostainings after treatment with 10 µM BDE-99 or co-treatment with 10 µM BDE-99 and 3 nM T3. All data are mean ± SEM of three independent experiments. Scale bar: 50 µm. *, p-value < 0.05.

Figure 6. Cellular thyroid hormone signaling in murine and human NPCs. (A) Quantification of migration distance after treatment with 3 nM T3 or 1 µM NH-3. Migration was determined after 48 h of differentiation. Real-time PCR analyses were quantified with a product-specific copy number standard. (B) Relative nestin expression in human and murine neurospheres after treatment with 3 nM T3 or 1 µM NH-3 normalized for ß-actin expression. (C) Number of copies of different THR isoforms in human and murine spheres per 1000 copies of ß-actin. All data are shown as mean ± SEM of three independent experiments. *, p-value < 0.05.

Figure 1

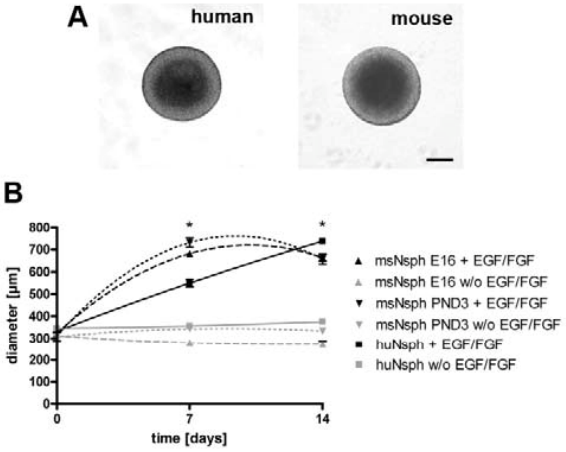

A
human
mouse
B
diameter [µm]
time [days]
msNsph E16 + EGF/FGF
msNsph E16 w/o EGF/FGF
msNsph PND3 + EGF/FGF
msNsph PND3 w/o EGF/FGF
huNsph + EGF/FGF
huNsph w/o EGF/FGF

Figure 2

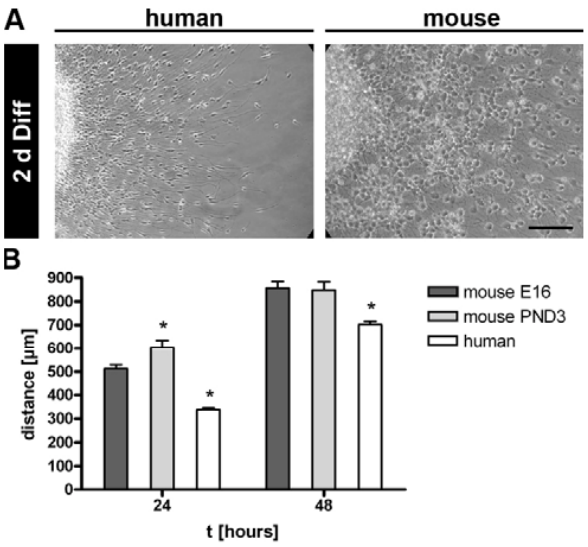

A
human
mouse
2 d Diff
B
distance [µm]
900
800
700
600
500
400
300
200
100
0
24
48
t [hours]
mouse E16
mouse PND3
human
*
*
*

Figure 3

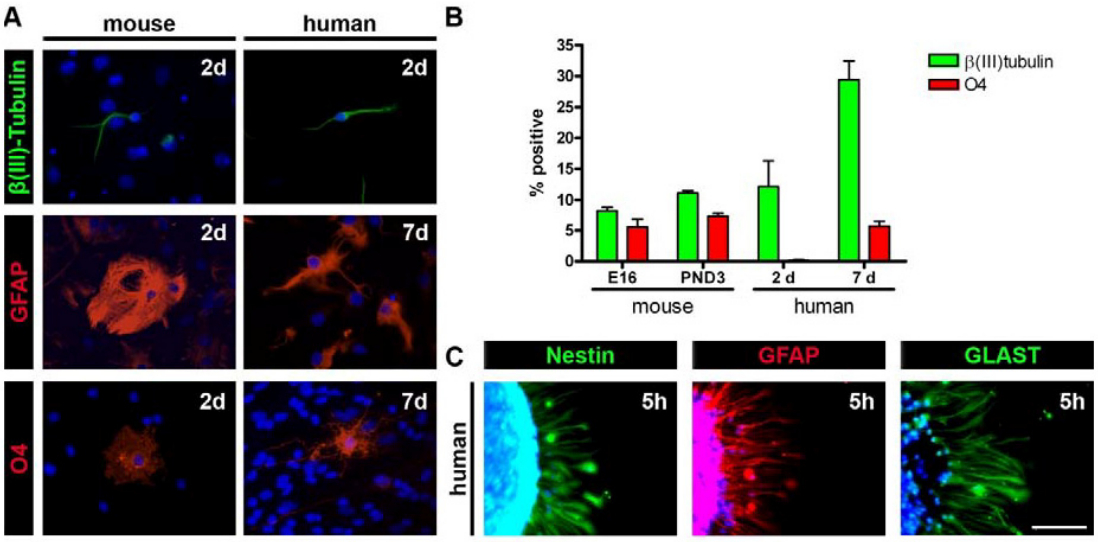

A
mouse
human
β(III)-Tubulin
GFAP
O4
2d
2d
2d
7d
2d
7d
B
% positive
35
30
25
20
15
10
5
0
E16
PND3
2 d
7 d
mouse
human
β(III)tubulin
O4
C
Nestin
GFAP
GLAST
human
5h
5h
5h

Figure 4

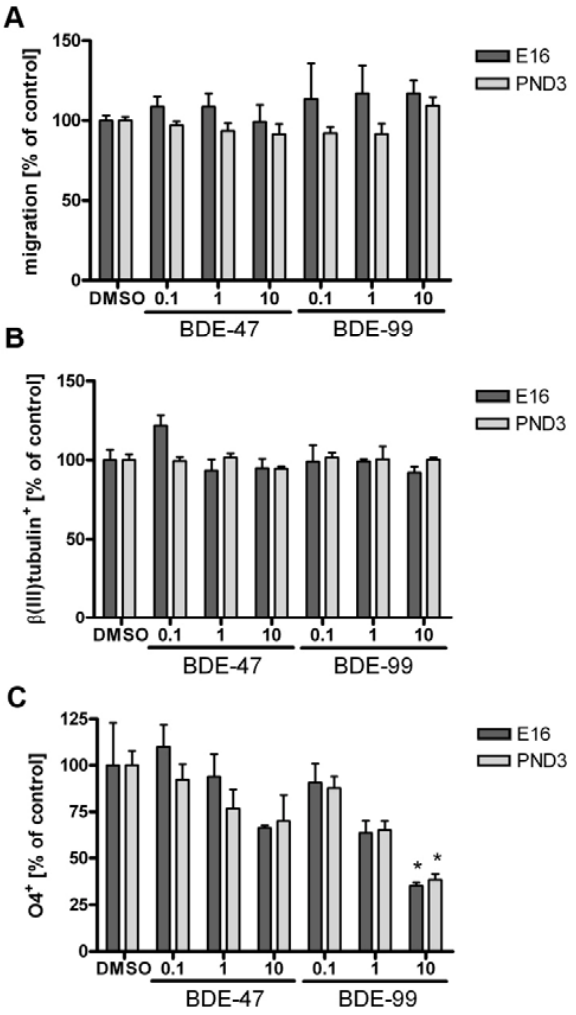

A
migration [% of control]
150
100
50
0
E16
PND3
DMSO
0.1
1
10
0.1
1
10
BDE-47
BDE-99
B
β(III)tubulin+ [% of control]
150
100
50
0
E16
PND3
DMSO
0.1
1
10
0.1
1
10
BDE-47
BDE-99
C
O4+ [% of control]
125
100
75
50
25
0
E16
PND3
*
*
DMSO
0.1
1
10
0.1
1
10
BDE-47
BDE-99

Figure 5

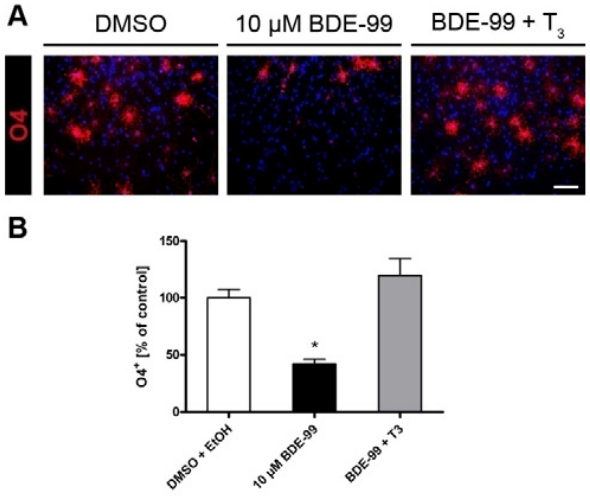
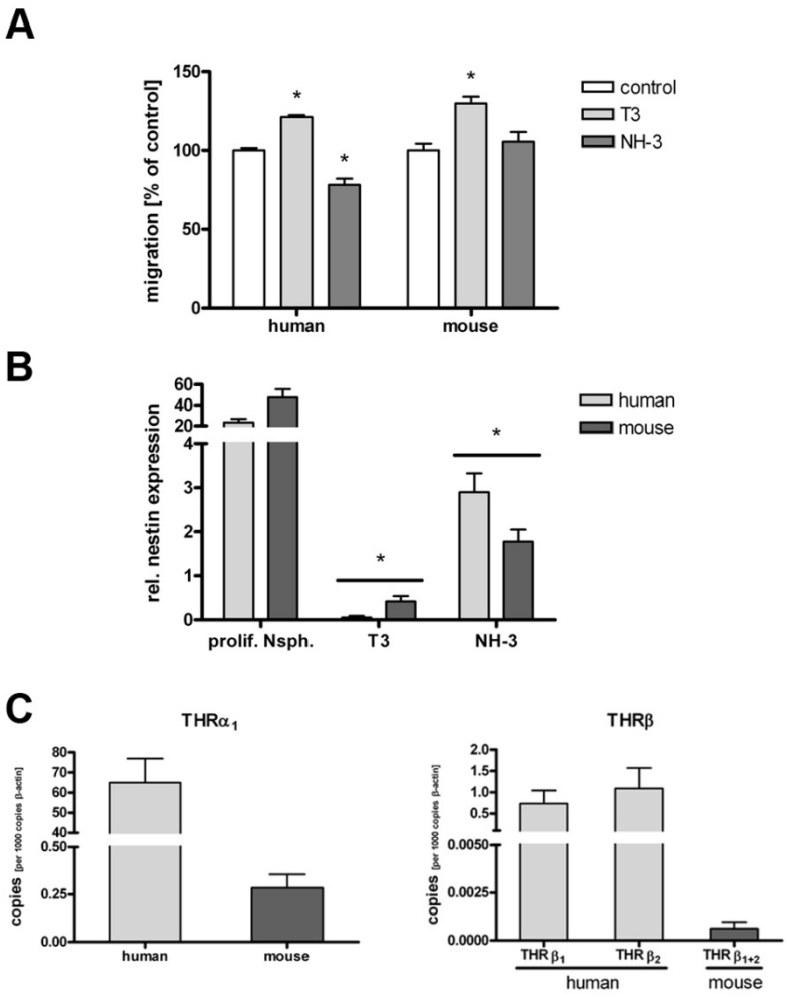

A
DMSO
10 µM BDE-99
BDE-99 + T3
O4
B
O4+ [% of control]
150
100
50
0
*
DMSO + EtOH
10 µM BDE-99
BDE-99 + T3

Figure 6


A
migration [% of control]
150
100
50
0
control
T3
NH-3
human
mouse
B
rel. nestin expression
60
40
20
4
3
2
1
0
human
mouse
prolif. Nsph.
T3
NH-3
C
THRα1
copies [per 1000 copies β-actin]
80
70
60
50
40
0.50
0.25
0.00
human
mouse
THRβ
2.0
1.5
1.0
0.5
0.0050
0.0025
0.0000
THR β1
THR β2
THR β1+2
human
mouse

Supplemental Material

Supplementary Movie 1. Living cell analysis of migrating murine and human neural progenitor cells. Murine (right) and murine (left) neurospheres were grown in a Focht Chamber System 2 installed in an inverted light microscope. Images were acquired every 2 minutes. During the first hours, the sphere settles onto the protein matrix. Subsequently, radial migration takes place. During the 24 hours of observation, cells of different morphology and migration speed can be followed. Furthermore, path finding with vast cell-cell- and cell-matrix interactions are visible.

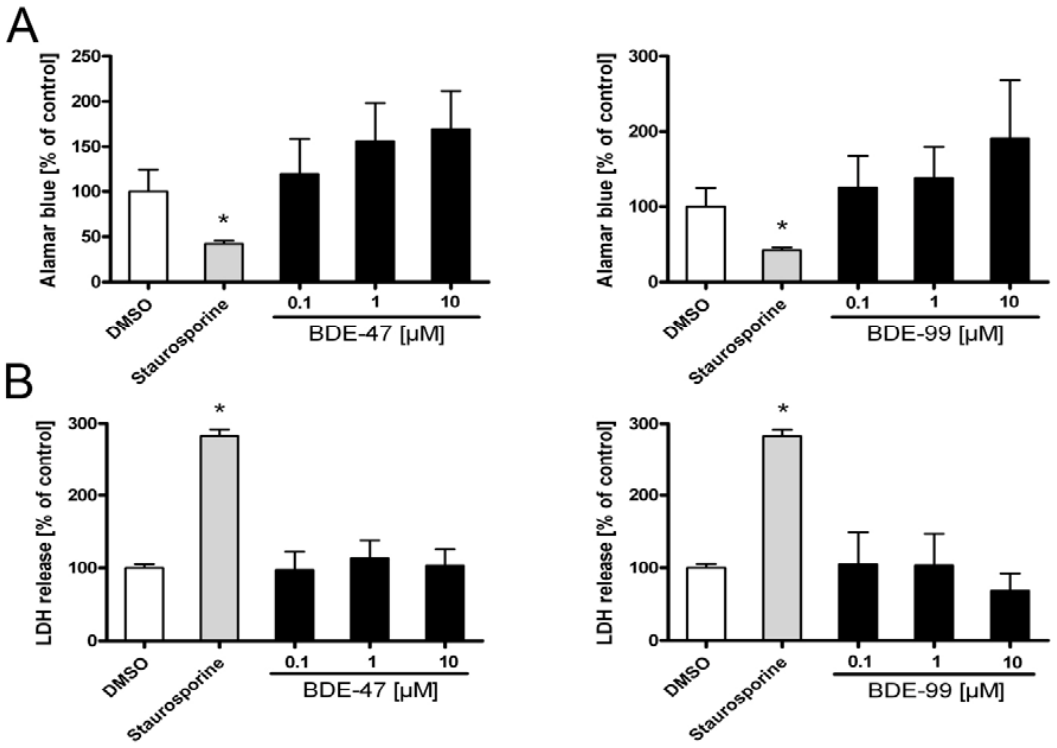
Supplementary Figure 1. PBDEs are not cytotoxic to msNPCs. After 2 days of exposure towards PBDEs in proliferation medium, spheres were plated onto a PDL/laminin matrix under mitogen withdrawal in further presence of PBDEs. Cytotoxicity of PBDEs were measured by the Alamar Blue Assay (A) or lactate dehydrogenase release (B). All data are mean ± SEM of three independent experiments (5 spheres/experiment). *, p-value < 0.05.

Supplementary Figure 2. PBDEs have no effect on msNPC proliferation. Neurospheres were cultured in proliferation medium in presence or absence of PBDEs. Proliferation was quantified by assessment of sphere diameter over time. Growth was determined as difference between the diameters after 0 and 7 days and shown as % of control. All data are mean ± SEM of three independent experiments (6 spheres/experiment). *, p-value < 0.05.

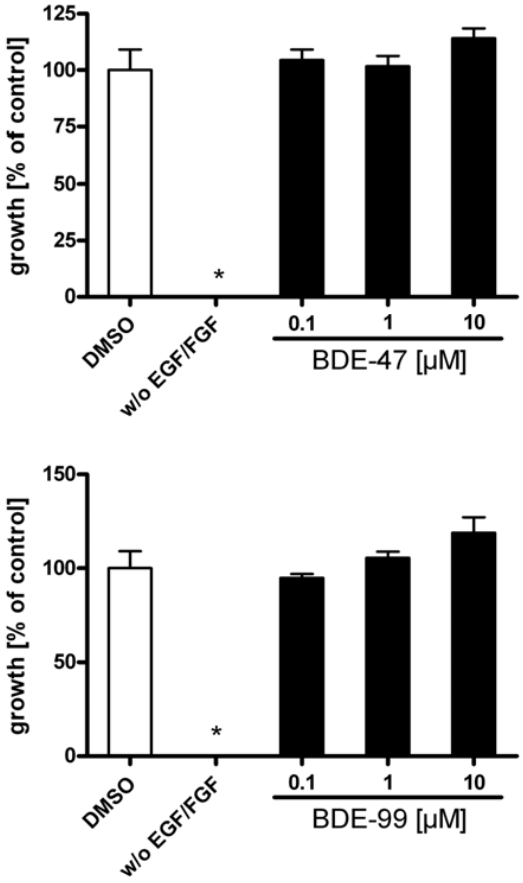
Supplementary Figure 3. PBDEs inhibit differentiation of hNPCs. (A) Quantification of immunostainings after treatment of hNPCs for 7 days with different amounts of BDE-99. (B) Quantification of immunostainings after treatment of hNPCs with 10 µM BDE-99 for indicated time points. All data are mean ± SEM of three independent experiments (5 spheres/experiment). Scale bar: 50 µm. *, p-value < 0.05.

Supplementary Figure 4. ^{14}C-BDE-47 accumulates in human and murine NPCs. After mitogen withdrawal neurospheres were allowed to attach to culture dish for 4h, afterwards cells were exposed to 1 µM ^{14}C-BDE-47 for 2 days (mouse) or 2 and 7 days (human) and half of the media was changed every 2 days. ^{14}C-BDE-47 concentrations were determined by liquid scintillation counting in residual medium and cell lysates. Intracellular ^{14}C-BDE-47 concentrations were calculated after background subtraction (same treatment without spheres) by a standard concentration curve and normalized to sphere volumes. Percent non-specific binding to the culture dish was determined by subtracting intracellular and media ^{14}C-BDE-47 from total ^{14}C-BDE-47 added to the cultures. All data are mean ± min/max of two independent experiments.

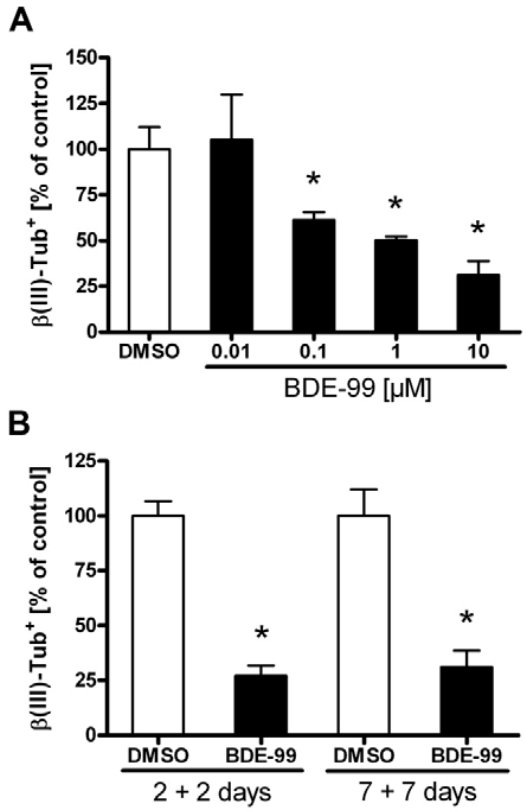
Supplementary Figure 1



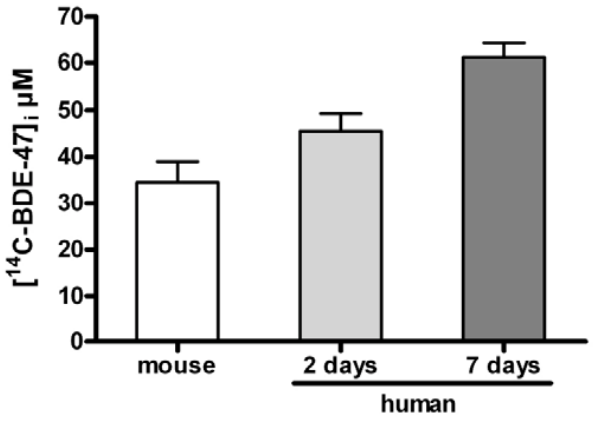
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Human Neurospheres as Three Dimensional Cellular Systems for Developmental Neurotoxicity Testing

Michaela Moors, Thomas Rockel, Josef Abel, Jason Cline, Kathrin Gassmann, Timm Schreiber, Janette Schuwald, Nicole Weinmann, Ellen Fritsche

[*Environ Health Perspect* **117(7)**:1131-1138]

Human Neurospheres as Three-Dimensional Cellular Systems for Developmental Neurotoxicity Testing

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BACKGROUND: Developmental neurotoxicity (DNT) of environmental chemicals is a serious threat to human health. Current DNT testing guidelines propose investigations in rodents, which require large numbers of animals. With regard to the "3 Rs" (reduction, replacement, and refinement) of animal testing and the European regulation of chemicals [Registration, Evaluation, and Authorisation of Chemicals (REACH)], alternative testing strategies are needed in order to refine and reduce animal experiments and allow faster and less expensive screening.

OBJECTIVES: The goal of this study was to establish a three-dimensional test system for DNT screening based on human fetal brain cells.

METHODS: We established assays suitable for detecting disturbances in basic processes of brain development by employing human neural progenitor cells (hNPCs), which grow as neurospheres. Furthermore, we assessed effects of mercury and oxidative stress on these cells.

RESULTS: We found that human neurospheres imitate proliferation, differentiation, and migration *in vitro*. Exposure to the proapoptotic agent staurosporine further suggests that human neurospheres possess functioning apoptosis machinery. The developmental neurotoxicants methylmercury chloride and mercury chloride decreased migration distance and number of neuronal-like cells in differentiated hNPCs. Furthermore, hNPCs undergo caspase-independent apoptosis when exposed toward high amounts of oxidative stress.

CONCLUSIONS: Human neurospheres are likely to imitate basic processes of brain development, and these processes can be modulated by developmental neurotoxicants. Thus, this three-dimensional cell system is a promising approach for DNT testing.

KEY WORDS: apoptosis, differentiation, DNT, human neurospheres, mercury, migration, proliferation. *Environ Health Perspect* 117:1131–1138 (2009). doi:10.1289/ehp.0800207 available via *http://dx.doi.org/* [Online 26 February 2009]

Developmental neurotoxicity (DNT) of environmental chemicals has been recognized worldwide as a serious threat to human health, and the resulting neurologic deficits negatively affect families and society (Goldman and Koduru 2000; Needleman et al. 2002). Current DNT testing guidelines (Organization for Economic Cooperation and Development 2007; U.S. Environmental Protection Agency 1998) propose investigations in rodents, mainly rats. Such a DNT *in vivo* testing strategy implies the use of 140 dams and 1,000 pups and is therefore extremely time- and cost-intensive (Lein et al. 2005). Relying solely on the existing guidelines to address current and anticipated future regulatory demands for DNT of the thousands of chemicals for which there are few to no DNT data would incur unacceptable costs in terms of animals and person-years (Lein et al. 2007). Therefore, according to the "3R principle" (reduction, replacement, and refinement) of Russel and Burch (1959), alternative testing strategies are needed to address animal welfare by refining and reducing animal experiments, and to create affordable, sensitive, and mechanism-based methods suitable for high- or medium-throughput screening (Collins et al. 2008). Furthermore, the inclusion of human-cell–based *in vitro* systems into an integrated DNT tiered testing approach has been recommended to circumvent species differences (Coecke et al. 2007).

To combine transatlantic strengths and avoid doubling of work, a partnership between the Johns Hopkins Center for Alternatives to Animal Testing (Developmental Neurotoxicity TestSmart program), the European Centre for the Validation of Alternative Methods, and the European Chemical Industry Council has been formed. This partnership follows the common goal of "incorporating *in vitro* alternative methods for DNT testing into international hazard and risk assessment strategies" (Coecke et al. 2007). Coecke et al. (2007) provided a comprehensive overview of the existing *in vitro* models and stated that, "although all the test systems described were not developed for regulatory purposes at this stage if they prove useful, we hope that this report will encourage their further development to render them amenable to high-throughput approaches."

Therefore, the aim of this work was *a*) to introduce the cell biological characteristics of human neurospheres as a three-dimensional cell system approach for DNT testing; *b*) to demonstrate that neurospheres are likely to mirror basic processes of brain development, such as proliferation, differentiation, migration, and apoptosis; and *c*) to demonstrate that these processes can be modulated by developmental neurotoxicants.

Materials and Methods

Chemicals. We obtained methylmercury chloride (MeHgCl) from Riedel-de Haën (Seelze, Germany); all other substances were obtained from Sigma Aldrich (Munich, Germany), unless otherwise stated.

Cell culture. Cryopreserved normal human neural progenitor cells (hNPCs; Lonza Verviers SPRL, Verviers, Belgium) were cultured at 37°C and 5% CO_2 as a suspension culture in proliferation medium consisting of Dulbecco's modified Eagle medium (DMEM) and Hams F12 (3:1) supplemented with B27 (Invitrogen GmbH, Karlsruhe, Germany), 20 ng/mL epidermal growth factor (EGF; Biosource, Karlsruhe, Germany), and 20 ng/mL recombinant human fibroblast growth factor (FGF; R&D Systems, Wiesbaden-Nordenstadt, Germany) (Moors et al. 2007). When spheres reached 0.7 mm in diameter, they were chopped up to passage 3 with a McIlwain tissue chopper. Differentiation was initiated by growth factor withdrawal in differentiation medium [DMEM and Hams F12 (3:1) supplemented with N2 (insulin, transferrin, sodium selenite, putrescine, and progesterone; Invitrogen)] and plated onto poly-D-lysine/laminin–coated chamber slides (BD Bioscience, Erembodegem, Belgium).

Chemical exposure. We exposed cells to indirubin (10 µM) in proliferation medium (28 hr), and to cAMP (200 µM), MeHgCl (250 nM to 1 µM), mercury chloride ($HgCl_2$; 500 nM to 10 µM, 48 hr) or staurosporine (0.1 and 1 µM), or hydrogen peroxide (H_2O_2; 0.1 and 1 mM) (24 hr) in differentiation medium. We chose concentration ranges of mercury according to Monnet-Tschudi

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et al. (1996), who found a concentration of 1 µM to be cytotoxic.

Migration analyses. Migration analyses were performed as previously described (Moors et al. 2007). For living cell migration analyses, neurospheres were grown in the Focht Chamber System 2 (Bioptechs, Butler, PA, USA) under temperature- and CO_2-controlled conditions. Images were acquired every 2 min by a Zeiss Axiovert 100 inverted microscope (Zeiss, Goettingen, Germany).

Immunohistochemistry. Proliferating or differentiating spheres were fixed in 4% paraformaldehyde for 30 min. After washing spheres in phosphate-buffered saline (PBS), they were incubated in a 25% sucrose solution (wt/vol) overnight at 4°C. Afterward, spheres were transferred to tissue-freezing medium (Jung HistoService, Nussloch, Germany). Cryostat sections (10 µm) were prepared for immunohistochemistry. Antibodies for staining were nestin (1:150; BD Bioscience), glial fibrillary acidic protein (GFAP; 1:100, Sigma Aldrich), or β(III)tubulin (1:100; Sigma Aldrich).

Immunocytochemistry/differentiation analyses. We performed immunocytochemistry as previously described (Fritsche et al. 2005; Moors et al. 2007). For quantification analyses, we used the Metamorph analysis software package (version 7.1.7.0; Universal Imaging Corp., West Chester, PA, USA). We determined the variation of protein expression by relating area of fluorescence signal to cell number in a region of interest within the migration area. Individual pixels were identified as "positive" if the fluorescence signal exceeded a determined color threshold [green, hue (H) 71–113, saturation (S) 10–255, intensity (I) 10–255; yellow/red, H 0–71, S 10–255, I 10–255)]. To determine the cell number, we selected images for positive 4′,6-diamidino-2-phenylindole (DAPI) staining (blue, H 152–180, S 10–255, I 10–255) and morphologic parameters (integrated morphology analysis: area, $10^4 > n > 10^7$; ellipsoid form factor, $0.1 > n > 1.8$).

Fluorescence-activated cell sorting (FACS)/cell cycle analyses. Proliferating neurospheres were grown in proliferation media with or without growth factors or exposed to indirubin. To obtain a single-cell suspension, neurospheres were washed once in PBS, incubated with Accutase (100%; PAA, Cölbe, Germany) at 37°C for 20 min, and then gently pipetted. The suspension was centrifuged (4°C, 1,400 × *g*, 5 min) and the pellet was resuspended in PBS containing 0.8% paraformaldehyde (Polyscience Inc., Eppelheim, Germany). Cells were fixed for 30 min at 4°C, centrifuged (4°C, 1,400 × *g*, 5 min), resuspended in PBS containing 0.15% saponin and 10 µg/mL RNase, and incubated for 30 min at 37°C. We then added 50 µg/mL propidium iodine 5 min before FACS analyses.

Cell viability, apoptosis, and proliferation assays. We measured cell viability using the CellTiter-Blue assay (Promega, Mannheim, Germany) as previously described (Moors et al. 2007). The assay is based on measurements of the mitochondrial reductase activity by conversion of the substrate resazurin to the fluorescent product resorufin by mitochondrial reductases, which can be assessed in a fluorometer. The lactate dehydrogenase (LDH) assay (CytoTox-One; Promega) assesses cell death by measuring LDH that leaks out of dead cells into the media. We performed the assay according to the manufacturer's instructions. Briefly, supernatants of treated cells were collected and incubated with an equal amount of CytoTox-One reagent for 4 hr before the detection of fluorescence (excitation, 540 nm; emission, 590 nm). Caspase-3/-7 activities were measured with the Apo-One Kit (Promega) according to the manufacturer's instructions. Briefly, cells were lysed and caspase activity was assessed by measuring the cleavage of a caspase-3/-7–specific fluorescent substrate (Z-DEVD-R110) with a fluorometer (excitation, 488 nm; emission, 538 nm).

For proliferation assays, spheres were cultured in proliferation medium supplemented with or without 20 or 100 ng/mL EGF in 96-well plates. We assessed cell viability as a measure for cell number using the CellTiter-Blue assay at different time points. Because the dye caused no acute cytotoxicity, spheres were washed with medium after fluorescence reading and then the same spheres were monitored over a period of 2 weeks. For determination of sphere size, we gauged sphere diameter optically with an object micrometer. We counted the number of cells/sphere after trypsination (0.25% trypsin; Invitrogen) for 2 min.

TUNEL assay. For terminal deoxynucleotidyl transferase 2′-deoxyuridine 5′-triphosphate (dUTP) nick end labeling (TUNEL) assays, we used fluorescein-coupled dUTP and the terminal transferase kit from Roche Diagnostics (Mannheim, Germany) to label DNA strand breaks; nuclei were counterstained with Hoechst 33258 (Invitrogen). Plated neurospheres were exposed to staurosporine (1 µM) or H_2O_2 (1 mM) after 48 hr of differentiation. After another 12 and 24 hr, cells were fixed with 4% paraformaldehyde, washed twice with PBS, covered with reaction mixture (2.5 mM $CoCl_2$, 5 µM fluorescein coupled dUTP, 5,000 U/mL terminal-transferase, 2 µg/mL Hoechst, 0.1% triton in 1× terminal transferase buffer), and incubated at 37°C for 1 hr. Slides were then washed with PBS three times and mounted with PBS/glycerol (1:1). Stained cells were analyzed with a fluorescence microscope.

Statistics. We used analysis of variance combined with the Bonferroni post hoc test for multifactor analyses (time and concentration effects), and the Student's *t*-test for two-group comparisons (treatment vs. control; two time points). The significance value was set at $p < 0.05$. To describe the associations between independent variables (diameter/cell number; diameter/fluorescence), we fitted curves up to the third degree. We used R^2 as a measure of goodness of fit.

Results

Human neurospheres grow floating freely in defined medium without addition of serum [see Supplemental Material, Figure 1A (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)]. Upon withdrawal of growth factor, cells migrate radially out of the sphere onto a poly-D-lysine/laminin matrix, thereby forming a migration area [see Supplemental Material, Figure 1B and the video (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)]. Each cell leaves the sphere edge in a 90° angle and travels away in a straight line. Moreover, cells move toward and away from each other.

To evaluate reproducibility and stability of neurosphere migration, we assessed dependence of migration speed on neurosphere size. Therefore, the distance between the sphere edge and the farthest outgrown cells was measured 24 hr after plating, dependent on different sphere diameters. Supplemental Material, Figure 1C (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf) shows that spheres with a diameter between 0.2 and 0.7 mm wander approximately 0.48 mm within 24 hr (e.g., 0.2 mm diameter, 0.48 ± 0.06 mm; 0.7 mm diameter, 0.48 ± 0.09 mm; mean ± SD), demonstrating that migration speed is independent of sphere size. Moreover, cells from different donors (0.3-mm-diameter spheres) also did not vary significantly in migration speed over 24 hr [see Supplemental Material, Figure 1D (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)].

Next, we analyzed the cellular composition of neurospheres. We sliced 10-µm cryostat sections of proliferating neurospheres and examined expressions of *a*) nestin, a marker protein for neural stem cells; *b*) β(III)tubulin, which stains neurons; or *c*) GFAP for glial cells. Immunocytochemical analyses revealed nestin-positive (+) cells were located mainly in the sphere periphery, whereas β(III)tubulin$^+$ and GFAP$^+$ cells resided in the sphere center (Figure 1A,B). This pattern disappeared after spheres were plated for differentiation. After 8 days of differentiation, β(III)tubulin$^+$ cells were located at the edge of the sphere, whereas nestin$^+$ and GFAP$^+$ cells were homogenously distributed throughout the sphere (Figure 1C,D).

In addition to the sphere itself, we investigated the cellular composition of the

migration area after 24 hr and 7 days of migration. Twenty-four hours after plating, nearly all migrated cells seemed to express nestin, showing that immature cells migrate out of the sphere. Furthermore, β(III)tubulin$^+$ and GFAP$^+$ cells were also located in the migration area. In contrast, 7 days after plating almost all cells lost nestin expression and became β(III) tubulin$^+$ or GFAP$^+$ (Figure 2A). Quantification of the number of pixels in the respective images revealed a 5.5-fold reduction in the number of nestin$^+$ pixels/nuclei after 6 more days of differentiation (570.9 ± 64 to 103.2 ± 29 pixels/nuclei; mean ± SD), whereas in the same time period the number of β(III)tubulin$^+$ and GFAP$^+$ pixels increased 4.7- and 1.9-fold, respectively [β(III)tubulin, from 118.7 ± 27.4 to 509.6 ± 55 pixels/nuclei; GFAP, from 250.8 ± 56 to 480.7 ± 198 pixels/nuclei; Figure 2A]. Furthermore, the immunocytochemical staining for β(III)tubulin suggests that neuronal cells may form connections and thus build neuronal networks (Figure 2A).

Another cell type emerging from neural precursor cells are O4$^+$ oligodendrocytes. They form within the neurosphere (Fritsche et al. 2005) and migrate out of the sphere over time. After 2, 4, and 7 days of differentiation, 3 ± 0.2, 52 ± 1, and 210 ± 5 O4$^+$ cells (mean ± SD), respectively, were located in the migration area (Figure 2B). They also changed morphology over time. Although after 48 hr most O4$^+$ cells were bipolar, we found more branching after 4 days; after 7 days of differentiation, multipolar and membrane sheet-forming cells were prominent (Figure 2C).

Next, we developed assays that identify changes in cell proliferation, differentiation, migration, and apoptosis by applying model chemicals, which are known to interfere with normal brain development (Grandjean and Landrigan 2006).

Cell proliferation in a neurosphere can be determined by counting the number of cells per dissociated sphere or by measuring the increase in sphere diameter over time. Figure 3A shows that there was a very good association between these two parameters

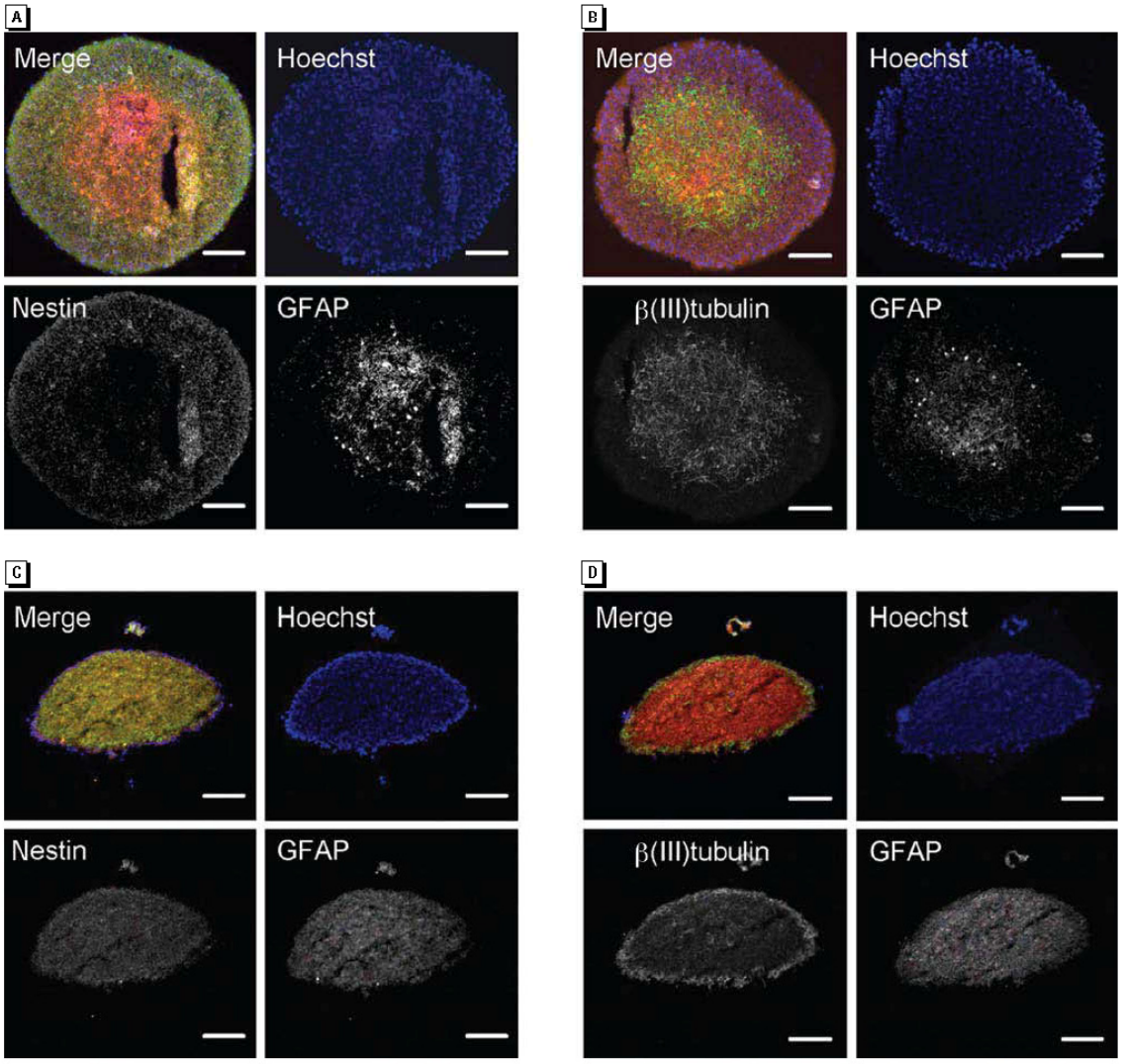

Figure 1. Cellular composition of human neurospheres shown in cryostat sections (10 µm) of proliferating (*A* and *B*) and differentiating (8 days after plating; *C* and *D*) neurospheres (representatives of five spheres for each developmental stage). Nuclei are stained in blue with Hoechst; nestin and β(III)tubulin are stained in green; and GFAP is stained in red. Individual antibody stainings are shown as contrast images. Bars = 100 µm.

(e.g., 2.6×10^3 and 5.3×10^4 cells for spheres 0.3 and 0.6 mm in diameter, respectively). We verified this observation and made the method suitable for high-throughput analyses by measuring viability of neurospheres dependent on sphere diameter with the CellTiter-Blue assay. Figure 3B demonstrates that viability of spheres correlates well with their sizes (e.g., spheres 0.3 and 0.6 mm in diameter had 4×10^3 and 8×10^3 relative fluorescence units, respectively). Growth of neurospheres in the absence or presence of 20 or 100 ng/mL EGF caused a 1.5 ± 0.4-fold or 2.4 ± 0.3-fold induction in viability (mean ± SD), respectively over 14 days, although the same spheres gained 0.08 ± 0.03 or 0.2 ± 0.04 mm in diameter, respectively, during this time (Figure 3C,D). We observed no differences in proliferation between spheres grown in EGF in the presence or absence of FGF (data not shown). Cultivation without growth factors as a negative control did not change size or viability. Thus, this assay can assess sphere proliferation.

We verified these data by FACS analyses for DNA content using propidium iodine staining. Among all stained cells, 97.75% showed only a G_0/G_1 peak (Figure 3F), whereas we found a typical cell cycle distribution for proliferating cells in only 2.72% of the population. About 35% of these were in G_2/M or S phase (Figure 3F, control), suggesting fast cell-cycling behavior. Indirubin, a G_2/M blocking agent that blocks signaling of cyclin-dependent kinases (Hoessel et al. 1999), increased the cell fraction in G_2/M phase from 14% to 37.8% (Figure 3F), whereas withdrawal of growth factors caused G_1 arrest.

To investigate effects of chemicals on differentiation, we exposed neurospheres to different Hg compounds. Immunocytochemical analyses after 48 hr revealed that migration areas of control cells consist of 10% β(III)tubulin$^+$ cells and 90% GFAP$^+$ cells (Moors et al. 2007). MeHgCl (500 and 750 nM) reduced the amount of β(III)tubulin$^+$ cells to 8 ± 0.17% (mean ± SD) and 2.3 ± 0.57%, respectively. Exposure to 4 μM $HgCl_2$ decreased the number of β(III)tubulin$^+$ cells to 4.7 ± 2.3%. In contrast, cAMP increased the formation of β(III)tubulin$^+$ cells to 165.4 ± 9% of control cells (Figure 4).

Next, we investigated the effects of Hg on cell migration with the neurosphere migration assay (Moors et al. 2007). Exposure to MeHgCl (500 nM) caused an inhibition of cell migration to 78.7% ± 7% of control values, which was further reduced by higher MeHgCl concentrations. $HgCl_2$ (4 μM) also reduced cell migration to 73.6 ± 13% of the controls (Figure 5A, B). Notably, cell migration was significantly affected by noncytotoxic Hg concentrations (Figure 5C).

To determine whether human neurospheres can be stimulated to undergo apoptosis, we exposed them to staurosporine, a potent inducer of the intrinsic apoptotic pathway via cytochrome c release followed by activation of the caspase cascade (Slee et al. 2000), or H_2O_2, a direct reactive oxygen species (ROS) donor, for 24 hr. LDH measurements of neurosphere supernatants indicate that staurosporine and H_2O_2 induce cell death in a concentration-dependent manner. However, the human neuroblastoma cell line SH-SY5Y (ATCC, Wesel, Germany) is more susceptible to staurosporine- and H_2O_2-induced LDH release than are the spheres, as indicated by a higher LDH release at lower concentrations, which we confirmed using phase-contrast microscopic images (Figure 6A,B). To explore whether staurosporine (1 μM) or H_2O_2 (1 mM) induced cell death via apoptosis, we performed TUNEL assays. Although the basal apoptosis rate of hNPCs after 3 days of differentiation was approximately 1% (data not shown), both treatments induced TUNEL-positive cells, showing that apoptosis is involved in staurosporine-induced and H_2O_2-induced cell death (Figure 6D). However, measurements

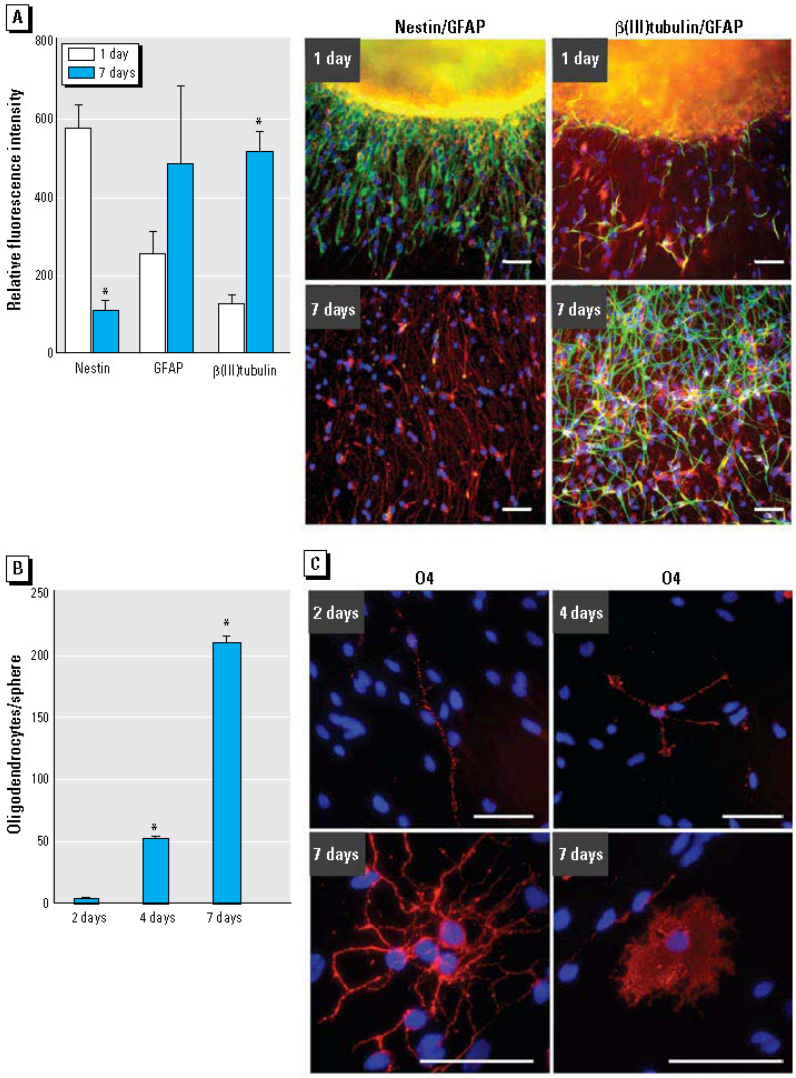

Figure 2. Cellular composition of the neurosphere migration area. (*A*) Quantification of nestin$^+$, GFAP$^+$, and β(III)tubulin$^+$ pixels after 1 and 7 days of differentiation (mean ± SD) and representative photographs [left: nestin (green), GFAP (red); right: β(III)tubulin (green), GFAP (red)]. Ten individual spheres were included in each calculation. (*B*) Quantification of migrated O4$^+$ cells, counted manually after 2, 4, and 7 days of differentiation in six individual spheres (mean ± SD). (*C*) Morphology of O4$^+$ cells at different time points. Bars = 100 μm.
*$p \leq 0.05$.

of effector caspase-3/-7 activities indicate that staurosporine-induced cell death is caspase dependent, whereas H_2O_2-triggered cell death is caspase independent (Figure 6C).

Discussion

In humans, DNT results in learning deficits and mental retardation (Hass 2006; Schettler 2001). Furthermore, various clinical disorders (e.g., schizophrenia, autism) are results of interference with normal brain development, and their etiologies are suspected to also imply environmental components (Rice and Barone 2000). To prevent harm, it is crucial to understand DNT potentials of chemicals, and thus testing is necessary. Therefore, we established and characterized this three-dimensional human neurosphere system that imitates the basic processes of brain development—proliferation, differentiation, and migration [Figure 2; see also Supplemental Material, Figures 1 and 2 (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)].

Individual spheres in single wells of a 96-well plate proliferated over time, and FACS analyses of propidium iodine–stained neurosphere single-cell suspensions revealed that approximately 2.72% of sphere cells went through S-phase of the cell cycle, confirming their proliferative capacity (Figure 3E,F). This is in agreement with Reynold and Rietze (2005), who found 2.4% of human neurosphere cells capable of proliferation as assessed by a single-cell clonogenic assay.

To illuminate the inside of the "black-box" neurosphere, we immunocytochemically stained proliferating spheres. Microscopic analyses illustrate a zonal distribution of nestin$^+$ hNPCs in the periphery and later GFAP$^+$ and β(III)tubulin$^+$ astrocytes and neurons in the center of the sphere (Figure 1). These findings are similar to data reported for murine neurospheres (Campos et al. 2004) and might be caused by a growth factor gradient from the sphere periphery to its inside. One could speculate that this zonal distribution resembles an "outside-in" brain structure, with nestin$^+$ cells representing the proliferative zone of the brain, which is in proximity to the growth-factor–containing liquor of the ventricles, and the GFAP$^+$ and β(III)tubulin$^+$ cells in the center of the sphere resembling superficial regions of the cortex (Campos et al. 2004). Whether the growth factor gradient is in fact responsible for zonal dissemination within a neurosphere is a subject for future investigations.

Growth factor withdrawal and presence of a poly-D-lysine/laminin matrix initiate cellular migration out of the sphere (Moors et al. 2007). Observation of initial migration over 24 hr by real-time phase-contrast microscopy illustrates that radial as well as tangential migration happens during this time [see Supplemental Material, Figure 2 (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)]. The cues causing cells to connect, disconnect, and move forward, backward, and even tangentially *in vitro* are so far unknown. Directed migration *in vivo* is motivated by chemical gradients of, for example, Netrin1/UNC6, semaphorins, or the reelin/dab1 pathway (Hatten 2002). Although some of these

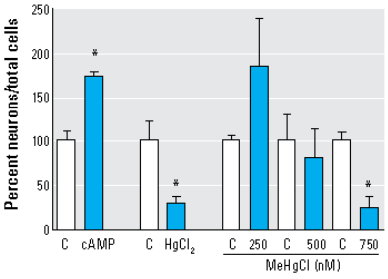

Figure 4. Chemicals disturb neurosphere differentiation. C, control. β(III)tubulin$^+$ cells/total nuclei were counted after exposing differentiating neurospheres to chemicals for 48 hr. Data are mean ± SD of at least three independent experiments (3–5 spheres per experiment).

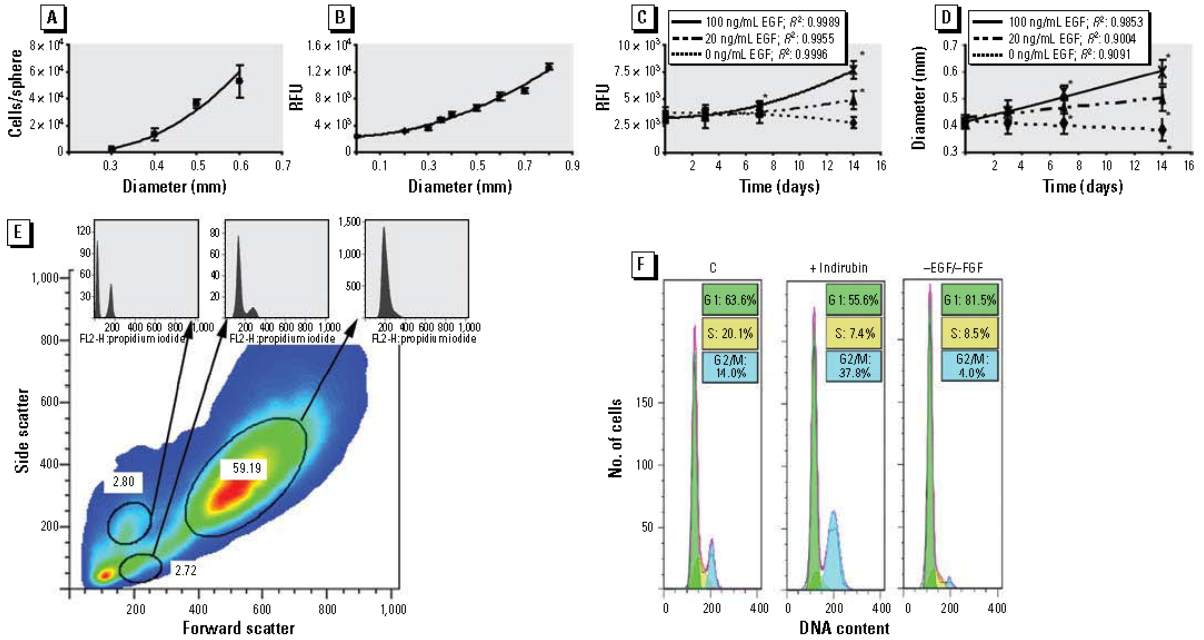

Figure 3. Assessment of neurosphere proliferation. RFU, relative fluorescence units. (*A*) Correlation between sphere diameter and number of cells/sphere (R^2 = 0.9926). (*B*) Correlation between sphere size and metabolic activity; reductase activity was measured with the CellTiter-Blue assay (R^2 = 0.983). (*C*) Measurement of sphere proliferation by assessing metabolic activity repetitively over time. (*D*) Assessment of proliferation in the same spheres shown in (*C*) by measuring sphere diameter over time. Results in (*C*) and (*D*) are typical representatives of three independent experiments at each time point shown as mean ± SD of 3–6 individual spheres. (*E*) FACS analysis of dissociated, fixed, and propidium iodine–stained neurospheres. The circled regions depict subpopulations; one contained proliferating cells (see second DNA histogram). (*F*) DNA content histograms of the proliferating cell population. The control histogram corresponds to cells cultured with EGF and FGF. A G_2/M or G_1 arrest was induced with indirubin (28 hr) or by withdrawal growth factor (96 hr).
*$p \leq 0.05$.

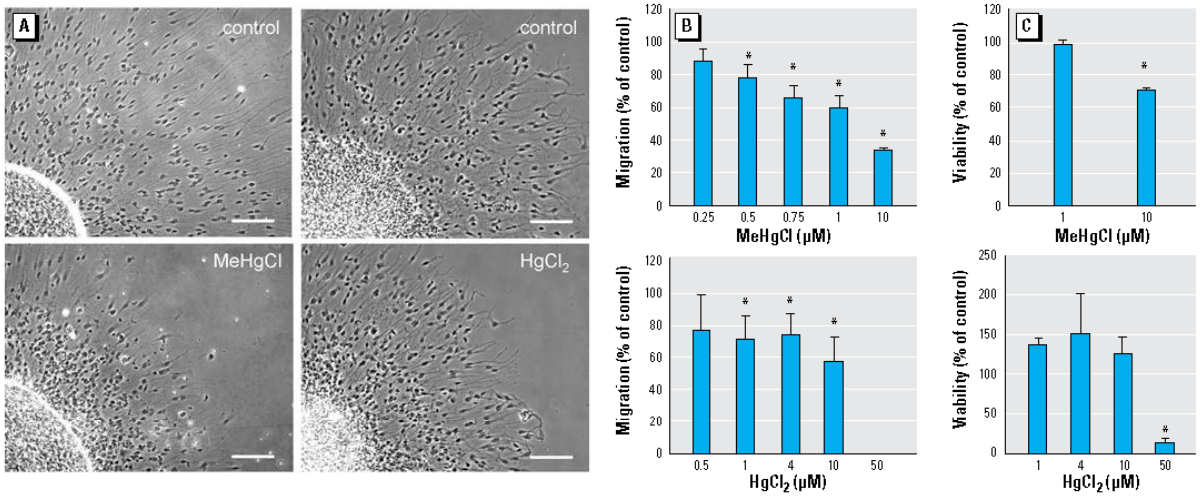

Figure 5. Mercury inhibits neurosphere migration. Phase-contrast images (*A*) and the respective quantifications (*B*) of cell migration (migration distance measured with an object micrometer from the edge of the sphere to the farthest outgrowth). In (*A*), bars = 100 μm. (*C*) Cell viability as assessed with the CellTiter-Blue assay. In (*B*) and (*C*), data are mean ± SD of at least three independent experiments (3–5 spheres/experiment).
*$p < 0.05$.

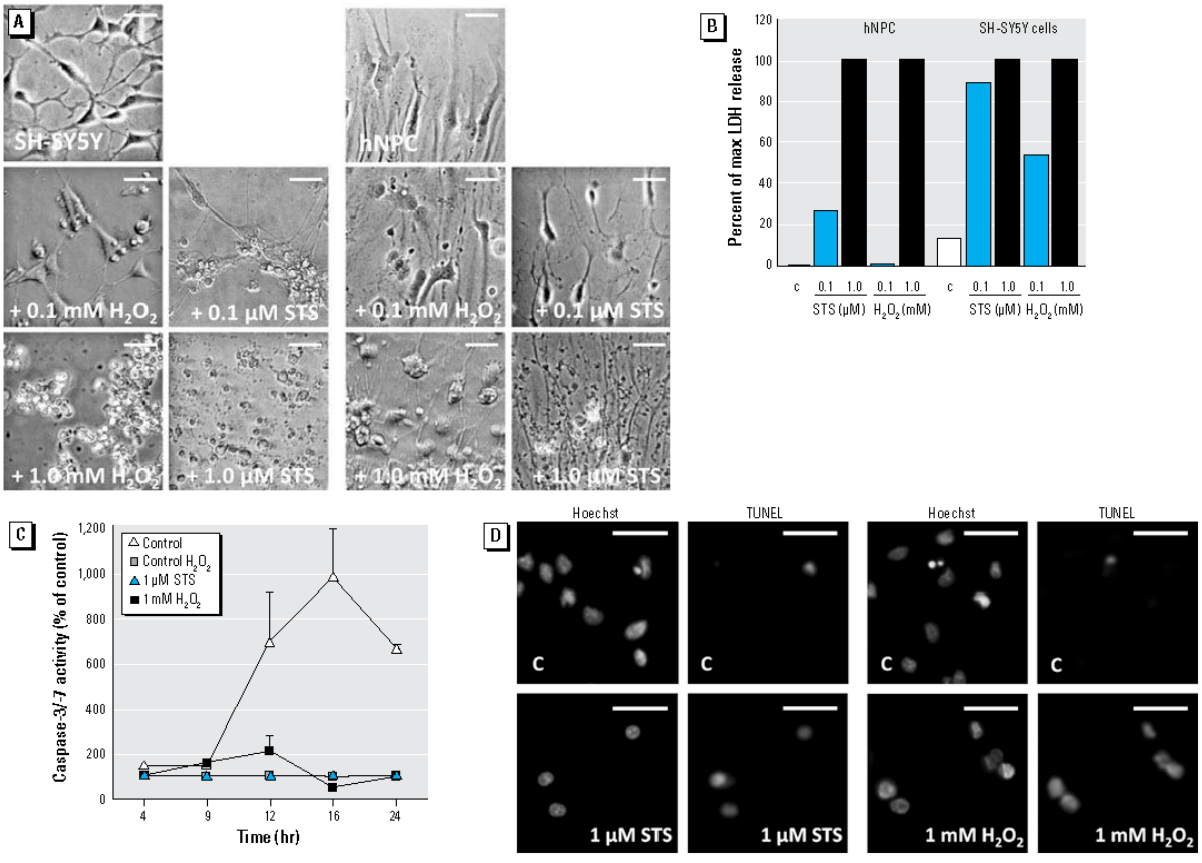

Figure 6. STS but not H_2O_2 induces caspase-dependent apoptosis. Abbreviations: C, control; max, maximum; STS, staurosporine. (*A*) Phase-contrast images and (*B*) corresponding LDH release of SH-SY5Y cells and hNPC after 24 hr of STS or H_2O_2 treatment. (*C*) Kinetic analyses of caspase-3/7 activity after STS or H_2O_2 treatment; values are typical representatives of two independent experiments (three spheres/experiment). (*D*) Cells showing positive TUNEL staining after STS or H_2O_2 exposure; nuclei are visualized with Hoechst. In (*A*) and (*D*), images are representative of two independent experiments; bars = 30 μm.

gene products, such as different semaphorins, are expressed in our neurospheres (Moors M, Fritsche E, unpublished data), whether such attractants or repellants are responsible for the directed migration we observe *in vitro* has to be further investigated. Nevertheless, the distance that differentiating hNPCs migrate over time is highly robust and reproducible. Migration speed is independent of neurosphere size and does not differ between three independent donors tested so far [gestational weeks 16–19; see Supplemental Material, Figure 1 (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)]. A faster migration speed (1 mm/24 hr) was reported for neurospheres that were prepared from postnatal brain cortices of premature infants (gestational weeks 23–25; Flanagan et al. 2006). This difference might be due to distinct culture conditions or ages of individuals.

During cellular outgrowth, hNPCs differentiate into GFAP$^+$, O4$^+$, and β(III)tubulin$^+$ glial- and neuronal-like cells while losing nestin staining (Figure 2A). The ratio of approximately 10% neuronal and 90% glial cells that we counted after 2 days of differentiation (Moors et al. 2007) resembles the physiologic distribution of brain cells in humans (Baumann and Pham-Dinh 2001). Furthermore, we found O4$^+$ oligodendrocyte precursor cells in the migration area that increase in number and degree of morphologic maturation over time (Figure 2) (Baumann and Pham-Dinh 2001). These differentiation results point to culture maturation.

For the development of *in vitro* assays that identify chemicals with DNT potential, we employed Hg, which is a human developmental neurotoxicant (Grandjean and Landrigan 2006). Prenatal Hg poisoning causes developmental delays, mental retardation, and adverse effects on memory and motor skills in children (Sanfeliu et al. 2003; Schettler 2001). Neuropathologic examinations revealed microcephaly and global brain disorganization resulting from disturbances in cell migration and division (Clarkson 2002; Schettler 2001). Moreover, postmortem brains had a decreased number of nerve cells (Castoldi et al. 2001; Choi 1989). We mimicked these effects *in vitro* by treating neurospheres with organic Hg and inorganic Hg, which were identified in human brain sections (Clarkson 2002). Hg decreased the migration distance (Figure 5A,B) and increased the glial cell/neuron ratio (Figure 4). We observed these effects at noncytotoxic concentrations, pointing to a target-cell–specific effect (Figure 5C). How do these findings correspond to Hg exposures in humans? From an Hg poisoning incident in Iraq, a lowest observed adverse effect level (LOAEL) for brain MeHg content in intoxicated mothers was calculated to be 800 ng/g, a level that caused neurologic symptoms in children (Clarkson 1993). Considering the measurements of Lewandowski et al. (2003), who determined cellular *in vitro* concentrations relative to corresponding medium Hg concentrations, this LOAEL is equivalent to an *in vitro* medium concentration of approximately 266 nM. MeHg accumulation in the fetus is higher than in adult organs, implying that the LOAEL is under- rather than overestimated. *In vitro* exposure of rodent neural stem cells to $HgCl_2$ (7–18 μM) or MeHg (2.5–5 nM) also resulted in reduced neuronal differentiation (Cedrola et al. 2003; Tamm et al. 2006). Although the sensitivity toward inorganic Hg was similar in human compared with mouse spheres (Cedrola et al. 2003), rodent stem cells treated for 7 days were more sensitive toward organic Hg than were human cultures treated for 2 days (Tamm et al. 2006). Although both mercuric compounds exert adverse effects by binding to sulfhydryl groups of proteins (Clarkson 1972), one further mode of action of organic Hg is the induction of oxidative stress (Sarafian and Verity 1991; Yee and Choi 1994). Antioxidant defenses are low in human embryonic brains and evolve during development (Buonocore et al. 2001). Furthermore, there might also be species differences in defense capacities (Knobloch et al. 2008). Thus, the differences between our and previously published results for MeHg could be due to the age of cultures (stem vs. fetal cells), species differences, or varying exposure times.

In contrast to Hg, cAMP, a well-described compound for inducing neuronal differentiation (Deng et al. 2001), caused an increased number of β(III)tubulin$^+$ cells in differentiated hNPCs (Figure 4A), demonstrating the dynamic ability of the cell system.

Deregulation of apoptosis results in developmental brain pathology or neurodegenerative diseases (Rodier 1995). Furthermore, oxidative stress induces apoptosis in many different cells types. Therefore, we attempted to trigger ROS-induced programmed cell death in hNPC cells. Although staurosporine induced caspase-dependent apoptosis, 1 mM H_2O_2 induced TUNEL$^+$ apoptotic cells without caspase-3/-7 activation, indicating that neurospheres undergo caspase-independent apoptosis (Figure 6). This is in concert with studies in primary rat cerebellar granule cells, which also responded with caspase-independent apoptosis to H_2O_2 (Dare et al. 2001). Furthermore, comparison of LDH activity of human hNPCs with the human neuroblastoma SH-SY5Y tumor cell line suggests that hNPCs are less sensitive toward oxidative stress than are SH-SY5Y tumor cells. These data also support observations that cancer cells are more susceptible to various stressors than are normal cells (Aykin-Burns et al. 2008; Hileman et al. 2004). Besides inducing apoptosis, preconditioning of mouse NPCs with a low concentration of H_2O_2 (5 μM) is cytoprotective (Sharma et al. 2008). Whether this is also true for hNPCs or even tumor cell lines needs to be investigated.

In summary, we have shown that *a*) proliferation, migration, differentiation, and apoptosis of human neurospheres can be quantified; *b*) *in vivo* effects of the developmental neurotoxicant Hg are imitated *in vitro*; and *c*) the methods applied are suitable for medium-throughput screening. Thus, our three-dimensional neurospheres offer a new, human, system-based method for DNT hazard identification. However, their applicability is limited to basic processes of brain development, because they do not resemble complex higher brain structure development such as formation of cortical layers. Moreover, they are limited in their potential to perform drug metabolism, as is fetal tissue *in vivo*. For including "maternal metabolism" in the *in vitro* system, strategies such as incubation with S9 mixes or hepatocyte coculture have to be established.

In the future, more chemicals known to cause DNT will be tested for their potential to interfere with human neurosphere performance to develop this method into a validation process and make it applicable for testing needs.

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Species-specific differential AhR-expression protects human neural progenitor cells against developmental neurotoxicity of PAHs

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Species-Specific Differential AhR-Expression Protects Human Neural Progenitor Cells Against Developmental Neurotoxicity of PAHs

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Abbreviations

AhR	arylhydrocarbon receptor
AhRR	arylhydrocarbon receptor repressor
ARNT	AhR nuclear translocator
B(a)P	benzo(a)pyrene
bHLH/PAS	basic Helix-Loop-Helix/Per-ARNT-Sim
CNS	central nervous system
CYP	cytochrome P450
DEPC	diethylpyrocarbonate
DNT	developmental neurotoxicity
DREs	dioxin responsive elements
EGF	epidermal growth factor
ERK	extracellular-signal regulated kinase
FACS	fluorescence-activated cell sorting
rhFGF	recombinant human fibroblast growth factor

IQ	intelligence quotient
LDH	lactate dehydrogenase
3-MC	3-methylcholanthrene
MEFs	mouse embryonic fibroblasts
MeHgCl	methylmercury chloride
MNF	3'methoxy-4'nitroflavone
NPCs	neural progenitor cells
PAHs	polycyclic aromatic hydrocarbons
PCBs	polchlorinated biphenyls
PDL	poly-D-lysine
POPs	persistent organic pollutants
SD	standard deviation
SEM	standard error of the mean
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin

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Abstract

Background: Due to their lipophilicity, persistent organic pollutants (POPs) cross the human placenta, possibly affecting central nervous system (CNS) development. The majority of POPs are known AhR ligands and activators of AhR signaling. Therefore, it has been proposed that AhR activation causes developmental neurotoxicity (DNT).

Objective: Whether AhR-activation is the underlying mechanism for reported DNT of POPs in humans is not known. Thus, effects of AhR ligands on basic processes of brain development were studied in two comparative *in vitro* systems.

Methods: We employed neurosphere cultures based on human and mouse (wildtype and AhR deficient) neural progenitor cells (hNPCs & mNPCs) and studied the effects of different AhR agonists and an antagonist on neurosphere development. Moreover, we analyzed expression of AhR and genes involved in AhR signaling.

Results: In contrast to wildtype mNPCs, hNPCs and AhR deficient mNPCs are insensitive to AhR agonism or antagonism. While AhR modulation attenuates mNPC proliferation and migration, hNPCs and AhR deficient mNPCs remain unaffected. We also show that species-specific differences result from non-functional AhR-signaling in human NPCs.

Conclusion: We demonstrate that in contrast to wildtype mouse, human NPCs are protected against PAH-induced DNT due to absence of the AhR which explains species-specific differences in sensitivity towards POPs.

Introduction

Persistent Organic Pollutants (POPs) bioaccumulate through the food chain and pose a risk of causing adverse effects to human health and the environment. Main substance classes are polycyclic aromatic hydrocarbons (PAHs), like 3-methylcholanthrene (3-MC) and benzo(a)pyrene (B(a)P), dioxins like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated biphenyls (PCBs). Due to their lipophilicity, POPs cross the human placenta exposing the fetus to the contaminant body burden of the mother. This results in increased adverse health outcomes, possibly affecting central nervous system (CNS) development (rev. in Wormley et al. 2004). Besides other toxicants like nicotine, one potential source for *in utero* exposure to POPs, especially to PAHs, is maternal cigarette smoking during pregnancy (Polanska et al. 2009; Rodgman et al. 2000). An inverse relationship between maternal smoking during pregnancy, offspring intelligence (IQ), and cognitive ability has been described in numerous cohort studies (Batty et al. 2006; Fried et al. 1998; Olds et al. 1994). Additionally, behavioral problems and psychiatric disorders in offspring have been associated with smoking during pregnancy (Thapar et al. 2003; Weissman et al. 1999; Fergusson et al. 1998). In addition to smoking, *in utero* exposure to PCBs and/or dioxins results in cognitive deficits in children (rev. in Grandjean and Landrigan 2006; Jacobson and Jacobson 1997; Patandin et al. 1999). These alterations are thought to involve the POP-activated arylhydrocarbon receptor (AhR), an evolutionary highly conserved member of the basic Helix-Loop-Helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors. Ligand binding to this cytosolic receptor induces nuclear translocation (Gasiewicz and Bauman 1987) and heterodimerization with another bHLH/PAS protein, ARNT (AhR nuclear translocator; (Reyes et al. 1992)). The resulting complex recognizes and binds specific DNA sequences, i.e. dioxin responsive elements (DREs), within gene promoter regions and modulates subsequent transcription of AhR-dependent genes (Fujisawa-Sehara et al. 1987). Most of the

above discussed substance classes like PAHs and dioxins are known AhR ligands and activators of AhR signaling. It has therefore been proposed that AhR activation causes developmental neurotoxicity (DNT). This hypothesis is supported by studies in invertebrate (*C. elegans*) and vertebrate (zebrafish, chicken, rat, monkey) species where dioxins and related compounds cause morphological abnormalities of brains or deficits in cognition and/or behaviour (Henshel et al. 1997; Hill et al. 2003; rev. in Kakeyama and Tohyama 2003; Qin and Powell-Coffman 2004). Whether AhR-activation is the underlying mechanism for the reported DNT effects after POP exposure in humans is not known.

To investigate potential species-specific differences, we employed comparative *in vitro* test systems for brain development based on neurosphere cultures from human and mouse neural progenitor cells (hNPCs and mNPCs, respectively). These three-dimensional cell systems mirror basic processes of fetal brain development such as proliferation, migration, differentiation and apoptosis. Moreover, they detect developmental neurotoxicants *in vitro* (Fritsche et al. 2005; Moors et al. 2007; Moors et al. 2009). We here report that in contrast to wildtype mNPCs, hNPCs and AhR deficient mNPCs are insensitive to AhR agonism or antagonism due to non-functional AhR-signaling indicating that humans are protected towards AhR-dependent, POP-induced DNT. Knowledge about such species-specific differences is of utmost importance with regard to chemical testing and hazard assessment for humans.

Material and Methods

Chemicals

The AhR antagonist 3′methoxy-4′nitroflavone was kindly provided by G. Vielhaber (Symrise, Holzminden, Germany). Methylmercury chloride (MeHgCl) was obtained from Riedel-de

Haën (Seelze, Germany) and TCDD was purchased from LGC Standards (Wesel, Germany). All additional chemicals used (unless otherwise noted) were purchased from Sigma–Aldrich (Munich, Germany) and were of the highest purity available.

Cell culture

HNPCs used in this study were purchased from Lonza (Verviers SPRL, Belgium). Data presented in this study was generated with hNPC from a single male individual gestational week 16. Similar results were obtained in a second individual of gestational week 18 with unknown sex. For mouse neurosphere culture, brains of wildtype and AhR knockout C57/BL6 mice (Charles River) were removed at embryonic day (E) 15.5-17.5 and transferred to phosphate buffered saline (PBS). Age of the embryos was determined according to the staging criteria of Theiler, in which E16 correspond to Theiler stage 24 (Bard et al. 1998). Brains of embryos were dissected, transferred to DMEM and mechanically dissociated. Trypsin/EDTA solution was added and incubated for 30 min at 37°C in a humidified atmosphere. Afterwards, the tissue suspension was triturated to obtain a single cell suspension which was centrifuged with 800 rpm for 5 min. Pellets were resuspended and plated in 10 cm petridishes. AhR deficiency of mice (Fritsche et al. 2007) was confirmed by PCR. Presented data of wildtype mNPC are derived from four independent preparations while data of AhR deficient mNPCs were obtained from two different preparations.

Both, hNPCs and mNPCs were cultured in proliferation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/ml EGF (Biosource, Karlsruhe, Germany), 20 ng/ml rhFGF (R&D Systems, Wiesbaden-Nordenstadt, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin) in a humidified 92.5% air/7.5% CO2 incubator at 37 °C in suspension culture. Differentiation was initiated by growth factor withdrawal in differentiation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with N2 (Invitrogen), 100 U/ml penicillin, and

100 µg/ml streptomycin) and plating onto poly-D-lysine (PDL)/laminin coated chamber slides.

Cell Viability

Cell viability was measured using a lactate-dehydrogenase (LDH) assay (CytoTox-One, Promega) according to the manufacturer's instructions. Briefly, supernatants of treated cells from the migration, mRNA-expression and proliferation assessments were collected at the respective timepoints and incubated 2:1 with the CytoTox-One reagent for 4 h prior to detection of fluorescence (Ex 540 nm/Em 590 nm). Complete lysis of cells with the included lysis buffer for 2 h at room temperature serves as a positive control.

Proliferation Assays, Cell Cycle Analyses and Migration Assay

Proliferation was assessed with a combination of CellTiter-Blue Assay (Promega, Madison, USA), which measures mitochondrial reductase activity, and microscopical determination of sphere diameter as described previously (Moors et al. 2009). Cell cycle was analysed by FACS. Therefore, neurospheres were exposed to the different chemicals for 48 h, dissociated to single cells with accutase, fixed with paraformaldehyde and stained with propidium iodine (Moors et al. 2009). Spheres cultured in proliferation medium with 20 ng/ml EGF and FGF serves as positive control (PC) and spheres cultured without these growth factors are termed negative control (NC). Migration distance was measured microscopically after 48 hours as previously described (Moors et al. 2007; Moors et al. 2009). Exposure for 48h with 1 µM MeHgCl served as positive control.

Quantitative Real-Time PCR

NPCs were treated under differentiating conditions with 10 µM 3-MC, 1 nM TCDD or 0.1 % DMSO as solvent control. After indicated timepoints, RNA was prepared with the Absolutely

RNA Microprep Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Real-time reverse-transcriptase polymerase chain reaction (rtRT-PCR) was performed using the LightCycler instrumentation (Roche, Mannheim, Germany) with the QuantiTect SYBR green PCR Master Mix (Qiagen, Hilden, Germany) as previously described (Fritsche et al. 2007). Conditions for PCR amplifications were initial 15 min at 95°C, 40 cycles of 15 sec at 94°C for denaturation, 25 sec of primer annealing, 30 sec at 72°C for elongation, and 2 sec at 72°C for fluorescence detection. Intron-spanning primers were designed using PRIMER 3 Software to reduce genomic amplification. Primer sequences for *hACTB, hAhR, hAhRR, hCYP1A1, hCYP1B1* were described by Fritsche et al. (Fritsche et al. 2005). Other primer sequences are: ***hARNT***: (F) CCCTAGTCTCACCAATCGTGGAT (R) GTAGCTGTTGCTCTGATCTCCCAG ***hC-MYC***: (F) ACCACCAGCAGCGACTCTGA (R) TCCAGCAGAAGGTGATCCAGACT (56°C annealing), ***msActb***: (F) CTACAATGAGCTGCGTGTGG (R) TAGCTCTTCTCCAGGGAGGA (60°C annealing), ***msAhR***: (F) GACAGTTTTCCGGCTTCTTG (R) CGCTTCTGT AAATGCTCTCGT (60°C annealing), ***msArnt***: (F) TGCCTCATCTGGTACTGCTG (R) GAACATGCTGCTCACTGGAA (58°C annealing), ***msAhRR***: (F) GTTGGATCCTGTAGGGAGCA (R) AGACCAGAGGCTCACGCTTA (60°C annealing), ***msCyp1a1***: (F) GGCCACTTTGACCCTTACAA (R) CAGGTAACGGAGGACAGGAA (54°C annealing), ***msCyp1b1***: (F) ACATGAGTTTCAGTTATGGCC (R) TTCCATTCACTGCTGAGAGC (58°C annealing), ***msc-myc***: (F) TGTCCATTCAAGCAGACG (R) GCATTTTAATTCCAGCGCATAG (54°C annealing). Expression levels were normalized to the expression of beta-actin. Gene expression was evaluated using the Ct value from each sample. The method of calculation is based on the method of the ΔΔCt (Livak and Schmittgen 2001). For determining absolute copy numbers product-specific standards amplified from cDNA were used to generate standard curves. A

target gene was considered to be quantifiable if the ratio copy number target gene/copy number beta-actin x1000 exceeded 0.001.

Western Blotting

Proteins were isolated from hNPC or HepG2 cells as described in Moors et al. (2007). Whole cell lysates (40 µg) were separated applying 10% SDS-PAGE and transferred to an Amersham Hybond-P-membrane (GE Healthcare, Chalfont St Giles, Great Britain). The membrane was blocked in Tris-buffered saline containing 0.01% Triton (v/v) and 5% (w/v) non-fat dry milk for 1h at room temperature, followed by incubation in the same buffer containing a polyclonal anti-AhR antibody (1:2000, Enzo Life Sciences (Loerrach, Germany)) or an anti-GAPDH antibody (1:100 000, Biozol (Eching, Germany)) overnight at 4°C. As secondary antibodies we used ECL anti-rabbit IgG or ECL anti-mouse IgG horseradish peroxidase linked antibodies (both 1:5000, GE Healthcare). Chemiluminescence signals were detected with an X-ray film.

Statistics

All results are means ± SEM of at least two independent experiments. We used analysis of variance combined with the Dunnetts post hoc test for multifactor analyses (concentration effects), and the Student's *t*-test for two-group comparisons (treatment vs. control) Significance value was set at $p< 0.05$ (Hays 1994).

Results

AhR agonism or antagonism does not cause cytotoxicity in hNPCs and mNPCs.

Cells were exposed to the AhR agonists 3-MC (synthetic model compound for AhR-activating metabolized PAH), B(a)P (metabolized, environmentally relevant PAH), TCDD (non-metabolized, environmentally relevant PAH) or AhR antagonist MNF (model

compound) for 6h up to 14 days as indicated in the supplementary figure 1. Exposure times for cytotoxicity thereby reflect duration of treatments in the proliferation, migration or gene expression assays. Concentrations were chosen due to known AhR activation/antagonism in other cell types. None of the exposures induces cytotoxicity (LDH release) of human or mouse NPCs (Suppl. Material 1).

Proliferation of mNPC, but not hNPCs is inhibited by AhR blockage.

Figure 1A and B show quantification of the proliferation assays (sphere diameter and Celltiter-Blue Assay, respectively) of human neurospheres after exposure to 1 and 10 µM 3-MC, 1 nM TCDD or 1 µM MNF. There was no significant disparity between different exposures after 7 and 14 days. This was verified in a second individual (data not shown). After 7 and 14 days, all human neurospheres except the ones without growth factors had grown from a diameter of 444 µm ± 15 µm to a diameter of 653 µm ± 33 µm and 829 µm ± 17 µm, respectively. These results were confirmed by FACS analyses for DNA content using propidium iodine staining (Figure 1C). Independent of exposure conditions, we found a typical cell cycle distribution for proliferating cells in approximately 2.5% of all NPCs (Moors et al. 2009). Of those, the main number of cells was in G0/G1-phase (53.5% ± 3.4%), while 28% ± 1.8% were in S-phase, and 18.4% ± 2.8% cells were in G2/M-phase after 48h of exposure.

Proliferation of mNPCs was monitored over only 7 days due to a size-restricted halt in sphere growth beyond that time. In contrast to the hNPC data, there was a significant inhibition of mNPC proliferation after 7 days of 1 µM MNF exposure. While control, 3-MC and TCDD exposed spheres grew from a diameter of 386 µm ± 23 µm to a diameter of 551 µm ± 85 µm, MNF-exposed spheres remained at 405 µm ± 78 µm. This was due to AhR inhibition as proliferation of AhR deficient neurospheres was not disturbed by MNF treatment (Figure 1D and E). Effects of MNF on mNPC proliferation were confirmed by FACS analyses for DNA

content using propidium iodine staining (Figure 1F). As for hNPCs, we found a typical cell cycle distribution for proliferating cells in approximately 2.5% of all cells. In the solvent control, 56.1% ± 4.6% of these cells were in G0/G1-phase, 38.1% ± 4.3% were in S-phase, and 5.8% ± 0.4% cells were in G2/M-phase after 48h of exposure. 3-MC treated cells showed a similar distribution. However, MNF treatment reduced the number of cells in G2/M-phase to 2.0% ± 0.4% while the distribution of cells in G0/G1- and S-phase remained relatively constant at 51.8% ± 0.2% and 46.3% ± 0.4%, respectively.

Neural cell migration is affected by AhR stimulation in mouse, but not human NPCs.

Next, we investigated if 3-MC, B(a)P, TCDD or MNF influence human and mouse NPC migration. Therefore, neurospheres were exposed to 1 and 10 µM 3-MC, 10 µM B(a)P, 1 nM TCDD or 1 and 0.1 µM MNF while differentiating for 48h. 1 µM MeHgCl served as a positive control for inhibition of migration. Independent of exposures, all human neurospheres adhered to the PDL/laminin matrix and migrated radially out of the sphere with an average migration distance of 739 µm ± 61 µm after 48h (Figure 2A & C). This was verified in a second individual (data not shown).

In contrast to the results in hNPCs, AhR activation reduced mNPC migration distance by 16% ± 5% (1 µM 3-MC), 21% ± 13% (10 µM 3-MC) and 32% ± 10% (10 µM B(a)P) compared to solvent controls, while MNF had no effects (Figure 2B & D). This was due to AhR activation as migration of AhR deficient neurospheres was not disturbed by 3-MC or B(a)P (Figure 2D). Interestingly, TCDD did not disrupt wildtype mNPC migration despite AhR activation (Figure 3C). This might be due to the fact that - in contrast to 3-MC and B(a)P – TCDD is hardly metabolized and thus does not produce reactive intermediates.

AhR-dependent gene transcription is only inducible in mNPCs, but not in hNPCs due to low abundance of AhR and ARNT transcripts and absence of AhR protein in human cells.

Because 3-MC, B(a)P and MNF did not influence hNPC viability, proliferation or migration, but modulated proliferation or migration of mNPCs, we determined *AhR* and *ARNT* mRNA expression under proliferating and differentiating conditions in hNPCs and mNPCs. *AhR* and *ARNT* mRNAs were expressed at very low copy numbers in human (0.6-2.5 and 13/1000 copies beta-actin, respectively) and higher copy numbers in mouse (20-63 and 716-1045/1000 copies beta-actin, respectively) NPCs. That beta-actin was in this case valid to use as a housekeeping gene was demonstrated by normalization of proliferating vs. differentiating NPCs to three additional housekeeping genes (RPL27, RPL30, OAZ1; Supplementary material figure 2). Expression of the AhR target genes *AhRR*, *CYP1A1*, *CYP1B1* and *c-myc* was not inducible by 10 µM 3-MC after 6h, 12h, 24h and 48h of differentiation in hNPCs (Figure 3A) and their basal *CYP1A1* expression level was undetectable. The same results were obtained for a second individual after 6h of treatment (data not shown). In contrast, 10 µM 3-MC induced *Cyp1a1* and *Cyp1b1* mRNA significantly in mNPCs after 6h to 6.6 ± 1.7- and 2.5 ± 0.25-fold of controls, respectively (Figure 3C). Although 1 nM TCDD did not disturb neural migration, it induced AhR signaling as shown by a 21-fold *Cyp1a1* induction in wildtype mNPCs (Figure 3C insert).

Comparison of mRNA expression levels between human and mouse NPCs showed that genes of AhR signaling and AhR gene battery were generally expressed in higher copy numbers/1000 copies beta-actin in mNPCs than in hNPCs (Figure 3D). Lack of AhR protein in hNPCs was confirmed by Western blot (Figure 3B). These results demonstrate that greater expression levels of AhR signaling pathway gene products cause toxicity of the AhR agonists 3-MC and B(a)P and the AhR antagonist MNF in mNPCs as AhR deficient mNPCs are protected against these effects.

Discussion

The development of cell-based, non-animal testing strategies for hazard assessment of chemicals is currently one of the most important tasks in toxicological research. In this regard, it is most important to choose appropriate model systems which are truly predictive for humans (National Research Council. 2007; Krewski et al. 2009). Human tumor cell lines which are easily accessible in large quantities bear the restriction that they do not represent cellular metabolism and signal transduction of normal cells. On the contrary, primary cells are often obtained as *ex vivo* cultures from rodents. Such primary cultures are regarded as superior over tumor-derived cells. However, species-specific differences limit their application. One example of how rodent primary cells can indeed misclassify hazards for humans is given in this study, where mouse-derived primary cells were shown to be more susceptible towards AhR modulation than their human counterparts. With regard to chemical testing, it is fundamental to be aware of such differences to not over- or underestimate hazards that chemicals pose to humans and thereby protect man and allow industry production and development of chemicals at the same time.

Specifically, in this study we discovered that proliferation of hNPCs was not affected by AhR agonists or antagonist (Figure 1A-C), while proliferation of mNPCs was completely blocked by AhR antagonism (Figure 1D-F). Our results of the proliferation analysis in hNPCs are in contrast to findings obtained in human liver or neuroblastoma tumor cells which show that exogenous AhR activation by TCDD or other AhR ligands inhibits cell proliferation and induces cell cycle arrest (Jin et al. 2004; Marlowe and Puga 2005). On the contrary, high AhR content promotes proliferation in a human MCF breast cancer cell line which was blocked by the AhR antagonist MNF or selective AhR knockdown via siRNA (Wong et al. 2009). In human umbilical vascular endothelial cells though, AhR activation by 3-MC also exerted antiproliferative effects as was seen in the tumor cell lines (Pang et al. 2008). These data demonstrate that not only the condition normal/vs. tumor cells determines if AhR activation

causes cell cycle progression or arrest, but also different cell types of the human body react differently towards AhR activation or inhibition.

In contrast to hNPCs, proliferation of mNPCs is completely blocked by MNF (Figure 1D-F). Also in the murine hepatoma cell line 1c1c7 flavone and alpha-naphthoflavone or AhR defect inhibit cell proliferation (Reiners, Jr. et al. 1999). Moreover, mouse embryonic fibroblasts (MEFs) from AhR knockout mice grow more slowly than wild-type cells (Elizondo et al. 2000). On the contrary, AhR knockout animals show accelerated proliferation in different organs like skin, hair follicles and liver blood vessels. Thus, as in humans, AhR effects on proliferation are also cell type-dependent in rodents and, as our data indicate, cell type-specificity is not consistent throughout species.

Besides progenitor cell proliferation, migration is another essential process in brain development. To address the role of the AhR in this process, we employed the neurosphere migration assay (Moors et al. 2007). AhR modulation by 3-MC, B(a)P, TCDD or MNF did not affect hNPC migration (Figure 2A & C). These data are in contrast to the two existing publications on this topic in human tumor cells showing increased motility and migration of MCF-7 cells after TCDD and 3-MC treatment in a scratch assay (Diry et al. 2006) and in a transwell migration assay (Seifert et al. 2009). In contrast, AhR stimulation reduces migration of mNPCs (Figure 2B & D). This is supported by an earlier *in vivo* study where prenatal exposure to the AhR ligand 7,12- dimethylbenz[a]anthracene disrupts cerebellar cytoarchitecture in rats (Kellen et al. 1976). Conversely, immortalized mouse mammary fibroblasts from AhR-null mice had decreased migration capacities in culture which was accompanied by inhibition of signaling pathways that regulate cell migration like focal adhesion kinase and mitogen activated protein kinase ERK1 (Mulero-Navarro et al. 2005). That ERK-dependent pathways are also necessary for normal migration of hNPCs was recently shown by our group (Moors et al. 2007). However, the AhR does not seem to determine ERK-dependent migration in hNPCs as migration distance does not change in

presence of AhR modulators (Figure 2A & C) but is impaired by ERK inhibition (Moors et al. 2007). Taken together, existing data on AhR-dependent migration implies that – as for proliferation – modulation of cell migration by AhR presence or AhR ligands differs between cell types and species.

To address the underlying reason for the observed species-specific differences in response to AhR modulation between human and mouse NPCs, we quantified copy numbers of genes which belong to the AhR machinery as well as genes which are AhR-regulated. Both, human and mouse NPCs express *AhR*, *ARNT*, *AhRR*, *CYP1B1* and *c-Myc* (Figure 3A & C). However, AhR and ARNT copy numbers are close to detection limit in hNPCs (and 8- to 100-fold lower than in mNPCs), hAhR protein is not detectable by Western blot and thus only mNPCs express quantifiable amounts of *Cyp1a1* and respond to 3-MC treatment with a time-related induction of *Cyp1a1* and *Cyp1b1* mRNA (Figure 3). Moreover, human AhR ligand affinity is approximately 10-fold lower than C57/BL6 mouse AhR-ligand attraction (Harper et al. 1988) attributable to an amino acid substitution in the human AhR ligand-binding domain (Ema et al. 1994). This mutation is also responsible for divergent toxic potencies of TCDD between responsive C57/BL6 and non-responsive DBA mice strains (Ema et al. 1994). The irresponsiveness of hNPCs towards AhR modulation is hence likely due to a function of very low *AhR* expression combined with a human low affinity receptor for ligand binding. Since a couple of reports revealed the existence of several polymorphisms in the human AhR gene, interindividual differences in ligand responsiveness between hNPC from different donors can not be excluded. However, even though single AhR polymorphisms (e.g. at codons 517 or 570) found in people of African descent were under suspicion to affect the expression of single genes like CYP1A1, it is widely accepted that none of the AhR polymorphisms described so far is of any functional consequence regarding the overall outcome of AhR response (Harper et al. 2002; Connor & Aylward 2009).

Species-differences similar to those reported here were observed in a comparative study of human and mouse palate organ cultures. This earlier work was driven by the fact that TCDD induces cleft palate in mouse embryos and that the risk for humans to develop such malformations upon *in utero* exposure towards TCDD or related compounds was not known. The authors from this study concluded that it seems highly unlikely that human embryos are exposed to sufficient amounts of TCDD to cause interruption of palatal differentiation because human palate organ cultures express several hundred-fold less *AhR* mRNA than the mouse cultures and human palates required 200 times more TCDD to produce a cleft palate *in vitro* than in the respective mouse model (Abbott et al. 1999). Our results support those findings in a different organ system, the developing brain, and indicate that studies on AhR-dependent developmental neurotoxicity in C57/BL6 mice *in vivo* overestimate the risk for disturbances of human brain development resulting from AhR activation. Our data also imply that the use of toxic equivalency factors (TEF), which correspond to the relative potency of a chemical to generate AhR-mediated effects compared to TCDD, based on data derived from rodents for risk assessment of POPs may not be necessarily useful for humans.

How can the epidemiological evidence for POP-related DNT in humans be explained if the AhR is not involved in chemically-induced DNT? Kodavanti (2005) comprehensively reviewed three alternative mechanisms how POPs might interfere with human brain development. One possibility may be POP-induced changes in neurotransmitters like dopamine or serotonine which could change among others learning and memory. As these endpoints extend beyond the basic processes of brain development, the basic 'neurosphere assay' (Breier et al. 2009) cannot detect such changes. Second, PCBs were found to alter intracellular phosphokinase C (PKC) signaling and Ca^{2+} homeostasis in rodents. As PKC modulation by the inhibitor BisI or the stimulator PMA causes inhibition and stimulation of hNPC migration, respectively (Moors et al. 2007), it is unlikely that the POPs used in this

study affect PKC signaling. Third, effects on thyroid hormone (TH) balance might contribute to POP-induced DNT. Thereby, some POPs can elicit direct biological effects on target organs like the brain by interfering with cellular TH signaling *in vivo* and *in vitro* (Fritsche et al. 2005; Schreiber et al. 2010; Zoeller and Crofton 2000). As the 'neurosphere assay' is able to detect endocrine disruption of TH signaling by PCBs and polybrominated diphenyl ethers (Fritsche et al. 2005; Schreiber et al. 2010), this mechanism is hence unlikely to be involved in AhR-independent DNT of 'classical' AhR ligands like 3-MC or TCDD in humans. The most likely mechanism how POPs interfere with human brain development is via systemic effects on TH homeostasis by directly affecting the thyroid gland and decrease the synthesis of TH, reducing blood TH levels by enhanced TH metabolism, or displacing natural ligand (T4) binding to the TH plasma transport protein transthyretin. That TCDD affects systemic TH balance also in humans was recently shown by Baccarelli et al. (2008) who found that in the highly TCDD-exposed Seveso cohort maternal exposure produces effects on neonatal thyroid function (Baccarelli et al. 2008).

In summary, we show that in contrast to mouse, human NPCs are protected against PAH-induced DNT due to absence of AhR. An accumulating body of evidence now indicates that human AhR signaling is less operative than AhR function in most laboratory animals. This knowledge should be taken into account for risk assessment of TCDD and related xenobiotics in humans.

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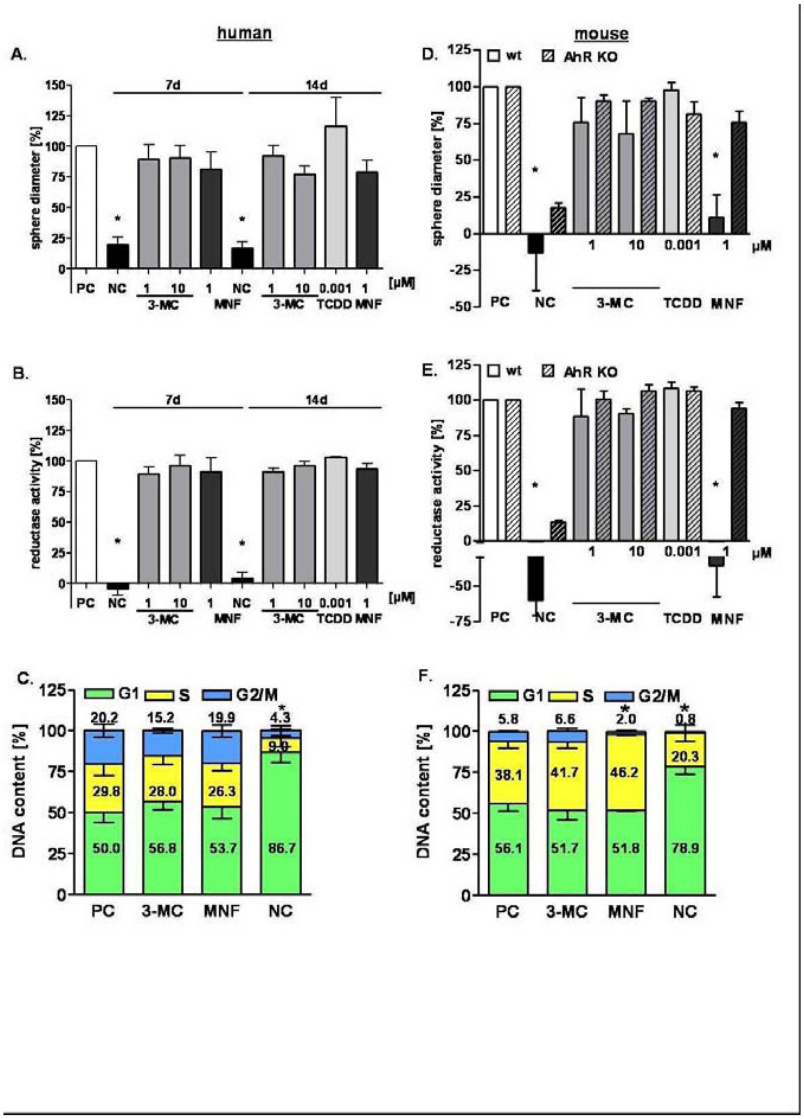
Figure Legends

Figure 1: MNF inhibits mNPC but not hNPC proliferation. NPCs were exposed to 3-MC, TCDD and MNF for 7 (mNPCs) or 14 days (hNPCs). After 7 or 14 days sphere diameters and mitochondrial reductase activities of human (A & B), wildtype and AhR KO mouse (D & E) neurospheres were measured. The results of the two endpoints were plotted in separate xy-diagrams and the gradient of the linear regression curve was assessed after 7 or 14 days. Data represent the mean gradients ± SEM of 2-4 independent experiments (5-6 spheres/exposure). Cell cycle phase distributions of dissociated, fixed, and propidium iodine–stained human (C) and mouse (F) NPCs were analyzed by FACS. Shown is the mean ± SEM of 3 independent experiments after 48h exposure to 10 µM 3-MC or 1 µM MNF. PC (positive control) = 0.1% DMSO with 20 ng/ml EGF and FGF; NC (negative control) = 0.1% DMSO without growth factors. (* = $p < 0,05$ vs. PC).

Figure 2: AhR agonists shorten mNPC but not hNPC migration. Human (A & C) and wildtype and AhR KO mouse NPCs (B & D) were exposed to 3-MC, B(a)P, TCDD, MNF or MeHgCl during differentiation for 48h. Migration distance from the edge of the sphere to the furthest outgrowth was measured. Data represent mean ± SEM of 2-5 independent experiments (5-8 spheres/exposure). * = $p < 0,05$ vs. 0.1% DMSO. Scale bar = 500 µm

Figure 3: 3-MC induces cyp mRNA expression in mNPCs. hNPC (A) and mNPC (C) mRNA expression of the indicated genes were analysed 6, 12, 24, 48h after 10 µM 3-MC exposure under differentiating conditions. Shown as insert in (C) is the cyp1a1 expression after 6h 1 nM TCDD exposure in wildtype and AhR KO mNPCs. Copy numbers of the respective genes were normalized to beta-actin and are expressed as fold induction compared to vehicle control (0.1% DMSO). Shown are means ± SEM of 2-4 independent experiments

(* = $p < 0,05$ vs. vehicle control). Note the different y-axis scaling in (A) & (C). (B) hNPC lysates (40 µg protein/lane) were analyzed for AhR expression by western blot using an AhR specific antibody and HepG2 cell lysates (10 µg protein/lane) as positive control. As loading control, a GAPDH specific antbody was used. Shown is a representative example. (D) Comparison of human and mouse mRNA copy numbers/1000 copies of the beta-actin of proliferating and 24h differentiating NPCs. Data represents at least three independent experiments. To compare human and mouse mRNA expression levels, the ratio mouse/human is calculated (n.d. = not determinable).



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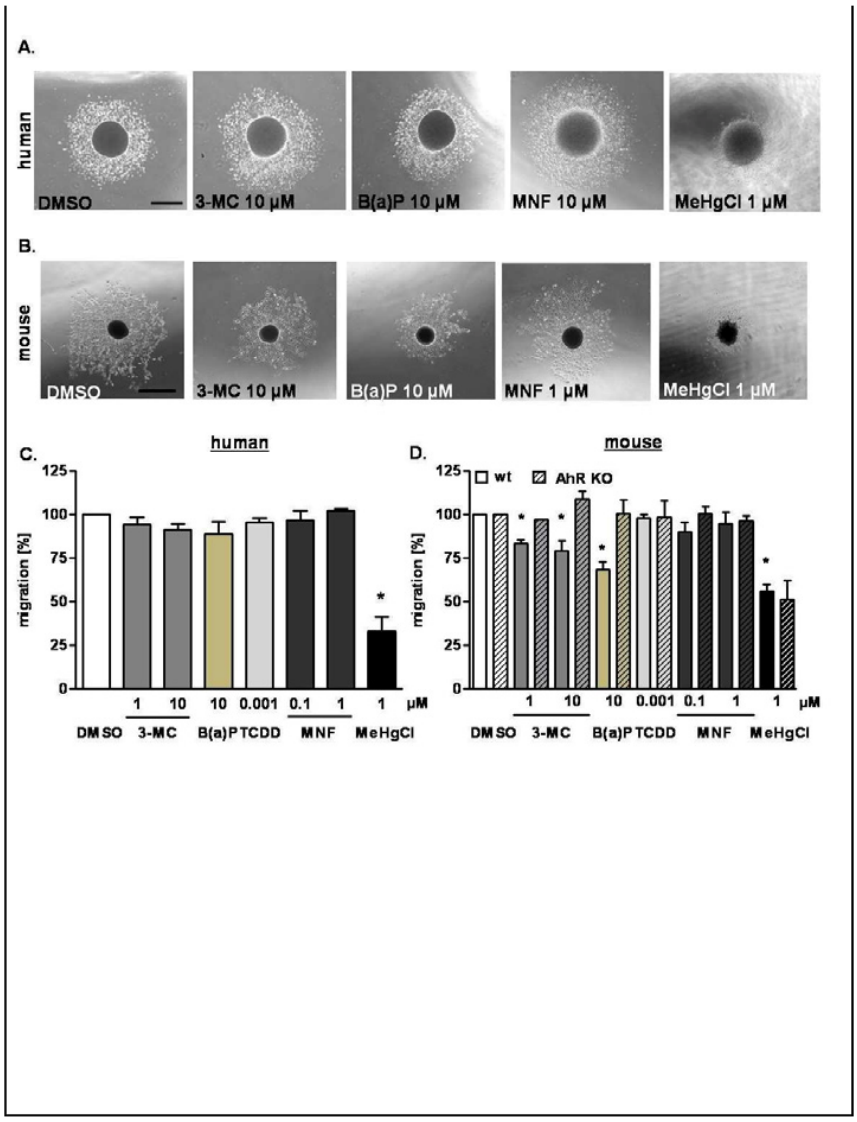

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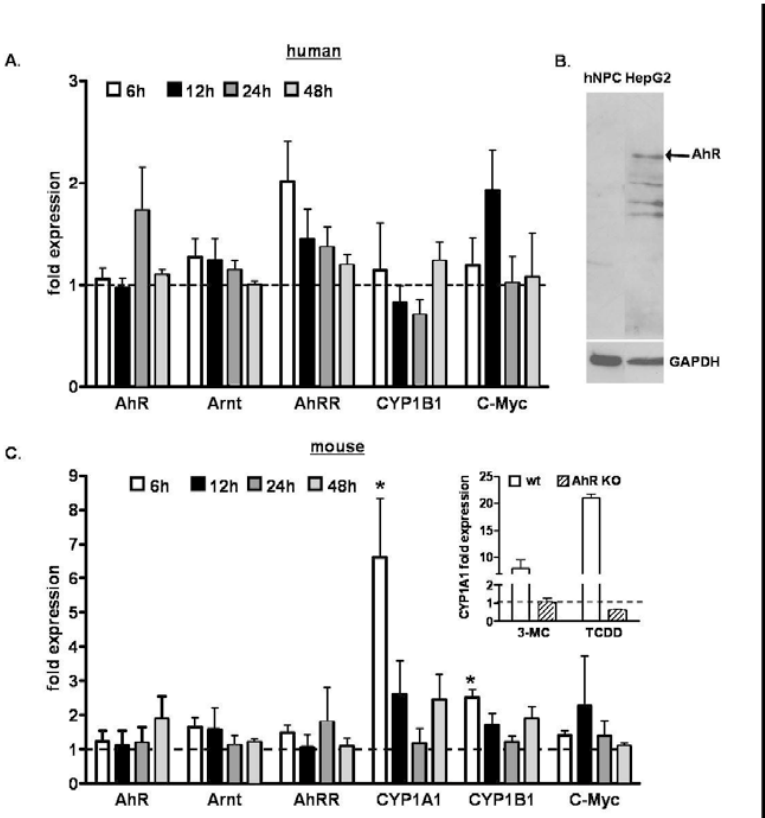


D.

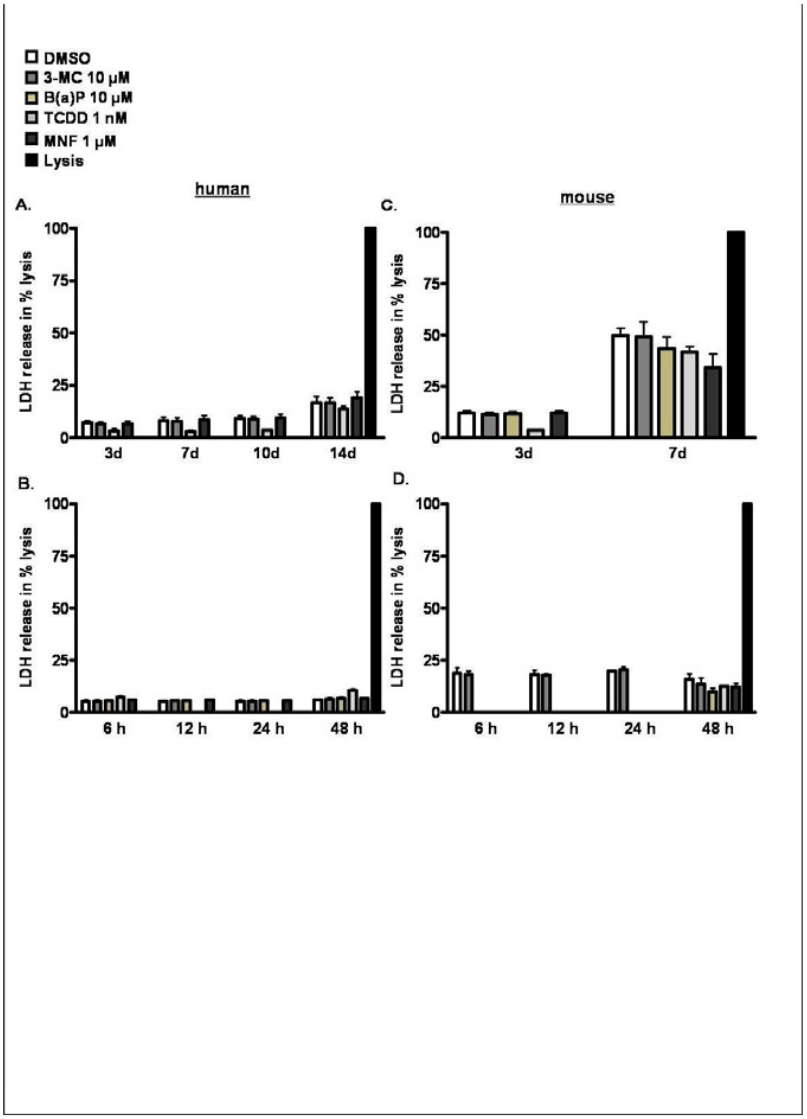
	human		mouse		ratio mouse/human	
	proliferating	differentiating	proliferating	differentiating	proliferating	differentiating
AhR	2.45	0.62	20.08	63.21	8.19	102.12
ARNT	12.95	13.55	716.26	1045.07	55.31	77.13
AhRR	0.94	0.61	79.21	387.55	84.24	638.85
CYP1A1	<0.001	<0.001	18.63	31.64	n.d.	n.d.
CYP1B1	0.01	0.06	146.24	834.91	16407.23	14990.75
C-MYC	5559.59	3017.13	14835.55	15967.72	2.67	5.29

Figure 3: 3-MC induces cyp mRNA expression in mNPCs. hNPC (A) and mNPC (C) mRNA expression of the indicated genes were analysed 6, 12, 24, 48h after 10 µM 3-MC exposure under differentiating conditions. Shown as insert in (C) is the cyp1a1 expression after 6h 1 nM TCDD exposure in wildtype and AhR KO mNPCs. Copy numbers of the respective genes were normalized to beta-actin and are expressed as fold induction compared to vehicle control (0.1% DMSO). Shown are means ± SEM of 2-4 independent experiments (* = $p < 0,05$ vs. vehicle control). Note the different y-axis scaling in (A) & (C). (B) hNPC lysates (40 µg protein/lane) were analyzed for AhR expression by western blot using an AhR specific antibody and HepG2 cell lysates (10 µg protein/lane) as positive control. As loading control, a GAPDH specific antbody was used. Shown is a representative example. (D) Comparison of human and mouse mRNA copy numbers/1000 copies of the beta-actin of proliferating and 24h differentiating NPCs. Data represents at least three independent experiments. To compare human and mouse mRNA expression levels, the ratio

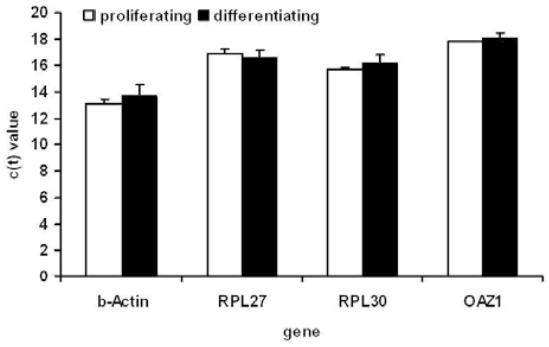
mouse/human is calculated (n.d. = not determinable).
103x137mm (300 x 300 DPI)

C.

	human		mouse		ratio mouse/human	
	prolif.	diff.	prolif.	diff.	prolif.	diff.
AhR	2.45	0.62	20.08	63.21	8.19	102.12
ARNT	12.95	13.55	716.26	1045.07	55.31	77.13
AhRR	0.94	0.61	79.21	387.55	84.24	638.85
CYP1A1	<0.001	<0.001	18.63	31.64	n.d.	n.d.
CYP1B1	0.01	0.06	146.24	834.91	16407.23	14990.75
C-MYC	5559.59	3017.13	14835.55	15967.72	2.67	5.29



Supplemental Material, Figure 1: Cytotoxicity determination by measurement of LDH-release of hNPCs under proliferating (A) and differentiating (B) conditions and of mNPCs under proliferating (C) and differentiating (D) conditions after 3-MC, B(a)P, TCDD and MNF treatment for depicted times. Complete lysis of cells with a lysis buffer for 2 h at room temperature serves as positive control. Shown are mean ± SEM of at least three independent experiments.
98x137mm (300 x 300 DPI)



Supplemental Material, Figure 2: Beta-actin is a valid housekeeping gene because it is stably expressed under different culture conditions. Beta-actin mRNA expression depicted as cycle threshold (ct) value of proliferating and differentiating hNPCs was compared to the expression of three additional housekeeping genes RPL27, RPL30 and OAZ1. Shown is the mean ± SD of one experiment performed in triplicate.
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