Molecular and functional analysis of toxicity pathways contributing to chemical induced developmental neurotoxicity in neural progenitor cells of human and rodent

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Molekulare und funktionelle Untersuchung toxischer Signalwege in der Entwicklungsneurotoxizität von Chemikalien in neuralen Progenitor Zellen von Mensch und Nager

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1 Introduction

1.1 Brain development

The human brain is the most complex organ in the body. The adult central nervous system consist of approximately 86 billion neurons, a roughly equal number of glial cells (Azevedo et al. 2009; Herculano-Houzel et al. 2016) and an estimate of 620 trillion to several quadrillion synapses (Silbereis et al. 2016). In order for those cells to form a functional adult brain they have to develop into the different subtypes of neurons and glial cells, position themselves in the right area at the right time and form a complex neuronal network. From the beginning of brain development in the early embryonic phase to its end in the mid-20s, this is ensured by a variety of strictly controlled cellular processes (Figure 1). As summarized in Stiles and Jernigan (2010) and Silbereis *et al.* (2016) human brain development begins with the recruitment of neural progenitor cells from the ectodermal layer during gastrulation. These cells form the neural tube as the first defined brain structure. During a phase of exponential growth, the neural tube expands to form the five brain vesicles that establish the primary organization of the central nervous system. Neurogenesis begins in the late embryonic phase at embryonic day 42 and continuous throughout the fetal phase and postnatally in certain brain regions (Bystron et al. 2008; Stiles and Jernigan 2010). It includes production of neurons from neural progenitors including radial glia cells, migration to the correct brain regions and postmitotic differentiation and maturation into several subtypes of inhibitory or excitatory neurons. Final positions and differentiation states of neurons are strictly controlled by gradients of cell fate-determining signaling molecules in different brain regions. Once positioned neurons start to extend dendrites and axons and form connections (synapses) with other neurons. The process of synaptogenesis begins during mid-gestation and leads to an excess production of neuronal connections (Silbereis et al. 2016). Subsequently, these connections are refined by strengthening some connections and eliminating (pruning) others. Oligodendrocytes and astrocytes are generated from radial glial cells shortly after the start of neurogenesis. Thereby glial precursors continue to proliferate, migrate, differentiate and mature during the first 3 years of postnatal development. Myelination, the process in which oligodendrocytes form the myelin protein sheets around neighboring axons to isolate them and facilitate a higher axonal conduction velocity, starts around birth and together with the synaptic pruning refines the brains functional network structures until

adolescence (Howard et al. 2008; Jakovcevski 2009; Miller et al. 2012). The exact interaction between neurons and oligodendrocytes/astrocytes during and after brain development are still not fully understood but there is no doubt that these cells play an important role in the development of a functional neuronal network.

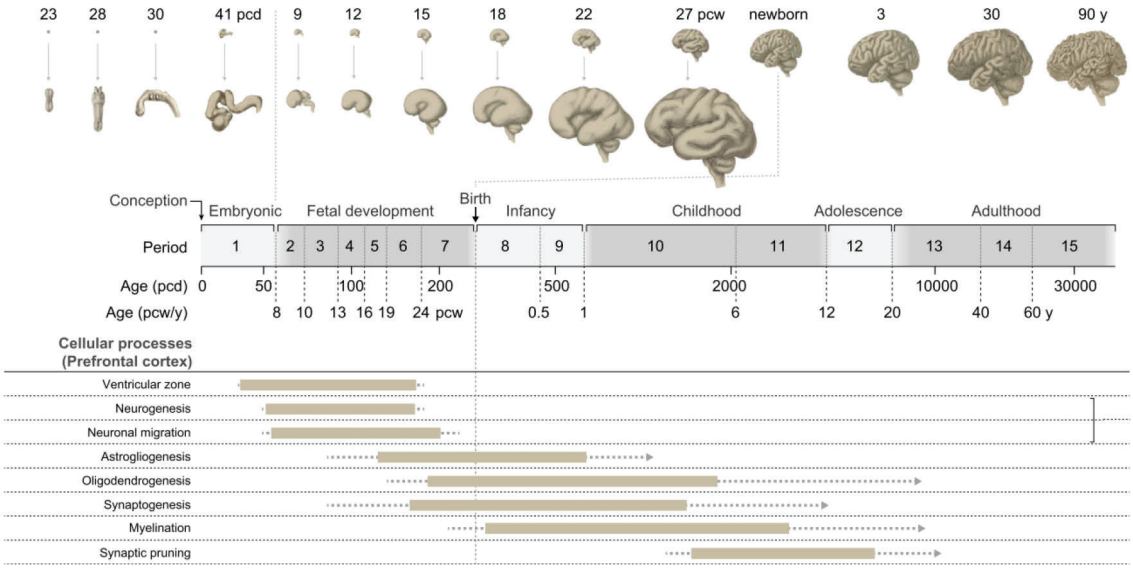

Figure 1. Cellular processes of human brain development from early embryonic phase to adulthood (adapted from Silbereis et al., 2016).

Altogether, the above-mentioned processes are guided by an interplay of proteins that act as signaling molecules, receptors, messengers or transcription factors whose expression is in turn regulated by genetic, epigenetic and environmental factors. In the end, the perfect interplay between intrinsic and environmental factors during the whole period of brain development determines the precise orchestration of brain developmental processes and produces a healthy and functioning human brain (Kandel et al. 2000).

1.2 Developmental Neurotoxicity (DNT)

Dysregulation of any neurodevelopmental process by environmental factors of chemical or physical nature (e.g. environmental chemical, drug, radiation) can lead to functional or morphological changes of the brain ultimately causing neurocognitive or neurobehavioral impairments such as learning disabilities, reduced IQ, dyslexia, Attention Deficit Hyperactivity Disorder (ADHD) or autism.

Bellinger (2012) for example estimated that a loss in IQ caused by some environmental chemicals is in the same order of magnitude than the IQ loss associated to medical events

such as preterm birth, traumatic brain injury, brain tumors or congenital heart disease. A decrease in the average IQ may not dramatically impact the individual but can cause a shift in IQ distribution leading to an increased percentage of people with mental retardation and a decrease in the percentage of people that are mentally 'gifted' (Figure 2). Overall the socioeconomic consequences can be tremendous and mainly consists of diminished economic productivity, higher cost and effort for people that need special care and education and an increased risk of antisocial and criminal behavior (Grandjean and Landrigan 2006).

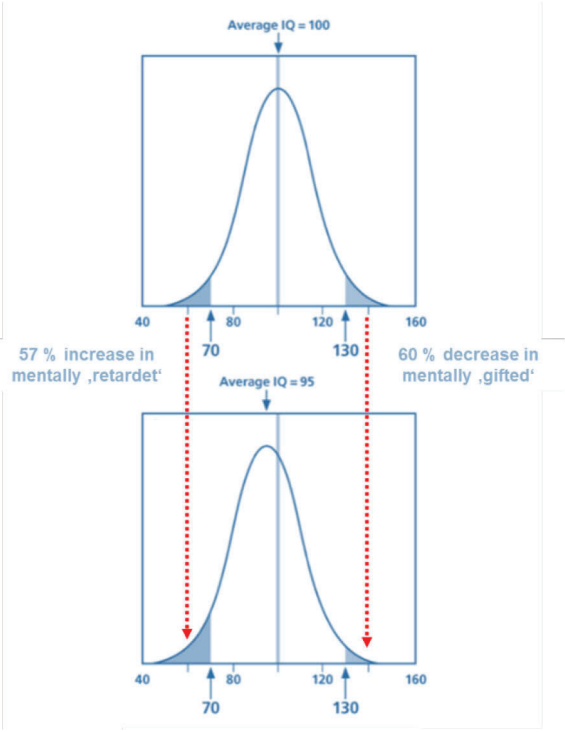

Figure 2. Significance of a loss in average IQ of a hypothetical population. It is estimated that a loss of 5 IQ points in a population of 260 million people with an average IQ of 100 leads to an increase of mentally 'retarded' from 6 to 9.4 million and a decrease in mentally 'gifted' from 6 to 2.4 million (Weiss, 1990; Figure is adapted from Schmidt, 2013)

It is estimated that methylmercury exposure leads to a yearly IQ loss of 600,000 IQ points in Europe and 264,000 in the US which corresponds to a loss of economic benefit of 8 and 5 billion US$ per year, respectively (Bellanger et al. 2013). Gould (2009) showed that exposure to lead is associated to a loss of 9.3 to 13.1 Million IQ points in children in the US, an increased number of children that needs special education, an increased incidence in ADHD cases and in increased crime rate due to social and emotional dysfunctions. The socioeconomic costs are estimated between 26.7 and 36.9 billion US$. It is generally assumed that the developing brain is more susceptible to adverse chemical actions than the adult brain. The main reason is that the variety of dynamic molecular and cellular

changes needs precise regulation over the whole developmental time period that are largely finished in adulthood. At the same time, the unborn is exposed towards compounds that in adults do not cross the blood-brain-barrier (BBB) via the mother's circulation. Higher child brain exposure for some compounds compared to the mother is due to the limited functionality of biological barriers, the BBB and the placenta barrier. The BBB protects the adult brain against many, especially hydrophilic chemical agents but does not offer full protection during development until early infancy (Zheng et al. 2003). Also the placenta offers only limited protection against compound exposure as many environmental toxicants cross this barrier. In the postnatal phase, the child is exposed towards mainly lipophilic chemicals by breastfeeding, which due to the high fat content of breast milk are generally found accumulated in this compartment (Needham *et al.*, 2011; Environmental Working Group, 2005).

From the chemical universe of more than 100,000 million substances (CAS - Registry 2015) only 15 Compounds have been identified as developmentally neurotoxic to humans. These are 9 environmental-, or industrial chemicals: lead, methylmercury, arsenic, polychlorinated and brominated biphenyls, toluene, fluoride, manganese, tetrachloroethylene; 3 pesticides: Chlorpyrifos, DDT and DDE; 1 medical drug: Valproic acid and 2 drugs of abuse: ethanol and cocaine (Aschner et al. 2016; Giordano and Costa 2012; Grandjean and Landrigan 2014; Kadereit et al. 2012). More than 200 Compounds are known to cause neurotoxicity (NT) in humans and there is experimental data for more than 1000 on their neurotoxicity in either *in vivo* or *in vitro* studies (Grandjean and Landrigan 2006). DNT testing is only mandatory for pesticides in the European Union the and required solely for substances that cause neurotoxicity or endocrine disruption in the United States, leaving a large variety of compounds so far untested for this endpoint (Schmidt et al. 2016). Given the high vulnerability of the developing nervous system there is concern that many more chemicals affect human brain development and that current safety margins derived from NT or other toxicity studies do not necessarily protect the developing brain. For example, methylmercury disturbs brain development with much lower exposure than it affects the adult brain in humans (Oken and Bellinger 2008). A recent review by Mundy *et al.* (2015) supports the aforementioned concerns showing that for 100 additional compounds there is some evidence on their developmental neurotoxic potential in animal in vivo studies with 22 of those having additional human evidence. Another challenge in DNT assessment is the identification of an association between early life exposure to often very small adverse effects that mainly occur at late life stages. Overall, the high vulnerability of the developing nervous system, the challenge to detect

DNT effects and the insufficient testing underline the importance and need to advance DNT testing.

1.2.1 DNT testing

Current DNT testing is performed according to the draft OECD Test Guideline (TG) 426 and EPA 870.6300 DNT Guideline (Epa 1998; OECD 2007). In both guideline studies exposure to the test compounds covers a period from early gestation until the end of lactation. The evaluation consists of observations on gross neurologic and behavioral abnormalities. These include tests that describe physical development, behavioral ontogeny, motor activity, motor and sensory function, learning and memory, the evaluation of brain weights and neuropathology in neonatal and young animals (preferably rat). Performing a DNT guideline study is highly resource-intensive because it takes approximately one year and costs up to one million US$ per chemical (Crofton et al. 2012). Moreover, these guideline studies are of particular ethical concern due to the high number of animals (approximately 1400 animals) that need to be sacrificed. Despite the large resource investment that needs to be done for performing a DNT guideline study, interpretation of data might be difficult because there are some uncertainties in the guidelines methodology, evaluation, and regulation. Furthermore, in vivo toxicity testing in rodents per se has uncertainties for the human situation due to considerable species differences (Knight 2007; Leist and Hartung 2013; Schmidt et al. 2016). In addition, they are limited in their capacity to test large amount of chemicals (Tsuji and Crofton 2012). Thus, only approximately 140 in vivo guideline studies (according to OECD 426 or EPA OPPTS 870.630) have been performed so far (EFSA 2016).

1.2.2 Species differences

It is increasingly recognized that laboratory animals do not necessarily resemble human physiology (Knight 2007; Leist and Hartung 2013; Schmidt et al. 2016). Olson *et al.* (2000) for example demonstrated that rodents identified only 43 % of 150 pharmaceuticals known to be toxic in humans. Knight (2007) summarized that out of 20 reviews that examined the contribution of an animal experiment for the development of human clinical intervention or deriving human toxicity classification, animal models were significantly useful or consistent with outcomes in humans in only 2 cases. Both findings well represent the limited predictive power of animals. The consequence are false predictions, ultimately causing failure in drug development or false risk assessment. Prominent examples are the sedative thalidomide or monoclonal antibody TGN1412. Both compounds did not show animal

toxicity but caused tremendous adverse health effects in humans (Attarwala 2010; Miller and Stromland 1999).

Limited predictive power of animal models might be explained by species-specificity toxicodynamics and toxicokinetics. In the field of neuroscience, species differences are especially apparent as it is the function of the human brain that makes us unique in the animal kingdom. Special features that distinguish the primate, particularly the human brain, from the rodent brain are for example a bigger neocortex in relation to body size or a seven times higher neuronal density in certain brain regions (Herculano-Houzel 2009). Concerning brain developmental processes the sequence of developmental events is comparable between species (Rice and Barone 2000). However, there are considerable difference in developmental timing, organization of brain structures as well as structure and distribution or even presence of certain cell types. The most obvious differences in developmental timing is the prolonged period of human brain development. While in rodents developmental processes take several days to weeks, similar processes can take years in human brain development. The process Neurogenesis for example takes place between GD9 and PND35 in rat and GW3 and 2.5 years in humans (Rice and Barone 2000; Workman et al. 2013). The delayed onset of neurogenesis in primates compared to rodents thereby facilitates a greater expansion of the progenitor cell pool leading to an increased neuronal output (Florio and Huttner 2014). Although humans have a much longer over all period of brain development, right after birth their brains are more mature than rodent brains possibly due to the relatively longer gestation time (Rice and Barone, 2000; Workman *et al.*, 2013; Florio and Huttner, 2014). In regional development, there are differences in the anatomical organization of primate and rodent embryonic cortices with some specific features in primates. One example is the outer subventricular zone, a proliferative layer, which gives birth to crucial computational components of the cortex that is absent in the rodent brain (Dehay and Kennedy 2007). Another obvious morphological difference is the highly convulsed (gyrencephalic) cerebral cortex with the large neocortex in the primate brain compared to the smooth cerebral cortex of the rodent (lissenciphalic) brain with a small neocortex. The high degree of gyrification is the evolutionary solution of the huge neocortical expansion in primates (Azevedo et al. 2009; Florio and Huttner 2014). Other species differences are apparent in the abundancy and structure of astrocytes (Bass et al. 1971; Oberheim et al. 2009; Zhang et al. 2016), the structure of dendrites or the proportion of GABAergic interneurons in the cortex (DeFelipe 2011). There are even certain cell types like the double bouquet cell that are very numerous in primates but absent in rodents (Yáñez et al. 2005). Altogether these differences could in some way affect species sensitivity towards chemicals and cause species-specific adverse outcomes

after compound action. Therefore, species-specificities need to be considered in human hazard and risk assessment processes.

1.3 Alternative toxicity testing

1.3.1 Paradigm shift in toxicology

The traditional concept of toxicity testing is the determination of a chemical effect in animal models based on an apical endpoint measures. For human risk assessment, a safe dose is calculated based on the dose that has no adverse effect in the animal and uncertainty factors that account for toxicodynamic and toxicokinetic inter-species differences as well as inter-individual susceptibilities. Low prediction, species differences and the increasing societal awareness of animal testing have been questioning the current concept of animal models for human risk assessment and drives the need to advance alternative testing approaches. In 2007 the National research council (NRC) of the United States published the report 'Toxicity testing in the 21st Century: A vision and a Strategy' in which they conclude that current testing is not adequate to fully protect human health. They further envision a major paradigm shift in future toxicology away from the apical endpoint measures of in vivo testing to alternative, toxicity pathway-based high throughput methods. Their proposal includes the utilization of new technologies in molecular and computational biology or biotechnology and the combination of alternative methods. In vitro methods (cell based or cell free), in silico methods or model organisms (C. elegans, zebrafish and drosophila) allow time- and cost-efficient screening of large amounts of compounds with a more mechanism-based hazard identification (Gibb 2008; Krewski et al. 2010; National Academy of Sciences 2007). Figure 3 presents a summary of the future toxicity testing approach envisioned by the NRC.

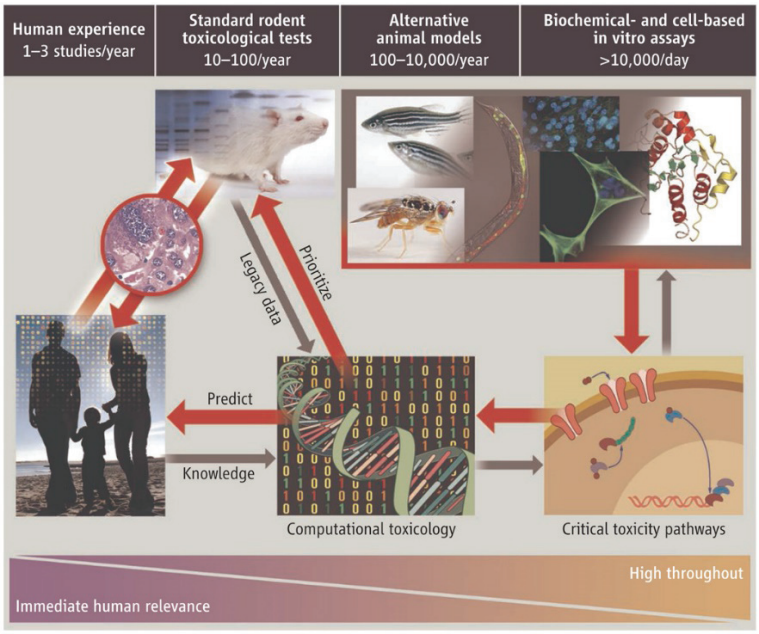

Figure 3. The future concept of toxicity testing. High throughput cell based in vitro assays are combined with alternative organisms and computational toxicology to allow the screening of large amounts of substances for compound prioritization or direct mechanism based human risk assessment (Collins et al. 2008).

1.3.2 The Adverse Outcome Pathway (AOP) concept

A framework for a mechanism-based toxicity assessment is the Adverse Outcome Pathway (AOP) concept. The AOP concept was first developed for future ecotoxicological risk assessment to face similar challenges as they have been described for human risk assessment by the NRC, namely an increasing demand to assess more chemicals, with greater speed and accuracy and a mechanism based risk assessment (Ankley et al. 2010). In the AOP concept existing or newly generated biological and toxicological data is summarized by linking all known events of chemical actions across the different levels of biological organization, from a Molecular Initiating Event (MIE) over multiple Key Events (KE) to the Adverse Outcome (AO; Figure 5; Ankley *et al.*, 2010; OECD, 2013).

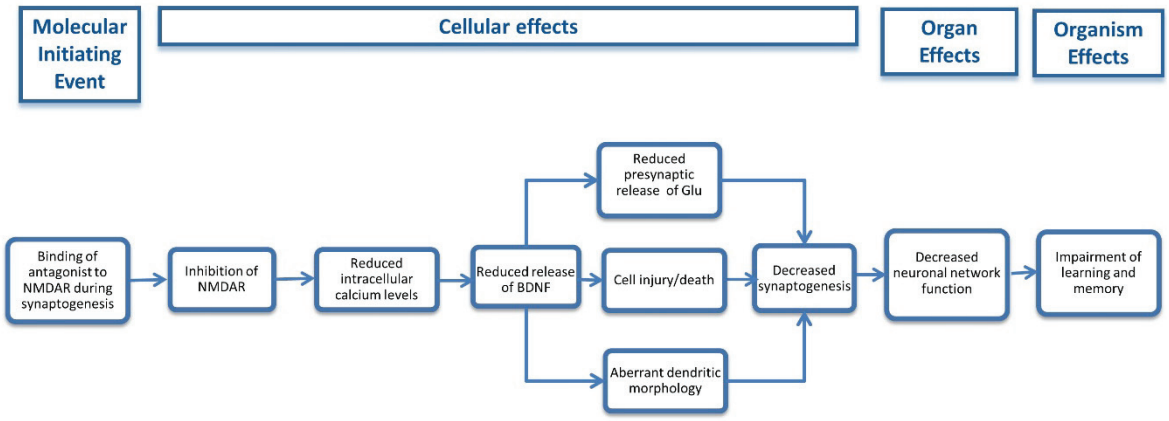

Figure 4 AOP on Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development (Sachana et al. 2016).

Each AOP is thereby characterized by a unique MIE followed by a chain of KE and an adverse outcome that can be common to different MIE. Organization of mechanistical information gathered from alternative models will generate more AOPs which subsequently inform the connection between an adverse outcomes (AO) in the alternative models compared to the AO in humans and thereby facilitates better interpretation of findings in vitro or in alternative organisms. Another key advantage of the AOP concept is the formation of chemical classes based on common KE's or a MIE for different compounds which will facilitate the prioritization in substances screening (Bal-Price et al. 2015a). Far from realization but a future vision is a purely AOP-based testing strategy in which a comprehensive collection of KE across multiple MIE will identify key processes for hazard identification based on chemical properties and inform human risk assessment.

1.3.3 DNT in vitro testing

As specified in Chapter 1, a variety of key neurodevelopmental processes is involved in the development of a fully functional human brain. If any of these events is sufficiently disturbed, it will lead to an adverse health outcome. Common key events that represent major neurodevelopmental processes over different developmental timings are listed in figure 5. In a DNT testing strategy, a selection of alternative assays need to be set up in a testing battery that in combination are able to cover all major key events necessary for brain development. Thereby individual alternative methods need to closely resemble human physiology and developmental timing.

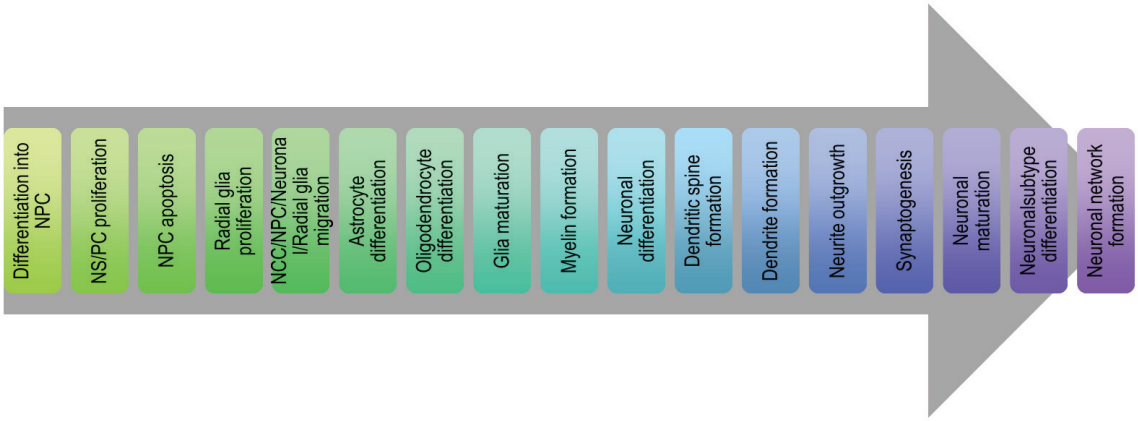

Figure 5. Key neurodevelopmental processes (Fritsche 2016).

Advancements in the field of in vitro testing in the last decades produced to a huge selection of alternative methods that resemble different developmental processes in different species, cell types, brain regions or developmental stages (Fritsche et al. 2015). Although rat primary cells, for historical reasons, are the most abundant and best studied cell systems with most processes studied, aforementioned species differences call for human based systems (EFSA 2016; Fritsche et al. 2015). A recent report on the availability and suitability of in vitro test systems names 6 cell models based on human stem and progenitor cells (hESC, hiPSC, hNPC, ReNcell CX, hUCBSC and LUHMES cells) that show to be suitable to assess 16 neurodevelopmental key events. However, for some key events (radial glia proliferation, glia maturation, dendritic spine formation, dendrite formation, axonal growth, and neuronal maturation, neuronal network formation?) there is still a lack of data concerning human based cell systems. For these endpoints, rodent based models may be considered until an appropriate human based models are developed (Fritsche 2016).

There are certain challenges in setting up a DNT testing battery. For most of the test systems there is a lack in harmonization of cell culture, compound treatment and endpoint evaluation protocols. Therefore, standard operation procedures (SOPs) for suitable test systems have to be set up to allow inter-laboratory reproducibility of test results. Another important step in model development is a thorough characterization of the test system. This is crucial to understand which processes or pathways of human brain development are represented by the respective system and will identify the application domain of the assay. In an ideal DNT testing battery the combination of test systems should represent all pathways relevant for brain development. A possible way to perform such characterization is by utilization of omics approaches like transcriptomes or proteomics that determine the molecular signature underlying changes over the course of cell

development (Hoelting et al. 2016). The use of model compounds or pharmacological modulators that specifically alter certain pathways facilitates a subsequent characterization of the response of a cell system. The last step is a validation of the assay with a training set of compounds. Such a training set contains positive and negative compounds, which are known to affect or not affect brain development, respectively. Challenging individual DNT assays with such a set of compounds provides information on substances' effects on individual endpoints including performance characteristics such as sensitivity and specificity of the individual assay. However, detailed information on the modes of action of such compounds is needed to actually assess assay performance. For DNT hazard assessment, single assays are not sufficient, one needs chemical testing in a DNT test battery covering neurodevelopmental processes comprehensively. Only the satisfying performance of such a battery will ultimately increase confidence in in vitro testing for human DNT hazard identification and promote regulatory acceptance of such methods for future risk assessment.

1.4 The 'Neurosphere Assay'

Primary neural progenitor cells (NPCs) can be used as an in vitro test system that is able to mimic early neurodevelopmental processes in a culture dish. Therefore, these cells growing in 3D as neurospheres are used in the 'Neurosphere Assay' to quantify effects of compounds on the major key neurodevelopmental processes NPC proliferation, migration, neuronal and oligodendrocyte differentiation according to SOPs (Gassmann et al. 2012; Baumann et al. 2014; Baumann, Dach, et al. 2015; Moors et al. 2009; Baumann et al. 2016). Such neurospheres are generated from whole human or rodent brains at comparable developmental timing. Workman *et al.* (2013) demonstrated that human fetal cells from GW16-18, as they are used for the 'Neurosphere Assay', correspond to rodent cells from postnatal day 1. In suspension culture and in presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF) NPC continue to proliferate and form three dimensional cell aggregates (Buc-Caron 1995; Chalmers-Redman et al. 1997; Reynolds et al. 1992; Svendsen et al. 1995). By regular mechanical dissociation neurospheres can be passaged and kept in culture over several weeks (rodent NPC) or month (human NPC; Svendsen *et al.*, 1997; Svendsen et al. 1998). After withdrawal of growth factors and when placed on an extracellular matrix cells migrate radially out of the sphere core while they start to express proteins of the main brain effector cells: beat-III-tubulin (TUBB3) for neurons, O4 for oligodendrocytes and glial fibrillary acidic protein (GFAP) for astrocytes (Figure 6 ;Brannen and Sugaya, 2000; Piper *et al.*, 2001; Reubinoff *et al.*, 2001; Lobo *et*

al., 2003). The progenitor cell properties, evident by nestin expression, thereby decreases with increasing differentiation time and migration distance (Schmuck et al. 2016).

With the 'Neurosphere Assay' effects of chemical exposure on NPC development is analyzed. Proliferation is assessed by incorporation of the thymidine analogue bromodeoxyuridine (BrdU), which is an indirect measure of DNA synthesis. An effect on migration is determined by measuring the migration distance from the sphere core to the edge of the migration area. The differentiation to neurons and oligodendrocytes is quantified as the number of TUB3 or O4 positive cells in relation to total nuclei in the migration area after immunocytochemical staining, respectively. The assessment of DNT specific endpoints is always accompanied by a measure of general cell viability and cytotoxicity at the identical time point in the same culture. For a measure of viability, the mitochondrial activity is determined with the alamar blue assay. Cytotoxicity is assessed as an indirect measure of membrane integrity with the lactate dehydrogenase (LDH) assay (Baumann et al. 2014, 2015a; Moors et al. 2009).

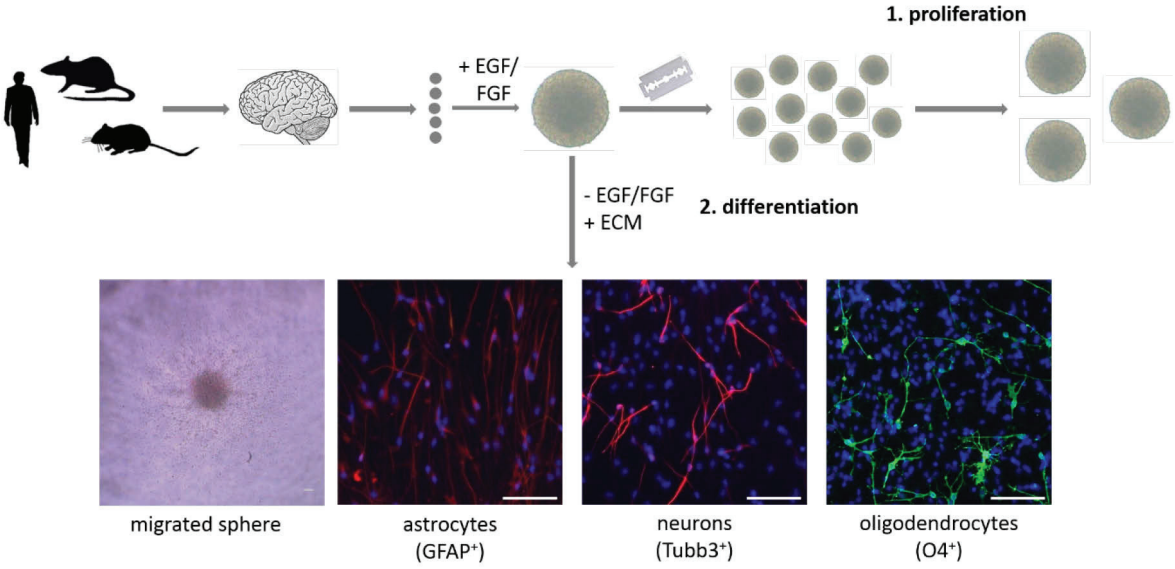

Figure 6. The 'Neurosphere Assay'. Neural progenitor cells from human (GW 16-18) and rodent (PND1) brains are cultivated with growth factors (EGF, FGF) and form 3D cell aggregates. Cells are passaged by mechanical dissociation. After withdrawal of growth factors and contact to the extracellular matrix (ECM) protein laminin, cells attach to the plate surface, migrate radially from the sphere core and thereby differentiate into the main effector cells of the brain, namely neurons, oligodendrocytes and astrocytes. Cell nuclei are shown in blue (Hoechst). Scale bar equals 100 µm.

High content image analysis (HCA) allows a fast and automated quantification of migration distance and neuronal differentiation and extends the 'Neurosphere Assay' to parameters

of neuronal morphology (number of neurites, neurite length, number of branching points), neuronal density distribution or quantification of nestin (NES)- and GFAP-positive cells (Schmuck et al. 2016). Other endpoints that can be assessed with the ‘Neurosphere Assay’ are general and cell type-specific apoptosis (Baumann et al. 2014) or maturation of oligodendrocytes (Dach et al. 2017) and astrocytes (Manuscript 2.1). These endpoints can be analyzed in a medium throughput set up allowing a fast and cost efficient screening of chemicals. The fact that NPCs differentiate into the major cell types of the brain forming a heterogeneous culture in a 3d set up and the possibility to generate them from multiple species are other key advantages of this assay. Especially the latter allows direct species comparison of compound action in vitro. According to the parallelogram approach (Baumann et al. 2015) this information can be used to extrapolate human in vitro findings to the human in vivo situation and thereby facilitate a mode of action-based and thus knowledge-driven risk assessment. Altogether above-mentioned advantages make the ’Neurosphere Assay’ a promising model system within a DNT testing battery.

1.5 Aim of this thesis

The developing nervous system is vulnerable to chemical insult raising the concern that a considerable amount of chemicals adversely affects human brain development. Because todays testing requirements are not sufficient to analyze the DNT potential of chemicals there is a huge data gap in DNT hazard identification across the chemical universe. There is consensus within the scientific community that this gap needs to be filled by compound screening in time- and cost-efficient, predictive manner by using alternative testing methods. Although these methods cannot fully replace current in vivo testing until more confidence is gained in their predictive capacities, they allow mechanism-based hazard identification and prioritization for further testing. The use of alternative assays for decision making on a regulatory level requires characterized and validated assays. Therefore, the main aim of this thesis is the biological characterization and pathway validation of 3D neurospheres generated from human and rodent brains as models for DNT screening and molecular and functional DNT pathway analyses.

The following goals were addressed in this thesis:

1) Molecular and functional characterization of human and rodent neural progenitor cells based on transcriptional changes and pharmacological modulation during NPC development.

2) Identification of assay performance across human and rodent neutrospheres with a training set of DNT positive or negative chemicals.

3) Investigation of the mode of action of Arsenic, a well-known developmental neurotoxic compound, in human and rat NPC.

2 Manuscripts

The present thesis consists of three manuscripts.

The first manuscript 2.1, ‘A transcriptome comparison of time-matched developing human, mouse and rat neural progenitor cells reveals human uniqueness’ provides a molecular characterization of developing neural progenitor cells in a species comparative manner. In this manuscript, we generated and compared mRNA expression profiles of developing human, mouse and rat neural progenitor cells. We further identified key regulators of fundamental neurodevelopmental processes and validated their pharmacological modulation on a functional level. The main aim of this work was the molecular characterization of the ‘Neurosphere Assay’ and its biological application domain.

In the second manuscript 2.2, ‘Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events’ the ‘Neurosphere Assay’ was challenged with a set of six well characterized DNT-positive and three DNT-negative compounds. Here the overall aim was to evaluate the general capability of the assay to correctly predict the DNT potential of this ‘test set’ of chemicals. The study further provides a comparison of species sensitivities towards the tested compounds.

In manuscript 2.3, ‘Arsenite Interrupts Neurodevelopmental Processes of Human and Rat Neural Progenitor Cells: the Role of Reactive Oxygen Species and Species-Specific Antioxidative Defense’ we applied the ‘Neurosphere Assay’ to evaluate the effects of arsenite, a well-known DNT compound, on neurodevelopmental key events and performed a mechanistic investigation of arsenite-mediated effects in human and rat neural progenitor cells. The main objective was thereby to demonstrate the suitability of the assay for mechanistic investigations and to better understand DNT relevant mechanisms of toxicity. All three publications share the overall goal to advance alternative DNT testing to enable a better human risk assessment and the reduction of animal experiments.

2.1 A transcriptome comparison of time-matched developing human, mouse and rat neural progenitor cells reveals human uniqueness

Stefan Masjosthusmann, Daniel Becker, Barbara Petzuch, Jördis Klose, Clara Siebert, Rene Deenen, Marta Barenys, Jenny Baumann, Simon Schliesky, Katharina Dach, Julia Tigges, Ulrike Hübenthal, Karl Köhrer, Ellen Fritsche

Nuclei Acids Research (NAR)

Es ist weitgehend akzeptiert, dass Nager nicht alle Aspekte der humanen Gehirnentwicklung repräsentieren können. Offensichtliche Gründe hierfür sind die evolutionäre Distanz sowie Unterschiede in der Physiologie. Aus diesem Grund unterliegt die Übertragung von tierbasierten Forschungsergebnissen auf die Situation im Menschen immer einer gewissen Unsicherheit und dem Risiko einer falschen Voraussage. In dieser Studie charakterisieren wir sich entwickelnde neurale Progenitor Zellen (NPC) von Mensch, Maus und Ratte anhand von Änderungen in ihrem Transkriptomsprofil im Laufe ihrer Differenzierung von proliferierenden NPC zu differenzierten Effektorzellen. Unsere Ergebnisse zeigen, dass die drei Spezies über die Differenzierungszeit sowohl qualitative als auch quantitative Unterschiede in ihrem Expressionsprofil aufweisen. Wesentliche Prozesse der Gehirnentwicklung wie Zellmigration, Neurogenese und Gliogenese, sowie die Entwicklung multizellulärer Organismen, sind allerdings konserviert in allen drei Spezies. Des Weiteren haben wir wichtige Regulatoren einiger dieser fundamentalen Prozesse identifiziert und durch pharmakologische Modulation funktionell charakterisiert. Diese pharmakologische Intervention offenbarte dabei verschiedene speziesspezifische zelluläre Effekte. Diese Studie unterstreicht die Wichtigkeit für das Verständnis von Speziesunterschieden und den Nutzen von auf menschlichen Zellen basierenden in vitro Modellen für die pharmakologische und toxikologische Forschung.

A transcriptome comparison of time-matched developing human, mouse and rat neural progenitor cells reveals human uniqueness

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ABSTRACT

It is widely accepted that human brain development has unique features that cannot be represented by rodents. Obvious reasons are the evolutionary distance and divergent physiology. This might lead to false predictions when rodents are used for safety or pharmacological efficacy studies. For translating animal-based research better to the human situation, human in vitro systems might be useful. In this study, we characterize developing neural progenitor cells from prenatal human and time-matched rat and mouse brains by analyzing the changes in their transcriptome profile during neural differentiation. Moreover, we identify hub molecules that regulate neurodevelopmental processes like migration and differentiation. Consequences of modulation of three of those hubs on these processes were studied in a species-specific context. We found that although the gene expression profiles of the three species largely differ qualitatively and quantitatively, they cluster in similar GO terms like cell migration, gliogenesis, neurogenesis or development of multicellular organism. Pharmacological modulation of the identified hub molecules triggered species-specific cellular responses. This study underlines the importance of understanding species differences on the molecular level and advocates the use of human based in vitro models for pharmacological and toxicological research.

INTRODUCTION

Pharmacological research as well as drug safety testing have mainly been based on studies in laboratory animals. Besides ethical concerns and high resource needs with regards to time and money, laboratory animals do frequently fail to predict beneficial or adverse compound effects for humans leading to high attrition rates when moving from preclinical research to clinical drug applications (1, 2), e.g. rodents only correctly identified 43% out of 150 pharmaceuticals known to be toxic in man (3). Also, an analysis of systematic reviews published in the 'Scopus' database on the human clinical or toxicological utility of animal experiments revealed that in only 10% of the reviews animal models were significantly useful (4). Several reasons were identified to be responsible for this unsatisfactory translation from animals to humans. For one, poor performance quality and reporting of animal studies impedes prediction (4–8). Moreover, species differences can hamper predictive value of animal in vivo studies (4, 8–10). Resulting false anticipations of compound's efficacy might result in economic damage and lack of treatment due to failures in drug development (10–12). Incorrect information on human safety or toxicity of substances is equally severe as it might result in serious human health effects as experienced with thalidomide or TGN1412, which did not show animal toxicity in the species tested but caused serious toxicities in humans (13, 14). While performance quality and reporting of animal studies

can be improved e.g. by introducing more stringent quality criteria (15), species differences in human vs. animal physiology and/or pathology cannot rapidly be overcome using in vivo whole animal studies.

To improve 'human relevance' in safety and efficacy studies, one can make use of human cell-based in vitro methods. Although such methods lack pharmaco-/toxicokinetics of the whole organism (absorption, distribution, metabolism, excretion), they are thought to maintain their pharmaco-/toxicodynamics of the target cell in vitro. There are many examples for species-specific differences in the molecular equipment of cells that can determine compound action (10, 16–19).

We have previously shown that such specificities in toxicodynamics across species are also maintained in primary, time-matched neurospheres from humans, mice and/or rats in vitro (20–22). Three dimensional (3D) neurospheres consist of neural progenitor cells (NPC) that allow assessment of compound-specific effects on NPC proliferation, radial migration, differentiation into neurons, astrocytes and oligodendrocytes as well as neuronal migration and neurite outgrowth (21–26). Moreover, modes of action of substances can be studied with this organoid cell culture method (22, 25, 27, 28). Due to these attributes, this 'Neurosphere Assay' is thought to be a valuable part of an alternative testing battery for developmental neurotoxicity (DNT) evaluation (29).

This transcriptome-based study was designed to understand the species-specific nature of immature brain cells, analyze the pathways underlying the neurodevelopmental functions that can be studied using the 'Neurosphere Assay' (21) and compare pathway functions between the three species: human, mouse and rat. These species were chosen due to human relevance, presence of transgenic animals and regulatory usage, respectively. Specific marker gene expression analyses identified human-specific traits in the neurospheres compared to rodent NPC. GO term clustering (30) and subsequent protein-protein interaction enrichment analyses (31, 32) were used to identify the functional pathways driving NPC functions computationally. These were then validated by functional studies in vitro using respective model compounds across the three species. The functionally validated microarray data defines the biological application domain of the 'Neurosphere Assay' and identifies species specificities in signaling relevant for neurodevelopmental functions.

MATERIAL AND METHODS

Chemicals

Bone morphogenetic protein 2 (BMP2) was purchased from R&D Systems (#355-BM; Wiesbaden, Germany). A stock solution (2.5 µg/mL) was prepared in B27 medium. N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and Epidermal Growth factor receptor (EGFR) -inhibitor PD153035 were purchased from Sigma-Aldrich (#D5942, #SML0564; Taufkirchen, Germany). For DAPT and PD153035 stock solutions of 40 mM and 10 mM were prepared in dimethyl sulfoxide (DMSO; Carl Roth GmbH; Karlsruhe, Germany), respectively. Working solutions were prepared in N2 with 0, 0.1, 0.13 or 0.25 % DMSO.

Cell culture

Human neural progenitor cells (hNPCs, male, gestational (GW) 16-19) were purchased from Lonza Verviers SPRL (Verviers, Belgium). Rat and mouse neural progenitor cells [rNPCs and mNPC, post-natal day (PND1)] were prepared time-matched to hNPCs (33) as described previously for rat (24) and for mouse (22). Human and rodent NPCs were cultured as neurospheres in proliferation medium consisting of DMEM (Life Technologies, Darmstadt, Germany) and Hams F12 (Life Technologies; 3:1) supplement with 2% B27 (Life Technologies), 1% penicillin and streptomycin (Pan-Biotech, Aidenbach, Germany), 20 ng/ml epidermal growth factor (EGF, Life Technologies), 20 ng/ml recombinant human fibroblast growth factor (FGF, R&D systems) for hNPC and mNPC and 10 ng/ml recombinant rat FGF (R&D systems) for rNPC. The culture was maintained at 37°C with 5% CO2. The culture was fed every two to three days by replacing half the medium with fresh medium and passaged every week by mechanical chopping of the spheres with a tissues chopper (McIlwain Tissue Chopper, Vibratome). To initiate differentiation, NPCs were plated on poly-D-lysine/laminin (Sigma Aldrich) coated dishes in differentiation medium for 3 and 5 days. The differentiation medium consists of DMEM (Life Technologies) and Ham F12 (Life Technologies; 3:1) supplement with 1% N2 (Life Technologies) and 1% penicillin and streptomycin (Pan Biotech). For differentiation of mNPC 1% hormone-free fetal calf serum (FCS; Biochrome, Berlin, Germany) was added to the culture medium.

The ‘Neurosphere Assay’

To analyze pathway modulation on a functional level we quantified cell migration, as well as neuronal- and oligodendrocyte differentiation in differentiating neural progenitor cells after treatment with the pharmacological modulators BMP2, DAPT and PD153035. Therefore, spheres with a diameter of 0.3 mm were plated on poly-D-lysine/laminin (Sigma Aldrich) coated 8 chamber slides in differentiation medium for 3 and 5 days as previously described (24). On day 3 half the medium was replaced with freshly prepared medium. Migration was analyzed 24h and 72h after plating as described in Baumann

et al. (2014). After 3 or 5 days spheres were fixed in 4 % paraformaldehyde for 30 min at 37°C and stained for neurons (βIII-tubulin positive cells) or oligodendrocytes (O^4 positive cells) as described in Baumann et al. (2014). Cell viability was analyzed by Alamar Blue assay (CellTitier-Blue assay, Promega, Mannheim, Germany) in the same chamber/well that was used to analyze the DNT specific endpoint (21, 24). Automatic counting of cell numbers in the migration area using the Omnisphero software (26) was an additional indicator for cell viability. For analyses of astrocytes maturation after BMP2 treatment, hNPC were also stained for astrocytes (GFAP positive cells) after 3 days of differentiation. Astrocyte maturation was quantified as migration distance of radial glia compared to total astrocyte migration distance because an increase in cell maturation increases the amount of mature stellate like astrocytes at the expense of radial glia (Fig. 6j). Apoptotic oligodendrocytes (O^4 positive cells with condensed staining around nuclei; Suppl Fig. 5l) were counted and normalized to the number of nuclei.

Generation of RNA samples

For proliferating conditions (0d), 75 neurospheres with a diameter of 0.3 mm were collected for each replicate. For differentiation conditions (3d, 5d) neurospheres were chopped to 0.1 mm and plated at a density of 440 pieces/well in a poly-D-lysine/laminin coated 6 well plate for 3 or 5 days, respectively. RNA was isolated from a total of 54 samples, 6 replicates for each condition (0d, 3d and 5d), and species (h-, r-, and mNPC). Isolation was performed with the miRNeasy kit (Quiagen, Hilden, Germany) according to the manufactures protocol. Total RNA was used for microarray analysis and qRT-PCR. Quality of total RNA was analyzed with the 2100 bioanalyzer (Agilent Technologies). In parallel to the generation of RNA samples we performed the 'Neurosphere Assay' to control if the cells from the same passage used for RNA analysis proliferate, differentiates and migrates according to our historical controls.

qRT-PCR analysis

For validation of microarray experiments we performed qRT-PCR of a set of 12-16 genes (Suppl. Fig. 6). Therefore, RNA from the microarray samples harvested on day 0, 3, 5 was transcribed to cDNA using the QuantiTect Rev Transcription Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocol. Quantitative polymerase chain reaction (qRT-PCR) was performed using the Rotor Gene Q Cycler (Qiagen) with the QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturer's protocol. All genes with the respective primer sequence are presented in Suppl. Tab. 16. Differential expression was calculated with the ddCT method using beta actin for normalization and 0-day samples (proliferating NPC) were used as a reference set to 1.

Affymetrix Arrays

For human NPCs synthesis of cDNA and subsequent biotin labelling of cRNA was performed according to the manufacturer's protocol (3´ IVT Plus Kit; Affymetrix, Inc.). Briefly, 100 ng of total RNA were converted to cDNA, followed by in vitro transcription and biotin labelling of amplified cRNA. After fragmentation, labelled cRNA was hybridized to Affymetrix PrimeView Human Gene Expression Microarrays for 16h at 45 °C, stained by strepatavidin/phycoerythrin conjugate and scanned as described in the manufacturer's protocol.

For rodent samples synthesis of biotin labelled cDNA was performed according to the manufacturer's protocol (WT Plus Reagent Kit; Affymetrix, Inc.). Briefly, 100 ng of total RNA were converted to cDNA. After amplification by in vitro transcription and 2nd cycle synthesis, cDNA was fragmented and biotin labelled by terminal transferase. Finally, end labelled cDNA was hybridized to Affymetrix Mouse / Rat Gene 2.0 ST Gene Expression Microarrays for 16h at 45 °C, stained by strepatavidin/phycoerythrin conjugate and scanned as described in the manufacturer's protocol.

Data analysis and statistics

Data analyses on Affymetrix CEL files was conducted in R'. Probes within each probe set were summarized by Robus multichip average (RMA) after quantile normalization of probe level signal intensities across all samples to reduce inter-array variability. The algorithms where provided by the R packages affy (34) for human samples and oligo (35) for mouse and rat samples. Differential gene expression (DEX) was statistically determined by one-way ANOVA followed by Tukey's range test (36). The significance threshold was set to $p \leq 0.01$ (FDR corrected; gene expression analyses). Probes that matched this threshold for no probe set were dismissed. The Affymetrix IDs were translated into gene symbols using a translation table containing all genes matching our Affymetrix IDs. It was built using the biomaRt R package (37). In a first step, a table with the gene symbols and expression data from their corresponding Affymetrix IDs was created. For genes, which matched to more than one Affymetrix ID, the mean of the differential expression per time frame was used. The p-values were combined applying Fisher's method (38).

The Principal Component Analysis (PCA) is based on the significance threshold filtered expression data. Therefore, we worked with 18 data points (6 replicates per time point) per gene per species. Division by its respective median (median for one gene over 18 samples) normalized the expression data of each Affymetrix Id. We chose all genes with identical gene symbol in all species and used the prcomp method from the R stats package (36) to perform the PCA. An adjacency matrix was calculated from the expression profiles of the genes over time using the scrime package (39). Hierarchical clustering was performed by hclust using unweighted pair group agglomeration method with arithmetic mean and cutree (36) resulting in 10 distinct clusters. These 10 clusters were manually summarized into the modules M1 – M4 according to their expression profiles over time. To allow a direct species comparison

of gene expression independent of differential gene expression, we defined genes as likely to be expressed (present) or likely to be not expressed (absent) based on criteria adapted from Kang et al. (2011). A gene is called present if the median log2 intensity value in 0-day samples is ≥ 6. Genes that do not meet this criterion are defined as absent. The expression profile of single genes in heatmap (Fig. 2g) was prepared with the Multiple Experiment Viewer (MeV 4.9.0; http://mev.tm4.org/).

All data analyses for functional endpoints were performed using GraphPad Prism 6.00 (GraphPad Software, La Jolla Carlifornia USA, www.graphpad.com). Statistical significance analyses were performed on data normalized to solvent control (except for cell migration, here we used raw data on migration distance) from at least 3 independent experiments by one-way ANOVA followed by Dunnett's multiple comparisons test. The significance cutoff was set to $p \leq 0.05$.

Biological process analysis/gene ontological annotation analysis

We used the Cytoscape (version 3.4.0) plug in ClueGO/CluePedia (version 2.2.5; 31, 32) for overrepresentation analyses of biological process GO clustering and network visualization. The statistical test for overrepresentation analysis was based on a two-sided hypergeometric option with a Bonferroni correction. We chose a p-value cutoff of 0.01 and a kappa score of 0.5 for GO clustering with at least two GO terms within one cluster (see parameters for analysis in Suppl. Tab. 17)

To generate gene interaction networks we used information from the STRING Protein-Protein Interaction database (33; http://string-db.org) within CluePedia, extracted the GO clusters cell migration, gliogenesis and neurogenesis and enriched all genes associated with the selected GO clusters with information on gene-gene or protein-protein interactions (binding, activation, expression and inhibition) with a confidence score of ≥ 0.75. Highly connected genes were identified as those genes showing at least three times (migration, neurogenesis) or two times (gliogenesis) the number of connection compared to the mean number of connection per gene within the respective cluster.

RESULTS

Analyses of changes in NPC mRNA expression profiles across time and species

To determine the changes in molecular equipment of NPC growing as neurospheres during the change from proliferation to differentiation (21), we first analyzed mRNA expression profiles of proliferating (0), as well as 3 and 5 days differentiating human, mouse and rat primary neural progenitor cells (hNPC, mNPC, rNPC; Fig. 1) by using microarrays (human PrimeView Array, mouse/rat Gene 2.0 ST Arrays, Affymetrix). Genes were called differentially expressed (DEX) if they were significantly ($p \leq 0.01$) up- or downregulated by at least 2-fold between any of the three time points (0 vs. 3, 0 vs. 5, or 3 vs. 5 days). In hNPC, mNPC and rNPC a total of 1684, 1979 and 2324 genes, respectively, are DEX across all time points. The number of genes regulated in the first 3 days of differentiation (0 vs. 3) is in the same order of magnitude between the three species (1121 in hNPC, 1196, in mNPC, 1033 rNPC). With continuing NPC differentiation and maturation, more genes are DEX at day 5 (0 vs. 5) with 1531 in hNPC, 1566 in mNPC and 2159 in rNPC. Of those, 971, 849 and 927 genes (>70 %), respectively, overlap with DEX genes at day 0 vs. 3 (Fig. 2 a-c). In rodent compared to human NPCs there is a higher number of DEX genes between 3 and 5 days (43 hNPC, 362 mNPC, 732 rNPC; Fig. 2d) indicating a stronger change in cultures over 5 days in rodent compared to human NPC.

We next determined the number of commonly regulated genes between the total of 1684, 1979 and 2324 DEX genes in hNPC, mNPC and rNPC, respectively, across all time points (0vs3, 0vs5 and 3vs5 days combined). Of these, only 186 (11%) genes share the same gene symbol in all three species (Fig. 2e). This small number of overlapping DEX genes during NPC differentiation can only in part be explained by the dissimilarities of array chips, because of the 1684 DEX genes identified in hNPC 83 % (1398) are present on both rodent array chips, yet are not DEX in the rodent cells (data not shown). To compare the magnitude of gene expression changes across the three species in addition to this qualitative difference of gene regulation during NPC differentiation, quantitative differences in magnitude of expression of all regulated genes ($p \leq 0.01$; 5571) with the same gene symbols across differentiation time and between the three species were evaluated by PCA (Fig. 2f). PC1 (33 % explained variation) reveals that for all species 0d samples cluster further from 3d than 3d from 5d samples, pointing towards largest gene expression changes when the cell program switches from NPC proliferation to neural differentiation compared to smaller changes during further maturation of differentiated cells from day 3 to day 5 in culture. For the species comparison, the PCA demonstrates that hNPC have a distinct differentiation dynamic compared to the rodent NPC as visible in the course of expressional changes over time (Fig. 2f, dotted lines). Differences in the differentiation dynamics are also obvious in the expressional changes of genes specific for certain cell types during brain development (Fig. 2g). Obvious examples are the neuronal marker *NRXN1* which is upregulated in hNPC (4.2 and 5.2 fold at 3 and 5 days, respectively), downregulated in mNPC (0.7 and 0.6 fold at 3 and 5 days, respectively) and not present on the microarray chip of rNPC, or the ventricular radial glia marker *ANXA1* which is downregulated in hNPC (0.3 and 0.4 fold at 3 and 5 days, respectively) and upregulated in rodent NPC (13.5 and 3.1 fold in mNPC, 2.6 and 2.6 fold in rNPC at 3 and 5 days,

respectively). To allow a direct species comparison of gene expression independent of differential gene expression, we defined genes as likely to be expressed (present) or likely to be not expressed (absent) based on criteria adapted from Kang et al. (2011; only present if median log2 intensity value in 0-day samples is ≥ 6). With this threshold we identified several genes that are only expressed in human cells *(HS6ST2*, *LRRC3B*, *CALB2* or *ARHGAP11B*; Fig. 2g and Suppl. Tab. 19). Taken together, the qualitative and quantitative expression data show that primary NPC from human and rodent origins obtain species-specific expression changes when differentiating to neural effector cells with only few DEX genes shared between human and rodent NPC and with some human specific marker genes for NPC development.

For further analyses of gene function behind these expression changes, we generated expression clusters using a hierarchical cluster analysis (HCA) for all DEX genes (Suppl. Fig. 1). We summarized these clusters according to their regulation pattern over time into 4 modules: module 1 (M1) and module 2 (M2) contain genes with the main expression changes, up and down, respectively, within the first 3 days of differentiation and no further regulation from day 3 to day 5 (Fig. 2h). These clusters contain similar numbers of genes across all species (M1: 611, 575, 504; M2: 667, 499, 404, for h-, m- and rNPC, respectively). We hypothesized that these are genes involved in the NPC program change from proliferation to neuronal and glial differentiation and the onset of cellular migration. Summarized in module 3 (M3) and module 4 (M4) are those genes, which are up- and down-regulated, respectively, mainly between day 3 and 5 in vitro. For these clusters, the numbers of genes differ between human and rodent NPC (M3: 191, 547, 887; M4: 215, 358, 428, for h-, m-, rNPC, respectively). These genes are thought to be mainly involved in processes of effector cell maturation and thus the fewer regulated genes in hNPC compared to their rodent counterparts might explain the lack in hNPC-derived neuronal maturation which seems to be enhanced in rNPC-derived neurons (41, 42). Mean expression change of the modules M1 and M3 (Fig. 2h) corroborate the observation that mNPCs and especially rNPCs show stronger expression changes between 0 and 5 days than hNPC (Fig. 2d).

Overrepresentation analyses (ORA) of Gene ontology (GO) biological processes (BP)

To computationally determine biological functions of genes in the temporal expression clusters, we performed ORA for the GO-terms BP using the Cytoscape plugin ClueGO (30). We first analyzed the biological functions of genes in clusters M1 and M2, where we expected the largest transcriptional changes due to the switch in cell program from proliferation to differentiation. From the 1278 DEX genes in hNPC, 1074 in mNPC and 908 in rNPC, respective 1070 (83.7 %), 904 (84.2 %) and 787 (86.7 %) were annotated to 155 (hNPC), 427 (mNPC) and 228 (rNPC) GO terms (Tab. 1; Suppl. Tab. 1-3). Although developing NPC from humans and rodents have only little overlap in DEX genes (11% of hNPC), 90 of the 155 (58%) GO terms enriched in hNPC were shared with rodent NPC. These GO terms are major BP involved in general organ development like e.g. animal organ development (GO:0048513), anatomical structure morphogenesis (GO:0009653) or multicellular organism

development (GO:0007275), organ specific developmental processes like head development (GO:0060322) and central nervous system development (GO:0007417), or GO terms specific to some major processes of brain development that are assessed by the functional 'Neurosphere Assay'. Here, the processes NPC proliferation, migration, neuronal and glial differentiation as well as cell death are represented by regulation of GO terms such as cell proliferation (GO:0042127), cell motility (GO:0048870), neurogenesis (GO:0022008), axonogenesis (GO:0007409), gliogenesis (GO:0042063) or regulation of programmed cell death (GO:0043067; comprehensively shown in Suppl. Tab. 4). To visualize how GO term enrichment compares in-between the three species, we summarized all GO terms into the following eight superordinate processes by expert judgment: (i) brain/organism developmental processes, (ii) neuronal/glial differentiation, (iii) migration/adhesion, (iv) proliferation, (v) cell death, (vi) cell signaling, (vi) other processes and (vii) processes related to other organs (Suppl. Tab. 5-7). Fig. 3a-c demonstrates that from the temporal expression cluster M1 and M2, GO terms associated to 'brain/organism developmental processes', 'neuronal/glial differentiation' are the most represented and together with 'migration/adhesion', 'proliferation and cell death' make up 60% of all GO terms in hNPC, while these GO terms represent only 34% and 41% in mNPC and rNPC, respectively. GO terms associated with processes related to cell signaling correspond to 14% of all GO terms in hNPC, while this is the largest group in rodent NPC with 26% (mNPC) and 32% (rNPC) of all GO terms. In total numbers, only 31 GO terms are associated to this group in hNPC, 92 in rNPC and 164 in mNPC. Some of the processes present in rodent but not in hNPC are e.g. cell-cell signaling (GO:0007267), response to steroid hormone (GO:0048545), negative regulation of cell communication (GO:0010648) or protein phosphorylation (GO:0006468; Suppl. Tab. 4). The remaining 26 % (hNPC), 40 % (mNPC) and 26 % (rNPC) GO terms are associated to other processes (e.g single-organism biosynthetic process, GO: GO:0044711 or ion transport, GO:0006811) and processes in other organs. One example for the latter group is the process heart development (GO: 0007507). Although this is prima vista not related to brain development, it shares 41 of the 62 genes (66%) with the process nervous system development (GO:0007399) in hNPC (Suppl. Tab. 1). This example demonstrates that GO terms of processes related to other organs might be overrepresented due to shared genes with nervous system development.

Next, we computationally analyzed the biological functions of genes in clusters M3 and M4. From the 404 DEX genes in hNPC, 906 in mNPC and 1417 in rNPC, respective 345 (85.4 %), 761 (84.0 %) and 1058 (74.7 %) were annotated to 79 (hNPC), 110 (mNPC) and 61 (rNPC) GO terms with 42 % shared GO terms between hNPC and mNPC and none between hNPC and rNPC (Tab. 1; Suppl. Tab. 8, 10-11). There are two- (mNPC) to three-fold (rNPC) more DEX genes present in rodent than in hNPC pointing to differences in culture maturation and/or species differences. This is supported by the magnitude fold change in DEX genes in rodents, mainly rat NPC, which display higher mean gene expression regulations compared to hNPC (mean regulation of M3: 3.5 fc in hNPC, 4.7 fc in mNPC, 7.1 fc in rNPC; Fig. 2h). Fig. 3d-f demonstrates that almost all GO terms in hNPC (91%) and more than half in mNPC (69%) are associated to the process of cell proliferation such as cell cycle (GO:0007049), chromosome organization (GO:0051276) or mitotic nuclear division (GO:0007067). Within these GO

terms 60-100% of all associated genes are down-regulated (Suppl. Tab. 8+10). This observation suggests that cell proliferation is still an important process during the onset of differentiation but strongly decreases between day 3 and 5 in hNPC and mNPC. Other GO terms in mNPC (18%) and more than half in rNPC (57%) are associated with the progression of cell differentiation and maturation. Here some GO clusters are cilium organization (GO:0044782) in mNPC, determination of left/right symmetry (GO:0007368) or microtubule-based process (GO:0007017) in both species and cilium morphogenesis (GO:0060271) or centriole assembly (GO:0098534) in rNPC (comprehensively shown in Suppl. Tab. 10-15). Because the majority of DEX genes in M3 and M4 of hNPC were dominated by downregulated genes, we performed an ORA for upregulated genes (M3) separately. ORA of hNPC M3 shows that 16 processes involved in maturation such as axonogenesis (GO:0007409) and dendrite development (GO:0016358) are overrepresented (Suppl. Tab. 9) in our data set.

Overall, the ORA of GO BP especially from M1 and M2 reflects (i) the multicellularity of the 3D neurospheres and (ii) specific neurodevelopmental processes during NPC development. These results demonstrate that molecular signatures of gene expression changes line the functional processes that are studied in the frame of the 'Neurosphere Assay' in vitro over NPC differentiation. It also indicates that many of these major processes of brain development and cell organization are conserved across species, yet with distinct molecular signatures. However, there are considerable species differences in the abundance of processes related to cell signaling, proliferation or the progression of cell maturation between the in vitro systems of the three species.

Identification of key regulators for human neurodevelopmental processes

To identify the underlying genes and pathways of the neurodevelopmental processes studied with the 'Neurosphere Assay', we performed a clustering based on shared genes between GO terms overrepresented in modules M1 and M2 of hNPC (Fig. 4) and rodent NPC (Suppl. Fig. 2-3). From the 19 GO clusters in hNPC we extracted those representing the major neurodevelopmental processes, i.e. cell migration, neurogenesis and gliogenesis. As the cluster neurogenesis was included in a cluster with general GO terms on (neuro)development, we manually removed all GO terms, e.g. GO terms like brain development (GO:0007420), regulation of cell morphogenesis (GO:0022604) or cell development (GO:0048468) that were not directly related to neurogenesis (Suppl. Fig. 4).

All genes within each cluster were enriched for their interaction (binding, activation, expression and inhibition) based on information from STRING protein-protein Interaction database (32); http://string-db.org; Fig. 5). We identified highly connected genes as key regulators (KR) or hubs for individual human neurodevelopmental processes. Genes that show at least three times (migration, neurogenesis) or two times (gliogenesis) the number of connections compared to the mean number of connections per gene within a cluster were defined to be highly connected. Thereby, we identified *BMP2*, *EGFR*, *MYC* and *NOTCH1* as KR across all three processes, *VEGFA*, *JUN* and *FGFR1* as KR for migration and neuronal differentiation and *EPHA2*, *LYN*, *PDGFRB*, *SRC* only for migration (Fig. 5; Tab. 2).

Species comparison revealed that for mNPC and rNPC only few genes (between 7 % to 14 %) of the processes migration, neurogenesis and gliogenesis with interaction data are shared with hNPC. From the KR only *VEGFA* is present in the two species, a finding that could be explained by the general low number of DEX genes shared between the species. The KR for rodent NPCs are *Agt*, *Cav*, *Flt*, *Fyn*, *Itga*, *Pdgfb*, *Ptgs2* for migration in mNPC, *Cx3cr1*, *Flt1, Itgb4* and *Ptk2* for migration in rNPC, *Bmp4* for neurogenesis in mNPC and *Ptk2* for neurogenesis in rNPC. For the process gliogenesis there was no KR for mNPC and the processes did not appear as a separate cluster in rNPC (Suppl. Tab. 20-24; Tab. 2).

For functional validation of some of the KR identified in hNPC transcriptomes, we analyzed the effects of their pharmacological modulation on migration, neuronal and oligodendrocyte differentiation in all three species. As KR we chose *BMP2*, *EGFR* and *NOTCH*, as they were predicted to modulate all three processes, i.e. hNPC migration, neuronal and glia differentiation (Fig. 5; Tab. 2). We modulated BMP2 signaling by addition of 0.01 - 100 ng/mL BMP2 during differentiation of human, mouse and rat neurospheres (Fig. 6). BMP2 does not affect migration of human and mNPCs, but induces migration of rNPCs after 72h (149.9 ± 7.2 % of control at 10 ng/mL; Fig. 6a). Furthermore, BMP2 does not affect differentiation into βIII-tubulin positive neurons in hNPC, while it induces and reduces neuronal differentiation in mouse and rNPC, respectively (202.1 ± 46.9 % of control at 50 ng/mL in mNPC and 62.2 ± 8.6 % of control at 5 ng/ml in rNPC; Fig. 6b). BMP2 reduces the differentiation to O4 positive cells in all species (48.7 ± 1.8 % and 50.8 ± 3.5 % of control at 1 ng/ml in human and rNPC, respectively and 54.2 ± 4.4 % of control at 5 ng/mL in mNPC; Fig. 6c). In addition, BMP2 induces maturation of GFAP positive cells, as indicated by a concentration-dependent increase of mature astrocytes at the expense of radial glia cell in the migration are. This effect was quantified by measuring the reduction of radial glia cell migration in comparison to the total migration of GFAP positive cells (from 82 ± 6.2 % of total migration at control to 62.4 ± 0.9 % of total migration at 1 ng/mL; Fig. 6j+k). Because migrating rodent NPC do not display typical radial glia morphology in our neurosphere culture (21), BMP2 effects on astrocyte maturation was only studied in hNPC. All effects described for BMP2 were at concentrations that did not affect overall cell viability measured by mitochondrial activity (Suppl. Fig. 5a+c).

NOTCH signaling was modulated by the addition of 0.08 – 5 µM of the NOTCH inhibitor DAPT. NOTCH inhibition does not affect migration of human and rNPCs, but inhibits migration of mNPCs after 72h (76 ± 5.7 % of control at 5 µM; Fig. 6d). Differentiation into βIII-tubulin positive cells after 72 h was not affect by NOTCH inhibition (data not shown). NOTCH inhibition induces neuronal differentiation only of human, not rodent NPC after 120h (175.2 ± 10.5 % of control at 5 µM, Fig. 6e), while after 72h DAPT exerts no effects on neuronal differentiation of either species. Differentiation of hNPC into O4 positive oligodendrocytes is inhibited by NOTCH inhibition (28.5 ± 4.3 % of control at 0.16 µM DAPT),,not affected in rNPC and induced to 255.4 ± 42.6 % of control in mNPC (5 µM DAPT, Fig. 6f). However, these additionally formed mouse O4 positive cells undergo apoptosis as identified in Suppl. Fig. 5k+l, leaving non-apoptotic oligodendrocytes at the same number than the control cultures. In differentiated

human or rat NPCs, no apoptotic O4 positive cells are present. The highest concentrations of DAPT (1.25-5 µM) reduces mitochondrial activity in all species (Suppl. Fig. 5e). Except for rNPC at 5 µM DAPT, this effect is not accompanied by a reduction in number of nuclei (Suppl. Fig. 5f), which lead us to the assumption that DAPT reduces mitochondrial activity rather than affect cell viability.

EGF signaling was modulated by addition of 0.5 – 10 µM of the EGFR inhibitor PD1530353. Inhibition of EGFR reduces migration of hNPCs after 24h and 72h (75.0 ± 5.9 and 77.5 ± 3.2 % of control at 10 µM) and does not affect migration in m- and rNPC (Fig. 6g). Differentiation to βIII-tubulin positive cells is induced in h- and rNPC (161.9 ± 7.2 % and 180.2 ± 7.0 % of control at 10 µM) and reduced in mNPC (41.4 ± 10.8 % of control at 10 µM; Fig. 6h). PD1530353 reduces the formation of O4 positive cells in hNPCs (66.3 ± 1.4 % of controls at 10 µM), while it does not affect the differentiation to O4 positive cells in m- and rNPCs (Fig. 6i). Effects on migration in hNPC and neuronal differentiation in all species is accompanied by a reduction in number of nuclei (59 ± 9.0 %, 72 ± 5.3 % and 78 ± 6.0 % of control in h-, m- and rNPC, respectively). In rNPC the 10 µM PD1530353 additionally affects viability after 72h (75.6 ± 9.0 % of control).

DISCUSSION

Within the paradigm shift of Toxicology in the 21st century the need for in vitro assays is voiced that reliably predict human toxicity (43, 44). One of the required toxicity endpoints with regard to chemical safety is reproductive and developmental toxicity currently assessed with the extended one-generation study (45). While giving valuable information for many different endpoints, this bioassay as well as the OECD TG426 specifically designed for DNT evaluation are not sufficient for identifying neurodevelopmental toxins (46, 47). Therefore, alternative assays predicting (neuro)developmental toxicity also for regulatory applications are urgently needed (46, 48). For any application, a thorough understanding of the alternative test system especially on the molecular level increases confidence in the method and might allow future usage in a broader context like the integrated approaches for testing and assessment (IATA; 49–51). This is why in this work the molecular equipment (transcriptome) of developing human NPCs over time (proliferating versus 3 and 5 days differentiated cells) was assessed; they were compared to time-matched (www.translatingtime.org; 52) mouse and rat NPC transcriptomes to identify species-specificities and pathways recognized as major regulating hubs were functionally validated for their impact on NPC migration, neuronal and oligodendrocyte differentiation across the three species (Fig. 1).

Recently, it was recognized that the uniqueness of higher cognitive and emotional functions in humans is largely determined by human-specific neurodevelopmental gene expression (52). On this basis we studied gene expression in neurodevelopmental in vitro systems of three different species. During NPC differentiation in vitro, 1684 human, 1979 mouse and 2324 rat genes were DEX (>2-fold up- or downregulated; $p<0.01$; Fig. 2a-c). To the best of our knowledge, this is the first study comparing transcriptomes of undifferentiated to differentiated human NPC in vitro. In a study that analyzed transcriptional changes of mouse differentiating NPC after treatment with brain derived neurotrophic factor (BDNF) or neurotrophin 4 (NT4) a total of 722 and 624 genes were differentially expressed at any of the three time points (24h, 48h or 96h; 54). The lower number of genes can be explained by the use of different microarray chips with less transcripts (13627) compared to the chips used in this study (>20000). Other similar studies were performed analyzing transcriptome changes of human embryonic stem cells (ESC) differentiating to cardiomyocytes (CM; 55). Between undifferentiated hESC and 48h or 12 days differentiation towards the CM lineage, respective 3579 (54) and 3035 (55) transcripts were found to be differentially expressed. These are around twice as many gene changes as we found in undifferentiated compared to 3 or 5 days differentiated hNPCs (Fig. 2a-c). This might be due to the fact that NPC are already on their way to neural tissue, while hESC are still omnipotent and thus differ more strongly from the terminally differentiated cell.

Principle Component Analyses using all genes that are significantly changed on the arrays ($p<0.05$) and present in all three species (5570) revealed that the majority (56.7%) of all variance between the condition and species can be described by the first two principal components (Fig. 2f). The PCA plot clearly shows the differentiation dynamics of the NPC in vitro system and indicates that the variance within experimental groups is relatively small compared to the variance between time points and species

(Fig. 2f). A similar PCA pattern was observed during hESC differentiation to CM. Here, 24 and 48h (54) as well as 6 and 20 days of differentiation (56) were clearly distinguishable from the stem cells of origin also pointing to highly dynamic in vitro systems with regards to differentiation capacities. Besides differences in gene expression over time within one species, we also observed well-defined distinctions in gene expression differentiation dynamics between the three species human, mouse and rat (Fig. 2f). Comparison of human and mouse ESC differentiated to embryonic bodies revealed that out of a total of 903 GO terms (biological processes), gene expression was only correlated between species in 395 and not correlated in 508 GO terms indicating substantial differences in transcriptional regulation of ESC-based embryonic body formation (57). This work supports the observed species differences in gene expression profiles during NPC development presented here.

One striking aspect of this study is that only 186 DEX genes (>2-fold, $p<0.01$; Fig. 2C) over developmental time were common in all three species (Fig. 2e), which is only approx. 10% of all DEX genes. This is a very small number considering that these in vitro systems are functionally very similar, i.e. migrating and differentiating primary NPC (21). These 186 genes cluster in 61 GO Terms (Suppl. Tab. 18) that contain some specific neurodevelopmental processes, but also a large variety of non-neurodevelopmental-related biological functions. A large variety of genes in these non-specific GO Terms, however, are generally involved in tissue and organ development pointing to the fundamental biological significance of these molecules in developing cell functions. Such include MYC (present in 18 of these GO Terms), which is engaged in cellular signaling including cell proliferation (58), PDGFRB (present in 16 of these GO Terms), which guides a variety of developmentally-relevant signaling pathways (59) and FGFR2 (present in 31 of these GO Terms), that obtains multiple functions during organ development (60) including the brain. A comprehensive list of the 186 genes and their grouping into GO Terms for biological processes can be found in Suppl. Tab. 18.

The DEX genes that differ between species, however, enrich in GO Terms for biological processes that could be assembled into analogous superordinate processes by expert judgment (Fig. 3). These GO terms in general reflect the neurodevelopmental processes that we study with the ‘Neurosphere Assay’ on a functional level in vitro (21, 24, 61) and were previously identified in the transcriptomes from developing brains in vivo (62). Despite the fairly similar grouping of enriched GO terms in superordinate processes the question remains why the majority of DEX genes diverge between the species. There are three major reasons that might explain why DEX genes of human, mouse and rat NPC differ considerably over time despite qualitative similarities in GO Term clusters. The first explanation might lie in species differences in developmental timing, the second one in differences in molecular equipment and/or regulation of equivalent cells and the third one in different cell type compositions of brains from different species. It is highly likely that the results presented in this study are motivated by a mixture of all three arguments and we will now provide examples for each of them.

Timing of brain development is known to follow different temporal traits in diverse species (33, 63, 64). This makes species-overarching comparison of gene expression during the neurodevelopmental period difficult. Especially, because during human brain development, changes in the transcriptome are largest

during the fetal period, i.e. 9 out of 10 genes are DEX between different developmental time points in vivo (samples taken every 2-5 post conceptual weeks (PCW) starting from PCW 4) and/or brain regions demonstrating high gene expression dynamicity during pre- and early postnatal development (40). Rodent brain development underlies similar gene expression changes than ontogenesis of the human organ, yet at a more rapid pace (65–67). In addition, neurodevelopmental processes that are guided by gene expression changes take place at species-specific speeds as exemplified by oligodendrogenesis, which takes 5 days in rodents, but 11-12 weeks in humans (68). Such species-dependent speed differences in e.g. the developmental process oligodendrogenesis leads to differences in oligodendrocyte-related gene expression between species also during NPC development in vitro when analyses are performed at the same time points (21, 22). Taken all these timing aspects together, it is not surprising that gene expression profiles from identical cell types like here the primary NPC from different species gained from brains at corresponding time points (52; Fig. 1) display distinct transcriptomes in their undifferentiated state as well as during in vitro differentiation over time (Fig.2f).

The second aspect underlying the observed species differences might be differences in molecular equipment and/or regulation of genes within cell types. A recent study analyzed cell type markers and functional classes of genes as cortical markers in specific cortical areas (69). These data revealed substantial cross-species differences between humans and mice with a dramatic shift of cortical layer-specific gene expression patterns between species indicating cross-species conservation and divergence of gene expression at anatomical and cell type levels (69). One example from this study is *CALB2*, which is preferentially expressed in the ventricular zone/sub ventricular zone-originated interneurons and was found enriched in human compared to mouse brains (69). Direct comparison of expression between species cannot be solely assessed by DEX genes, which describes expression changes between two time points and is a known issue when dealing with cross-species transcriptome comparisons (70). Therefore, we defined a gene as likely to be expressed (present) or likely to be not expressed (absent) based on criteria adapted from Kang et al. (2011; median log2 intensity value in 0-day samples is ≥ 6 = present, otherwise absent). With these combined methods we also found that *CALB2* expression and regulation over time is human NPC-specific in our data set (Fig. 2g and Suppl. Tab. 19). In addition, *PDGFD* acting through *PDGFRB* regulates cell-cycle progression and progenitor cell expansion in human, but not mouse, cortex (71). This instance is also reflected in our species-overarching in vitro methods with PDGFD reaching the ‘present’ threshold in hNPC, but not in mouse correlates (Fig. 2g and Suppl. Tab. 19). Surprisingly we observed rNPC to be similar to hNPC with regard to this marker, but to the best of our knowledge there is no literature available to compare our findings with. Concerning genes expressed during neuro-, astroglio- and oligodendrogenesis, we also found similarities and differences in gene abundance across species. The human astrocyte-specific gene LRRC3B, for example, is exclusively present and regulated in human compared to rodent cultures, while the astrocyte maturation markers *SPARCL1*, *GFAP* and *S100b* are present in NPC from all three species (71; Fig. 2g and Suppl. Tab. 19). These examples support our second notion that some of the transcriptome differences that we observe between human, mouse and rat neurospheres are due to qualitative species-specificities.

The third explanation for the large species differences in DEX genes identified in our study might root in a different cell type composition of human compared to rodent brains. One striking makroscopic distinction of human from rodent brains is the brain surface. While human brains are gyrencephalic, rodent brains contain no gyri and sulci and are thus lissencephalic. Gyrencephaly is a result of cellular expansion of a special type of progenitor cell, the basal progenitor (BP) or outer radial glia (oRG) cell in the human enlarged outer subventricular zone (OSVZ) of the developing cortex (73–77). This cell type is thought to be specific for human brains and can thus be identified by human-specific molecular markers. One of these markers is the recently identified oRG cell marker *FAM107A* (synonym for DRR1; 70, 74, 75), which we found to be expressed in human and absent in mouse NPC, supporting the previously published data. However, we also found this marker to be present and highly regulated in rat NPC. (Fig. 2g and Suppl. Tab. 19). One reason for the absence of expression and regulation of *FAM107A* in developing mouse and the expression and regulation in developing rat NPC could be the neuronal expression in mouse E 10.5–16.5 and rat E 18.5 brains (78). While neuronal *fam107a* expression is restricted to an early embryonic timeframe and the NPC used in this study are generated from PND1 brains, mNPC do not show any expression of this gene. In rat, neuronal *fam107a* expression starts on E 18.5 in vivo suggesting that our observed gene presence in developing rat NPC is due to neuronal and not radial glia cell expression, which needs further experimental confirmation. These data indicate, that a combination of developmental timing and cell type-specific expression might drive gene abundance in such mixed cell type systems.

In contrast to the oRG, ventricular zone RG (vRG) are supposedly more similar across species (79). This is reflected on the molecular level within this study as the vRG-specific *CRYAB* and *ANXA1* gene products (76) are present in NPC of all three species (Fig. 2g and Suppl. Tab. 19). Intermediate progenitor cells (IPC), however, are likely absent in the culture due to absence of the IPC marker *EOMES* (TBR2), despite *HES6* presence in mouse and human cells (76); Fig. 2g). *HES6*, however, seems to have additional, *NFκB*-dependent functions in migrating neurons during cortical development (80) suggesting that *HES6* expression in those neurospheres is likely due to expression in migrating neurons and thus independent of IPC. In addition, all NPC express *HES1*, *VIM*, *SLC1A3* (*GLAST*) and *NES* (73, 75, 81) supporting the concept that also a large variety of genes are expressed in a similar fashion across species (69). However, the here discussed data also provides evidence that some of the transcriptional species differences observed in this study are based on different cell types, i.e. oRG cells in humans (75, 76), which seem to be well-reflected in the neurospheres in vitro systems.

Besides the GO term grouping by expert judgment, we analyzed transcriptomes from each species across differentiation time using a GO term clustering in the Cytoscape plugin ClueGO (31; Fig. 4). The plots – and here especially the human data - illustrate that the transcriptomes mirror the *in vitro* functions of the 3D models, i.e. NPC proliferation, migration, differentiation into neurons and glia cells as well as apoptosis over 5 days of differentiation (Fig. 4, Suppl. Fig. 2+3). Comparison of the plots of the three species reveals differences in their appearance (Fig. 4, Suppl. Fig. 2+3). This is probably due to two reasons. For one, attributable to the approximate 90% differences of DEX genes between species (Fig.

2C), distinct GO terms were annotated from the data sets. Secondly, existing GO term annotations for humans, mice and rats differ in quality and quantity due to the different background information available for each species (82). As an example, the biological process 'cell signaling' is defined by four times more GO terms in mouse than in human NPC-derived transcriptomes (Fig. 2). This is probably owing to the large amount of existing signaling data generated in mice compared to humans including data from the many transgenic mice that dominate biomedical research (83).

For a pathway-to-function validation of the 'Neurosphere Assay' we next identified molecular key regulators possibly guiding cellular functions based on *in silico* annotations. The analyses revealed 11, 7 and 4 hub genes for the human neurodevelopmental processes NPC migration, neuro- and gliogenesis, respectively (Fig. 5; Tab. 2). We picked the three key regulators *BMP2*, *EGFR* and *NOTCH* that were computationally predicted to be involved in all three of these human processes (Tab. 2) for a functional *in vitro* validation and species comparison. The data of the consequences of *BMP2*, *NOTCH* and *EGFR* pathway modulation are shown in Fig. 6.

The transforming growth factor-β (TGF-β) superfamily member BMP2 signals through BMP receptors (BMPR) Type 1 (BMPR1a/Alk3) and (BMPR1b/Alk6) or Type 2 (BMPR2; 83). According to the human and rodent transcriptomes, with the exception of *bmpr1b* in rat NPC, these *BMPR* are present in proliferating and differentiating cultures (Suppl. Tab. 19). In contrast to the *in silico* prediction (Fig. 5a-c), BMP2 only acts on glia-related endpoints in human NPC by reducing oligodendrocyte differentiation (Fig. 6c) and accelerating astroglial maturation (Fig. 6j+k). In addition to reduction of oligodendrocytes, BMP2 induces migration and reduces neuronal differentiation of rat and induces neurogenesis in mouse NPC (Fig. 6a-c), yet BMP2 was not predicted as a modulator of any of these endpoints in the rodent cultures (Suppl. Tab. 21-24). Discrepancies of computational prediction based on GO terms and actual experimental data when using relevant cell systems might be built on the data behind GO annotations. Such data underlying GO terms are retrieved from different tissues, cell models, *in vitro* and *in vivo* analyses, species, and, in case of development, distinct developmental timing (82). For example, in our data set gene expression of *ID1* (*inhibitor of DNA binding/differentiation*) strongly increases during differentiation in hNPC (9 fold at 0vs3 days). Because BMP2 is a known transcriptional inducer of *ID1* (85) and both of them are annotated to the GO term cell migration (Suppl. Tab. 1), this annotation contributed to the computational identification of BMP2 as a hub gene for cell migration. Searching for the data behind this annotation, the information on *ID1* and migration is a 'traceable author statement' based on observations in endothelial cells (86) and is not related to migration in the developing brain. The information that *BMP2* is related to migration is inferred from sequence or structural similarity of homolog or ortholog genes, which is a hypothesis that has no experimental proof. Consequently, data sets behind GO terms need improvement with regards to specificity of cells, tissues, species and, if applicable, developmental timing. Nevertheless, we demonstrated that BMP2 effects on rodent NPC are similar than previously published and showed the new information that BMP2 induces rat NPC migration. BMP2 induces E13.5 (mouse) and E16 (rat) NPC differentiation into βIIItubulin+ neurons (87–89) or reduces this process in mouse E17 NPC (90) and mouse embryonic stem cells (91), yet in

all systems BMP2 impedes oligodendrogenesis and in all but the mouse E13.5 BMP2 promotes astroglial fate (summarized in Suppl. Tab. 25). Thus, our data reproduces the fact that BMP2 reduces oligodendrogenesis in rodents and adds so far unknown information that similar to BMP7 (21), BMP2 reduces oligodendrocyte differentiation and induces astrocyte maturation of human NPC. The differences in the published as well as in our own data concerning BMP2 effects on rodent neuronal differentiation (Suppl. Tab. 25, Fig. 6b) are difficult to explain, but might be due to preparation and/or cultivation of cells in presence or absence of FCS (92), brain region, origin of cells, plating in spheres or as single cells, co-treatment of BMP2 with or without FGF2, developmental age or species.

For mammalian NOTCH signaling, a phylogenetic very well conserved signaling pathway, activation of the transmembrane NOTCH receptors 1-4 by an extracellular ligand is crucial (93). According to the transcriptome data from this study, human NPC express *NOTCH1-3*, mouse NPC all four *notch* isoforms and rat NPC also *notch1-3* and all three species display expression of different isoforms of the *NOTCH ligands DELTA-LIKE* (*DLL*) and/or *JAGGED* (*JAG*; Suppl. Tab. 19). In contrast to the *in silico* prediction that *NOTCH* is guiding NPC migration, neuronal and glia differentiation (Fig. 5a-c), inhibition of NOTCH signaling by the NOTCH receptor inhibitor DAPT only induces neuronal and inhibits oligodendrocyte differentiation of human NPC, while it does not affect their migration (Fig. 6d-f). Although not computationally predicted as a modulator for rodent neurodevelopmental processes in our data set, DAPT reduces mouse NPC migration and induces mouse NPC oligodendrocyte formation (Fig. 6d+f), while rat NPC were not affected by NOTCH inhibition *in vitro*. Activation of NOTCH signaling in neural stem cells (NSCs) has been implicated in inhibition of neuronal differentiation and terminal differentiation into the astrocyte lineage (94) in several cell types and species including drosophila, early xenopus embryos, the developing chick retina, rat retinal progenitors and the developing mouse brain (95). Hence, the results obtained in the human neurospheres match the published data from other species, i.e. pharmacological inhibition of NOTCH with DAPT increases neuronal differentiation and inhibits oligodendrogenesis (94, 96, 97). Why mouse NPC acted in an opposite way than human NPC towards NOTCH inhibition could have several reasons. For one, in contrast to human and rat NPC, mNPC differentiation cultures contain FCS, which might be responsible for the different effects of DAPT on mouse compared to human NPC development (92). Moreover, NOTCH favors the fate specification of oligodendrocytes and astrocytes in stages were cells are not yet committed to neuronal or glial fate, yet it inhibits the subsequent specification to $O4^+$ cells in favor of $GFAP^+$ cells (98, 99). Possible differences in developmental timing between human and mouse NPC culture might therefore provide an explanation for the different DAPT effects in human and mouse NPC. We also observed an increase in apoptotic O4+ cells in the DAPT treated mouse cultures. This has previously been observed *in vivo*, where transgenic mice with an inactive Notch1 receptor show premature oligodendrocyte differentiation at E17.5 which are eliminated by apoptotic cell death before full differentiation (100). In addition it is striking, that rat NPC are not affected by NOTCH inhibition at all. This might be due to the low expression of NOTCH signaling pathway molecules (Suppl. Tab. 19) keeping this pathway inactive in the neurosphere culture.

Epidermal Growth Factor (EGF)-dependent signaling regulates NSC proliferation, migration, and differentiation into neurons and glia cells during development (101–104). Thereby, EGF exerts its action through the EGF-receptor (EGFR; 100, 103). The EGFR is expressed in NPC of the three species studied (Suppl. Tab. 19). Investigating the effects of EGFR inhibition by PD153035 in absence of externally provided EGF gives insight into auto- or paracrine functions of endogenously produced EGFR ligands. Such ligands, like HB-EGF and TNFα, are generated through enzymatic cleavage by ADAM17/TACE (tumor necrosis factorα converting enzyme), e.g. guaranteeing survival, proliferation and development of cells of the oligodendrocyte lineage during development in an EGFR-dependent manner in mice (104). Inhibition of the human NPC EGFR produced results as expected from the *in silico* prediction and the scientific literature, i.e. reduced NPC migration (27) and oligodendrocyte differentiation as well as induced neuronal differentiation (104; Fig. 6g-i) showing that hNPC recapitulate physiological EGFR functions *in vitro*. Again, *egfr* was not identified as a hub gene in rodent NPC. Yet, as expected from the literature, PD153035 increased neuronal differentiation of rat NPC (106), while mouse NPC responded with an inhibition of neurogenesis (Fig. 6h). This discrepancy in mNPC concerning the published literature (101) is possibly be due to the usage of FSC during differentiation in this study, while the C17.2 cells used by Ayuso-Sacido et al. (2010) were differentiated in absence of FCS. Studying the effects of PD153035 on human NPC differentiation in presence of FCS substantiated this hypothesis: similar to mNPC, the EGFR inhibitor reduced hNPC neuronal differentiation in presence of FCS (Suppl. Fig. 5m). Thus, FCS can convert cells' responses to pathway modulators implying that one has to be cautious when using FCS in cell culture medium for pathway analyses and check for human and/or *in vivo* relevance of data. Other than in human NPC, we did not observe inhibition of migration of rodent NPC in our study. This might be due to dissimilar EGF responses of the different glia types or maturation stages of glia in the differentiating NPC cultures of the three species (Fig. 2g; 21). Maturation and cell type-specific EGF responses due to asymmetric EGFR distribution as a mechanism for shaping brain regions or cell type diversity within brain regions was reported earlier (107). Also in contrast to human NPC and expected from the published literature (104, 108, 109), rodent NPC oligodendrocyte differentiation is not modified by inhibition of EGFR signaling in the neurosphere cultures. Possible explanations for these discrepancies might be developmental timing and or brain region, as rat PND0-1 hippocampal NSC's differentiation to oligodendrocytes is EGF responsive, while neuronal differentiation is not (109), which is opposite in the study presented here. Another explanation could be changing EGFR levels with developmental age (110) in a cell type-specific manner over time (107). It might be suggested that faster maturation of rodent NPC in comparison to human NPC determines responses to EGF.

Taken together, our transcriptome-based data clearly demonstrates that primary NPC from different species differ in their molecular equipment despite similar cellular functions, i.e. NPC migration, neuronal and glia differentiation. Functional pathway validation due to pharmacological modulation of pathways identified via transcriptome analyses also identified species variations. Although more species-specific functional analyses of neurodevelopmental pathways need elucidation to gain a more complete picture of the human-specific NPC connectome, this work already strongly supports the

concept of human cell-based in vitro analyses for neurodevelopmental toxicity or efficacy testing. Understanding such molecular pathways underlying cellular functions in in vitro systems is fundamental for understanding the assay's application domain. In addition, comprehension of similarities and differences of pathway functions between species is of high importance for pharmacology and toxicology because a high percentage of drugs fails when translating efficacy or safety from animals to humans (2).

AVAILABILITY

WebMeV (Multiple Experiment Viewer) is a cloud-based application supporting analysis, visualization, and stratification of large genomic data, particularly for RNASeq and microarray data. (http://mev.tm4.org/)

GraphPad Prism is a biostatistics, curve fitting and scientific graphing software. (www.graphpad.com)

STRING (Search Tool for Retrieval of Interacting Genes/Proteins) is a bioinformatics database containing information on direct and indirect interaction between genes and proteins. (http://string-db.org)

Cytoscape is and open source software for network data integration, analysis and visualization (http://www.cytoscape.org/)

R is is a free software environment for statistical computing and graphics. (https://www.r-project.org/)

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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TABLE AND FIGURES LEGENDS

Table 1: GO BP overrepresentation analyses.

SPECIES	HUMAN		MOUSE		RAT	
EXPRESSION CLUSTER	*M1+2*	*M3+4*	*M1+2*	*M3+4*	*M1+2*	*M3+4*
# GENES	1278	404	1074	906	908	1417
# GENES ANNOTATED	1070	345	904	761	787	1058
# GO TERMS	**155**	**79**	**427**	**110**	**228**	**61**
SHARED GO TERMS	*# (% human) of GO terms*					
EXPRESSION CLUSTER	*M1+M2*			*M3+M4*		
HUMAN-MOUSE	122 (78)			33 (42)		
HUMAN-RAT	98 (63)			0		
ALL SPECIES	90 (58)			0		
MOUSE-RAT	181			45		

Table 2: Highly connected genes as key regulators

		MIGRATION	NEUROGENESIS	GLIOGENESIS
HUMAN	MEAN #connections /Gene Human	4.3	5.7	3.4
	Key regulators (#connections) human	BMP2*(23), EGFR*(22), EPHA2(16), FGFR1(18), JUN*(20), LYN(14), MYC*(23), NOTCH1*(18), PDGFRB(18), SRC(44), VEGFA(26)	BMP2*(24) EGFR*(17), FGFR1(14), JUN(20), MYC*(25), NOTCH1*(26), VEGFA(26)	BMP2*(10), EGFR*(6), MYC*(10), NOTCH1*(14)
MOUSE	MEAN #connections /Gene (overlap human)	4.4 (13.6%)	2.7 (14.4%)	3.3 (7.1%)
	Key regulators (#connections) Mouse	Agt(17), Cav1(20), Flt1(14), Fyn(17), Itga(17), Pdgfb(14), Ptgs2(15), Vegfa(17)	Bmp4(10)	none
RAT	MEAN #connections /Gene (overlap human)	2.7 (8.2%)	2.5 (7.6%)	not present
	Key regulators (#connections) rat	Cx3cr1(10), Flt1(11), Itgb4(9), Ptk2(16)	Ptk2(9)	

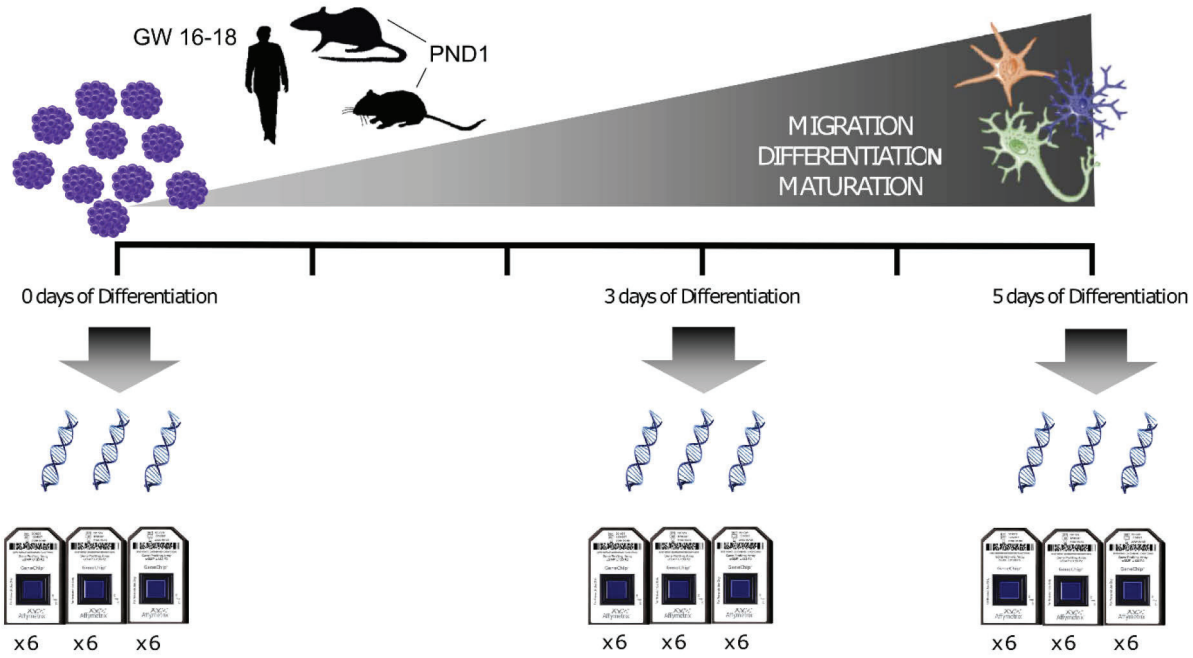

Figure 1. Experimental set up.

NPC were generated from fetal human brain (GW16-18) or post-natal mouse and rat brain (PND1) and cultivated as floating neurospheres. RNA was isolated from proliferating (0d), as well as three and five days differentiated human, mouse and rat NPCs, from 6 replicates per condition and species. Transcriptome analyses from these samples were performed using human PrimeView Array and mouse/rat Gene 2.0 ST Arrays from Affymetrix.

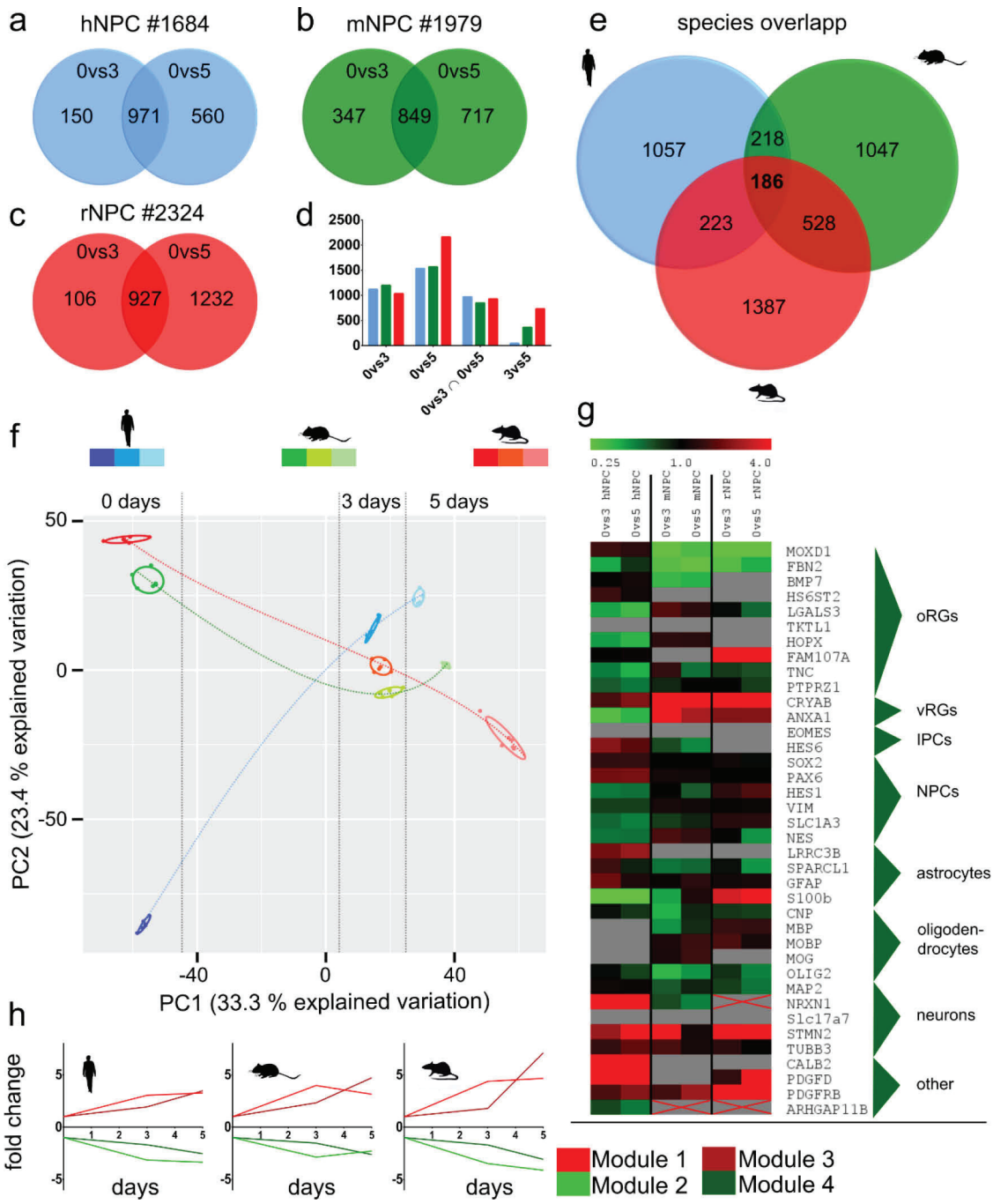

Figure 2. Qualitative and quantitative comparison of expression profiles across species.

Differential gene expression within each species and between time points was statistically determined by one-way ANOVA followed by Tukey's range test. Genes with $p \leq 0.01$ and fold change ≥ 2 were called differentially expressed (DEX). (a-c) Overlap of the number of DEX genes between 3 (0vs3) and 5 (0vs5) days of differentiation for human (a), mouse (b) and rat (c) NPCs. (d) Comparison of the number of DEX genes for each time point (0vs3, 0vs5, overlap between 0vs3 and 0vs5 and 3vs5) between human (blue), mouse (green) and rat (red) NPCs. (e) DEX genes (at any time point) that share the same gene symbol in human (blue), mouse (green), and rat (red) NPCs. (f) Principal component analysis (PCA) was performed based on the expression of all significantly regulated ($p \leq 0.01$) genes that shared the same gene symbol between species (5570) and compares the expression profile over

time (0, 3 and 5 days; dark to pale) between species (human, blue; mouse, green; rat, red). (g) Expression profile of single genes associated to specific cell types during neural development and in the CNS of human, mouse and rat NPCs. Genes, defined as not present are depicted in grey, genes depicted in grey with a red cross are not on the respective microarray chip. (h) Hierarchical clustering generated 10 distinct expression clusters (Suppl. Tab. 1), which were further summarized into 4 modules. Data is represented as mean DEX over time of all genes within one module for human mouse and rat NPCs.

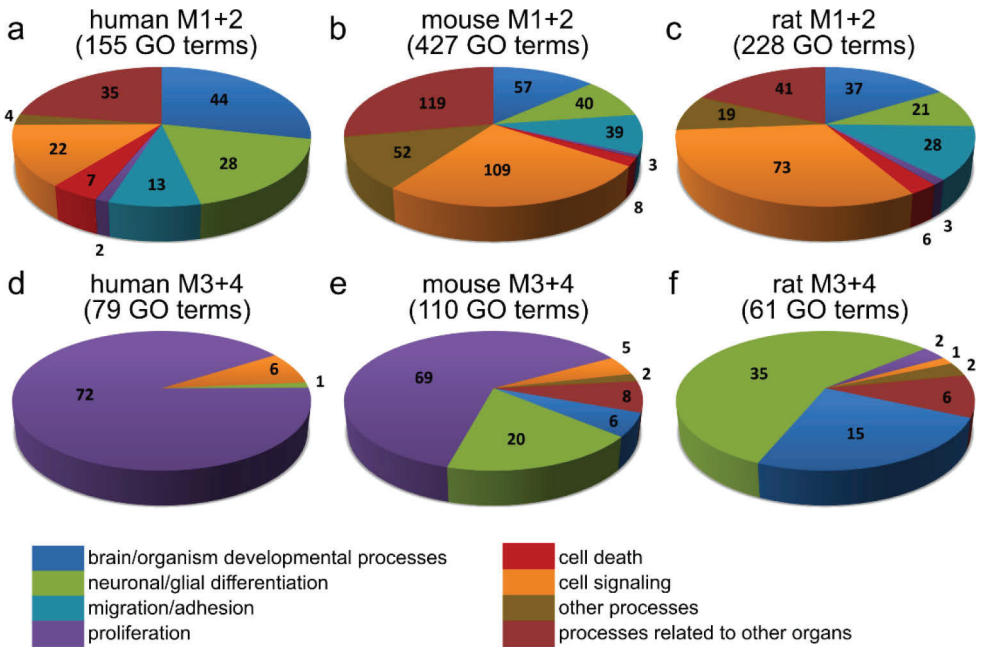

Figure 3. Classification of GO terms into superordinate biological processes.

ORA analysis was performed using the Cytoscape plugin ClueGO (Bindea et al. 2009). All overrepresented GO terms ($p \leq 0.01$ based on a two-sided hypergeometric option with a Bonferroni correction) for modules 1+2 (a-c) and modules 3+4 (d-f; also see Fig. 2) were summarized into 8 superordinate processes for human (a, d), mouse (b, e) and rat (c, f) NPCs based on expert judgement. Number in the pie chart represents the number of GO terms assigned to each superordinate processes.

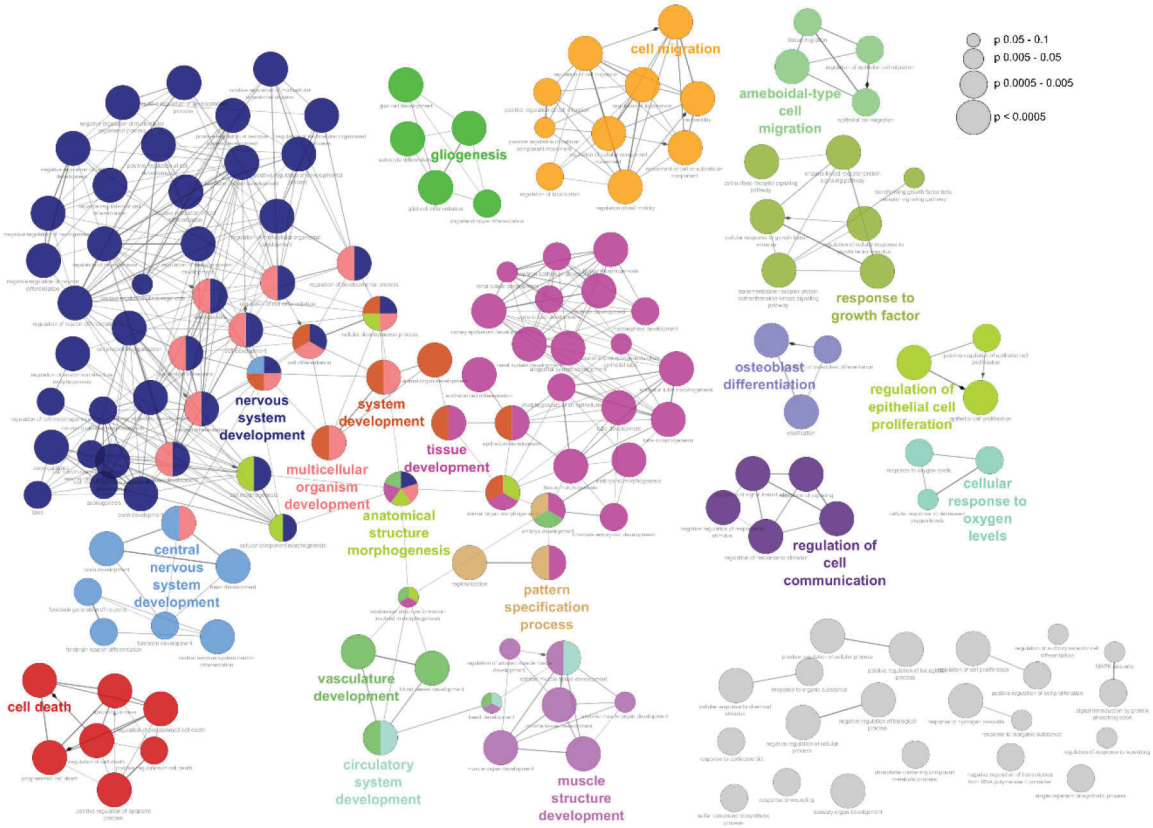

Figure 4. GO clustering in hNPC.

Overrepresented GO terms of modules 1 and 2 in hNPC were clustered according to gene overlap between GO terms with a kappa score threshold of 0.5 and at least three GO terms within one cluster using the Cytoscape plugin ClueGO (Bindea et al. 2009). Edge thickness represents similarity between GO terms. Node size represents significance of overrepresentation. The GO term with the highest significance determines the name of the respective GO cluster (bold; colored). Different colors represents different GO cluster. Grey nodes do not belong to a cluster. Significance thresholds of ORA was set to $p \leq 0.01$ based on a two-sided hypergeometric option with a Bonferroni correction.

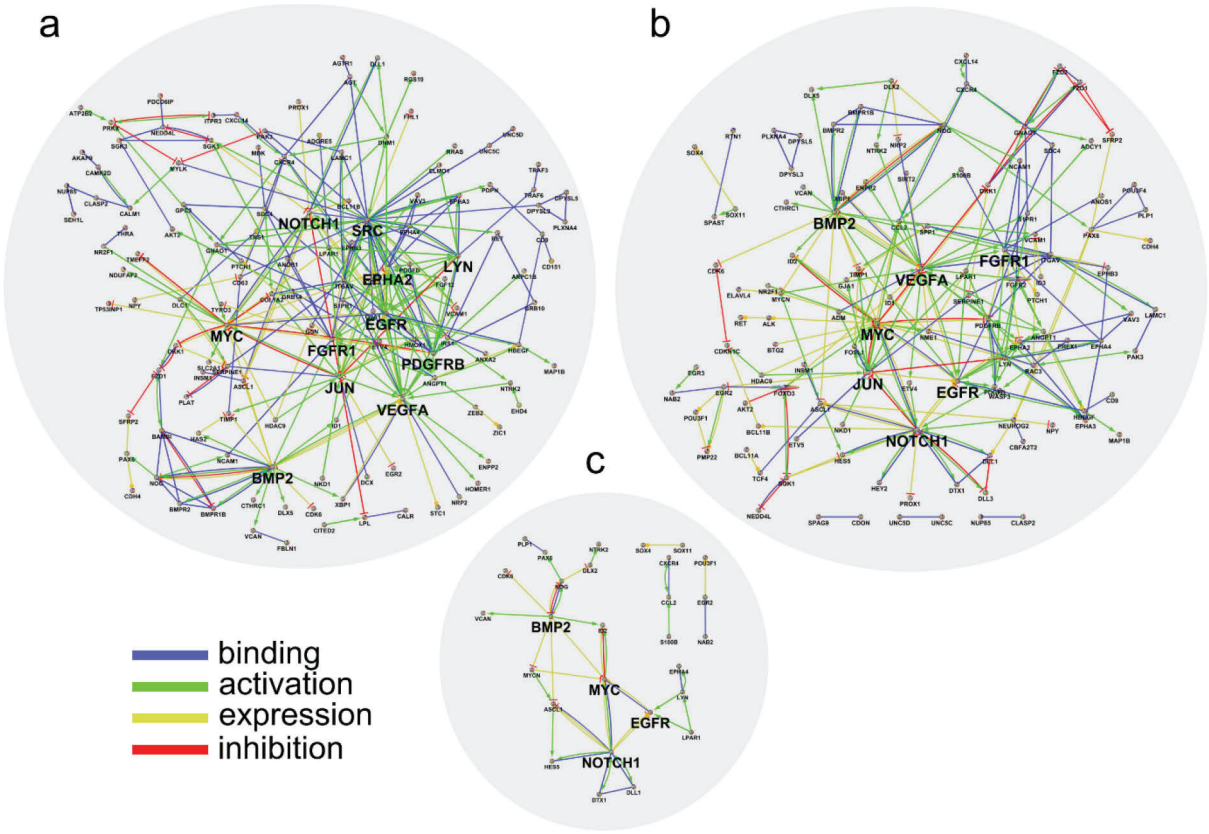

Figure 5. Gene-gene interaction networks of major neurodevelopmental processes overrepresented in hNPCs.

The cytoscape plugin GluePedia (Bindea et al. 2013) was used to enrich the GO clusters cell migration (a), neurogenesis (c) and gliogenesis (c) from the ORA of modules 1 and 2 of hNPC with information on gene-gene/protein-protein interactions (binding in blue, activation in green, expression in yellow and inhibition in red) from the STRING database (Szklarczyk et al. 2015) with a confidence score of ≥ 0.75. Highly connected genes (bold) were identified as those genes showing at least three times (migration, neurogenesis) or two times (gliogenesis) the number of connections compared to the mean number of connections per gene within the respective cluster.

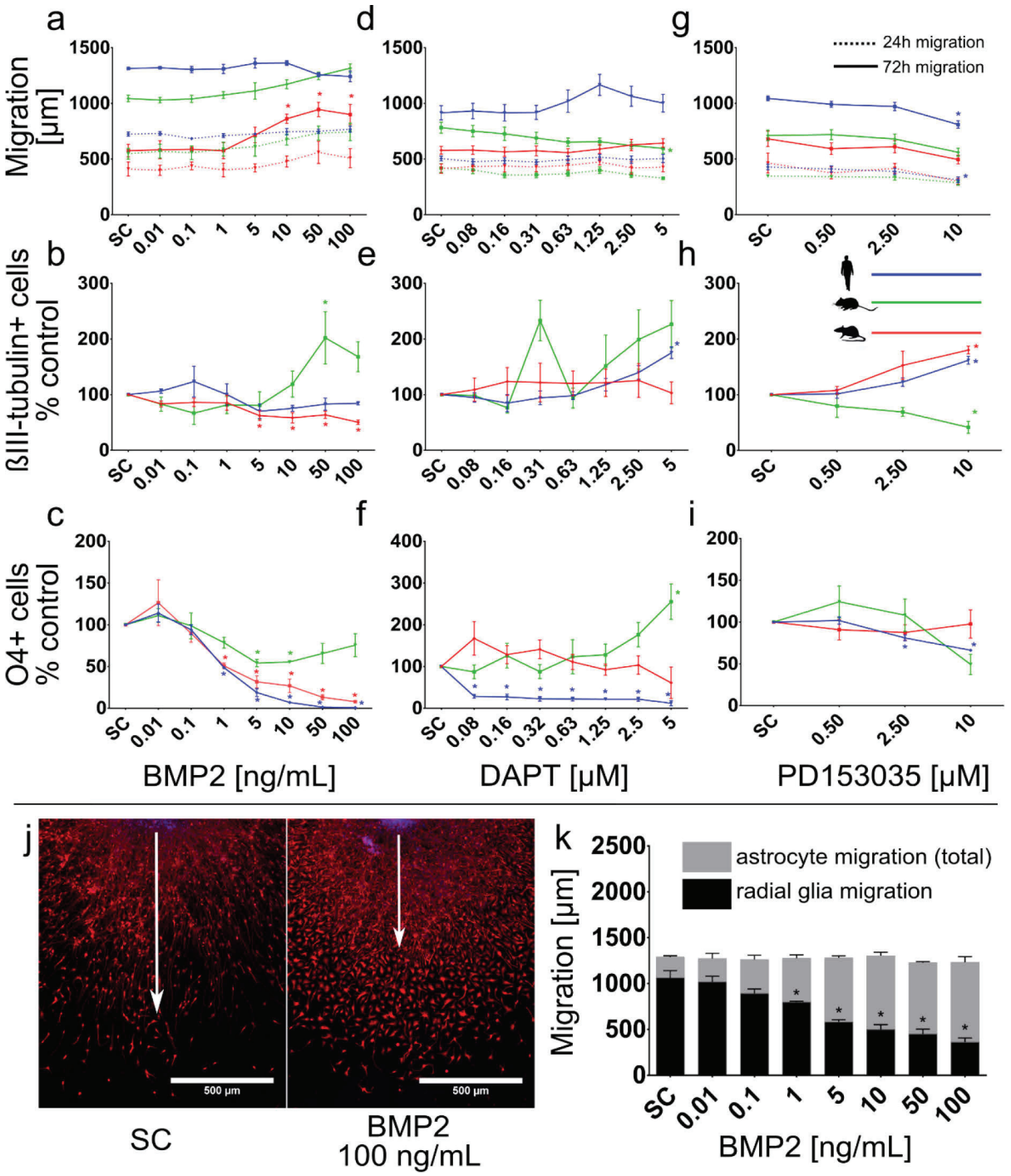

Figure 6. Pharmacological modulation of the BMP2, NOTCH and EGF pathway.

NPCs from human (blue), mouse (green; differentiated in FCS) and rat (red) were treated with increasing concentrations of BMP2 (a, b, c), DAPT (NOTCH inhibitor; d, e, f) and PD1530353 (EGFR inhibitor; g, h, i) and analyzed for migration (after 24h (dotted line) and 72h (solid line); a, d, g) neuronal differentiation (after 72h for BMP2, EGFRi and 120h for DAPT; b, e, h), oligodendrocyte differentiation (after 120h; c, f, i) and astrocyte maturation (only in hNPC after BMP2 treatment; j, k). Neurons and oligodendrocytes were immunocytochemically stained with ßIII-tubulin and O4, respectively, and quantified as percent of neurons/oligodendrocytes compared to Hoechst33258 counterstained nuclei. Astrocytes were stained with GFAP and maturation of radial glia cells was measured as migration of

radial glia compared to total migration of GFAP positive cells. (j) Representative pictures of immunocytochemically stained astrocytes after 72h BMP2 treatment of hNPCs. Scale bar represent 500 µm, white arrow marks radial glial migration. Except for migration distance (shown as raw migration distance in µm), data was normalized to the solvent control and is displayed as concentration response relationship with mean ± SEM of at least three independent experiments. *indicates a significant difference to solvent control based on one-way ANOVA ($p<0.05$) followed by Dunnett's multiple comparison test.

Supplementary Material

A transcriptome comparison of time-matched developing human, mouse and rat neural progenitor cells reveals human uniqueness

Nucleic Acids Research

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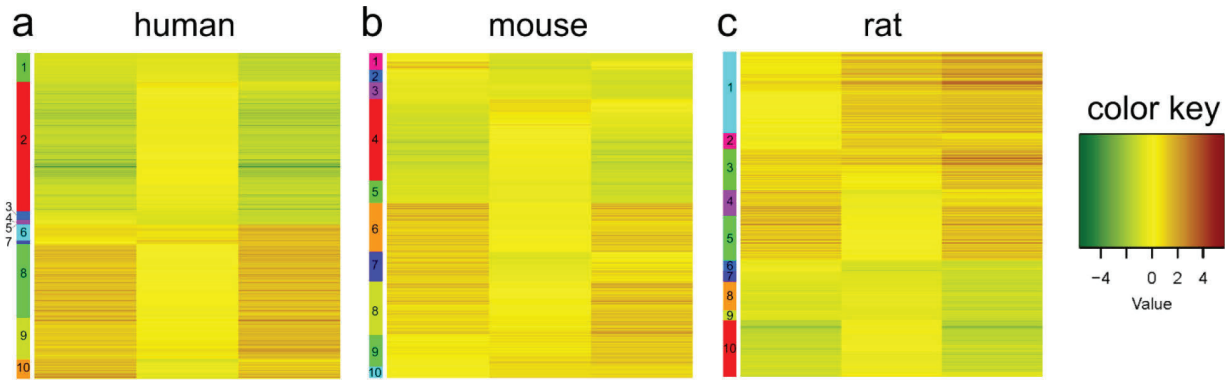
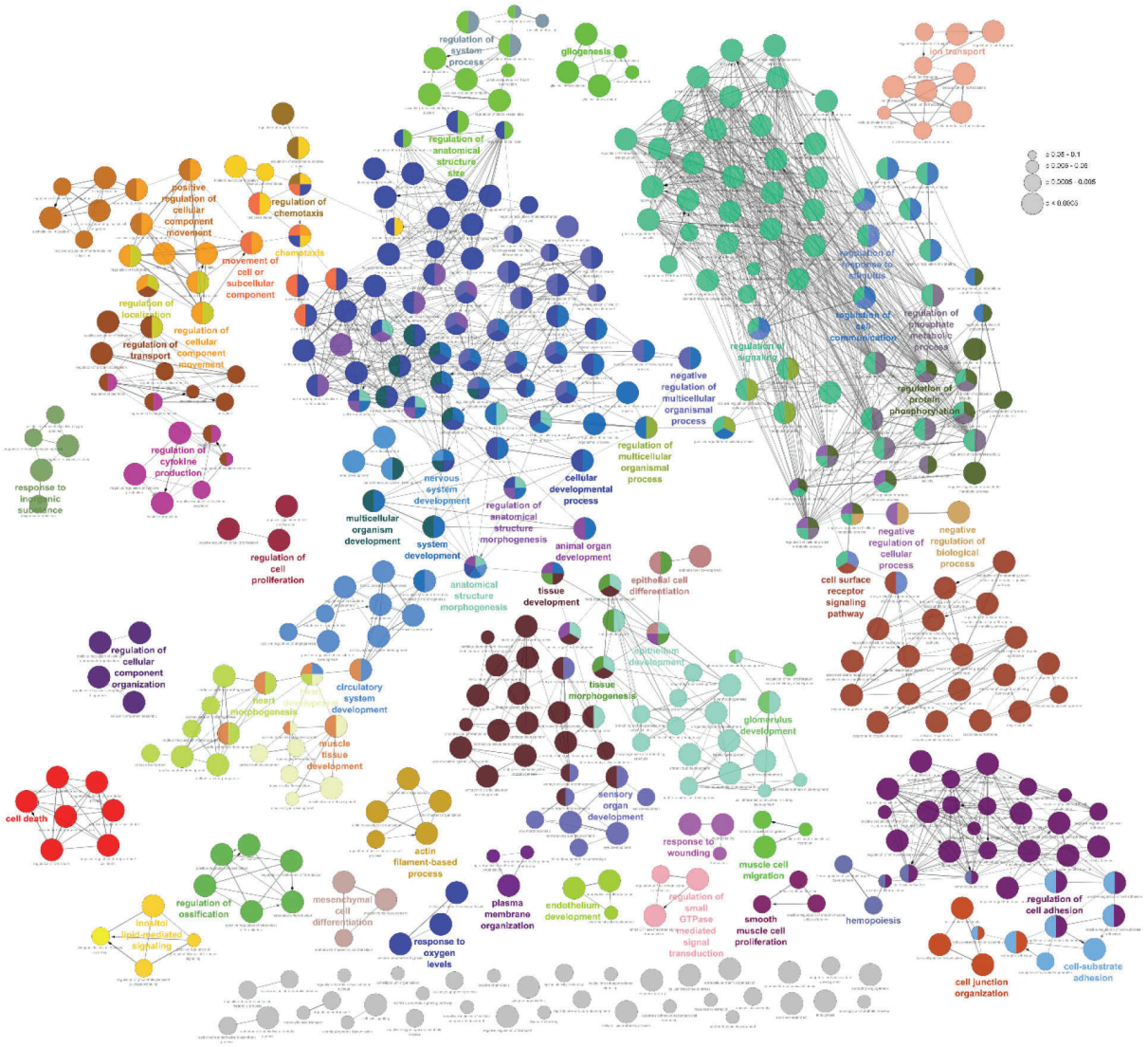


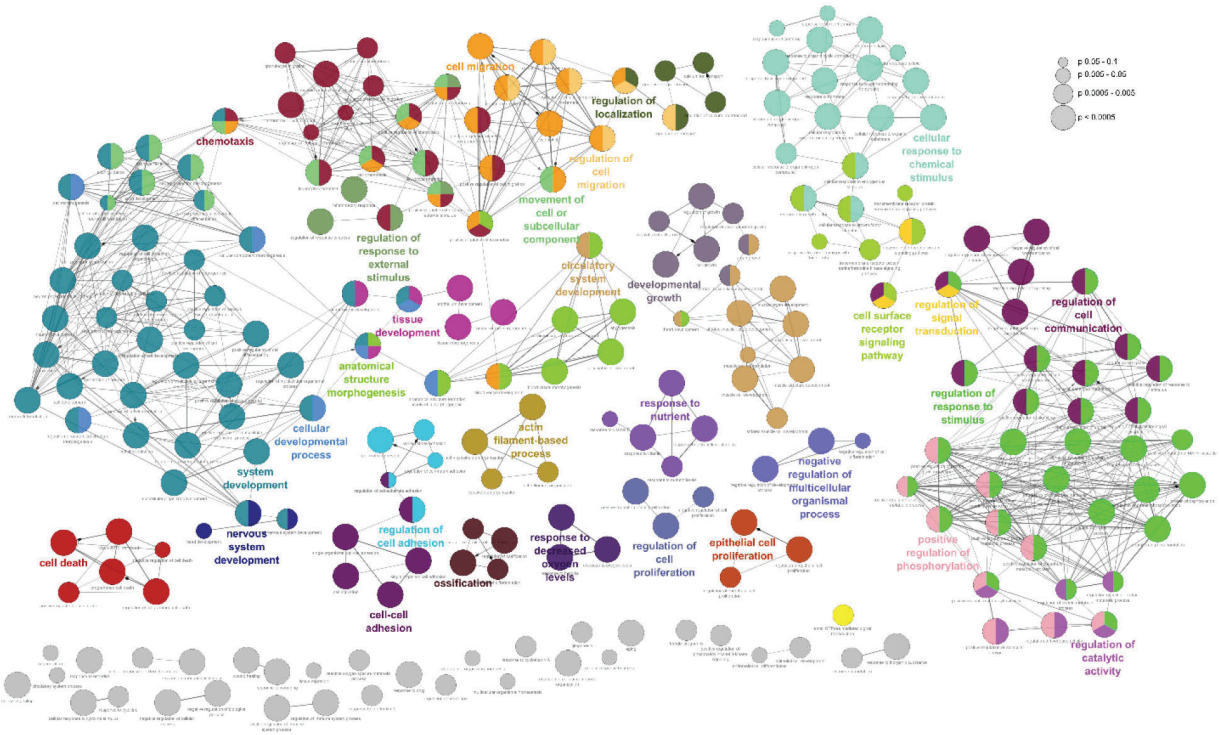
Suppl. Fig. 1. Hierarchical cluster analysis

Hierarchical clustering of all genes that were differentially expressed ($p \leq 0.01$ and fold change ≥ 2) between any two time points (0vs3, 0vs5 and 3vs5) in human (a) mouse (b) and rat (c) NPCs was performed using an unweighted pair group agglomeration method with arithmetic mean and cutree, resulting in 10 distinct clusters as indicated by colored bars (random coloring) to the left of each heatmap.



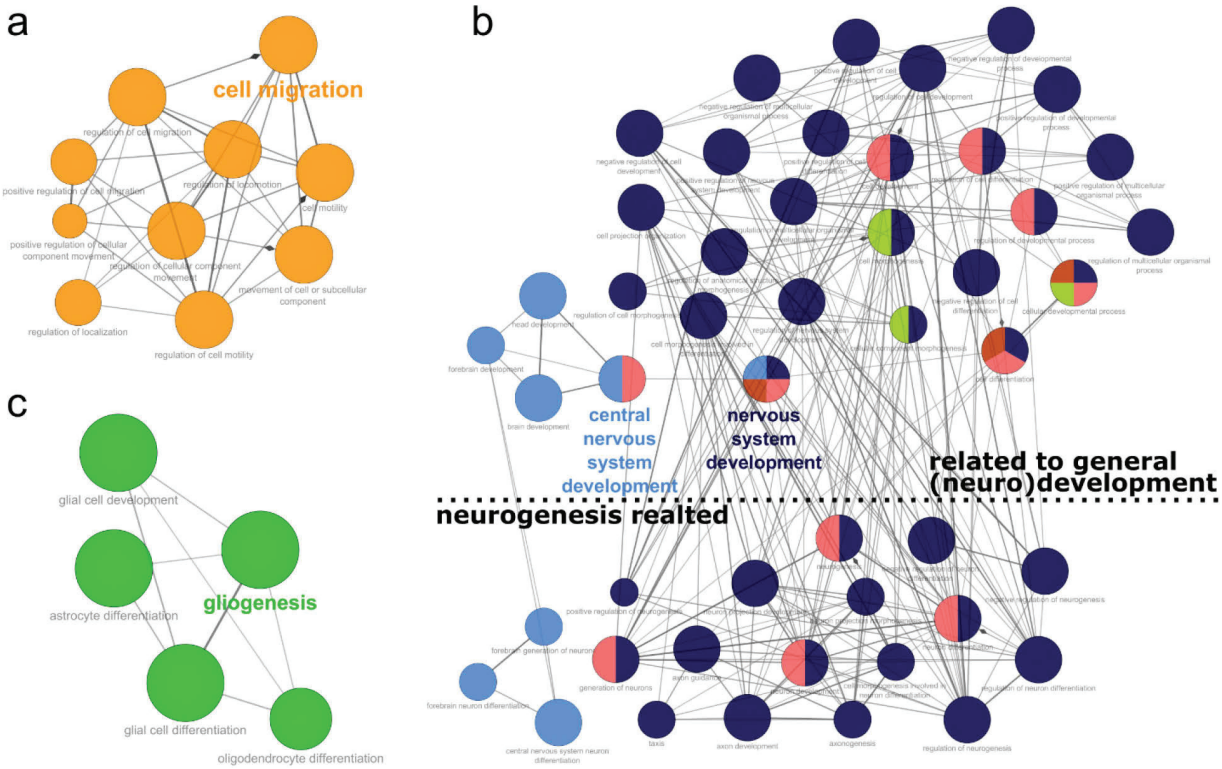
Suppl. Figure 2. GO clustering in mNPC.

Overrepresented GO terms of modules 1 and 2 (see Fig. 2h) in mNPC were clustered according to gene overlap between GO terms with a kappa score threshold of 0.5 and at least three GO terms within one cluster using the Cytoscape plugin ClueGO. Edge thickness represents similarity between GO terms. Node size represents significance of overrepresentation. The GO term with the highest significance determines the name of the respective GO cluster (bold; colored). Different colors represents different GO cluster. Grey nodes do not belong to a cluster. Significance thresholds of ORA was set to $p \leq 0.01$ based on a two-sided hypergeometric option with a Bonferroni correction.



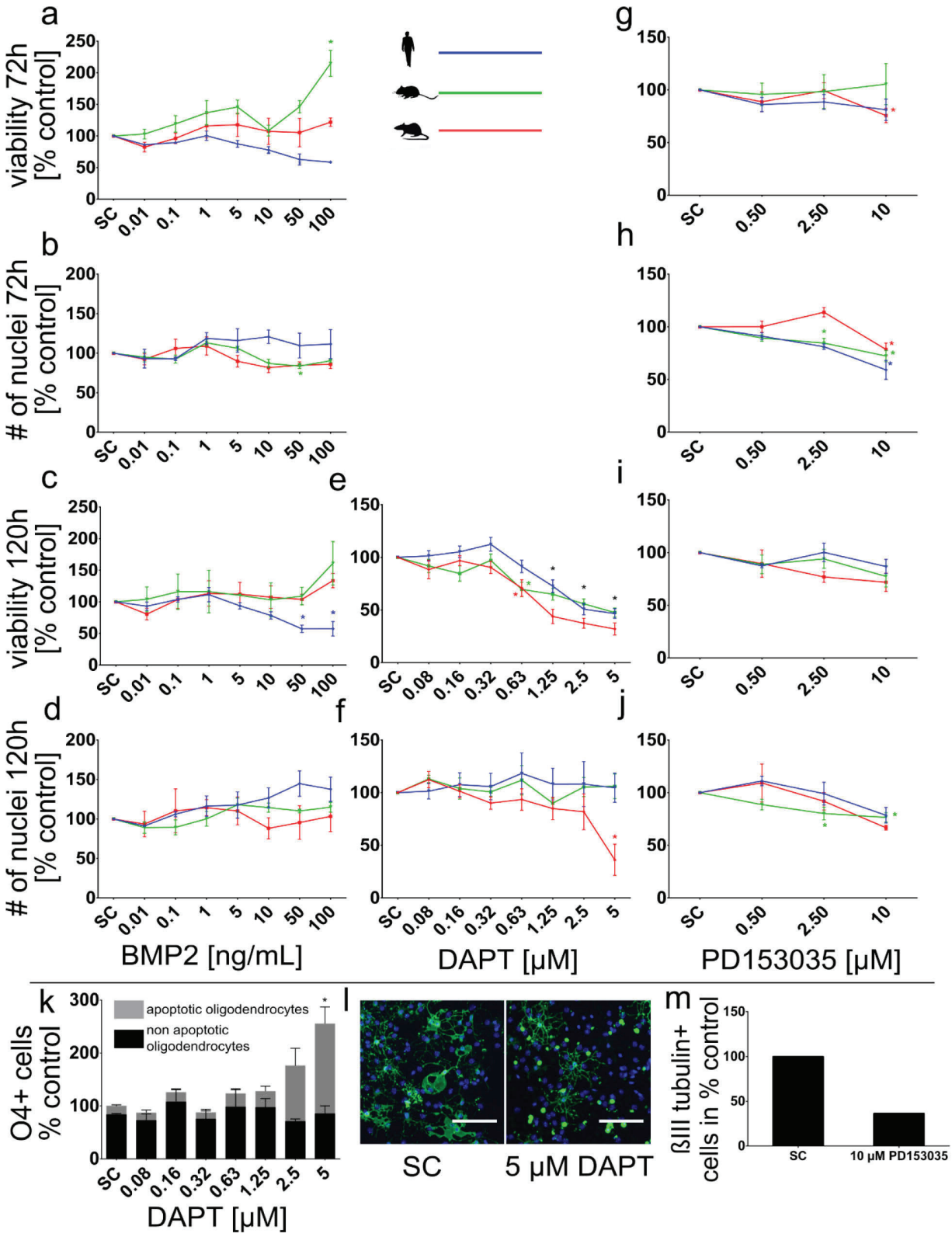
Suppl. Figure 3. GO clustering in rNPC.

Overrepresented GO terms of modules 1 and 2 in rNPC were clustered according to gene overlap between GO terms with a kappa score threshold of 0.5 and at least three GO terms within one cluster using the Cytoscape plugin ClueGO (Bindea et al. 2009). Edge thickness represents similarity between GO terms. Node size represents significance of overrepresentation. The GO term with the highest significance determines the name of the respective GO cluster (bold; colored). Different colors represents different GO cluster. Grey nodes do not belong to a cluster. Significance thresholds of ORA was set to $p \leq 0.01$ based on a two-sided hypergeometric option with a Bonferroni correction.



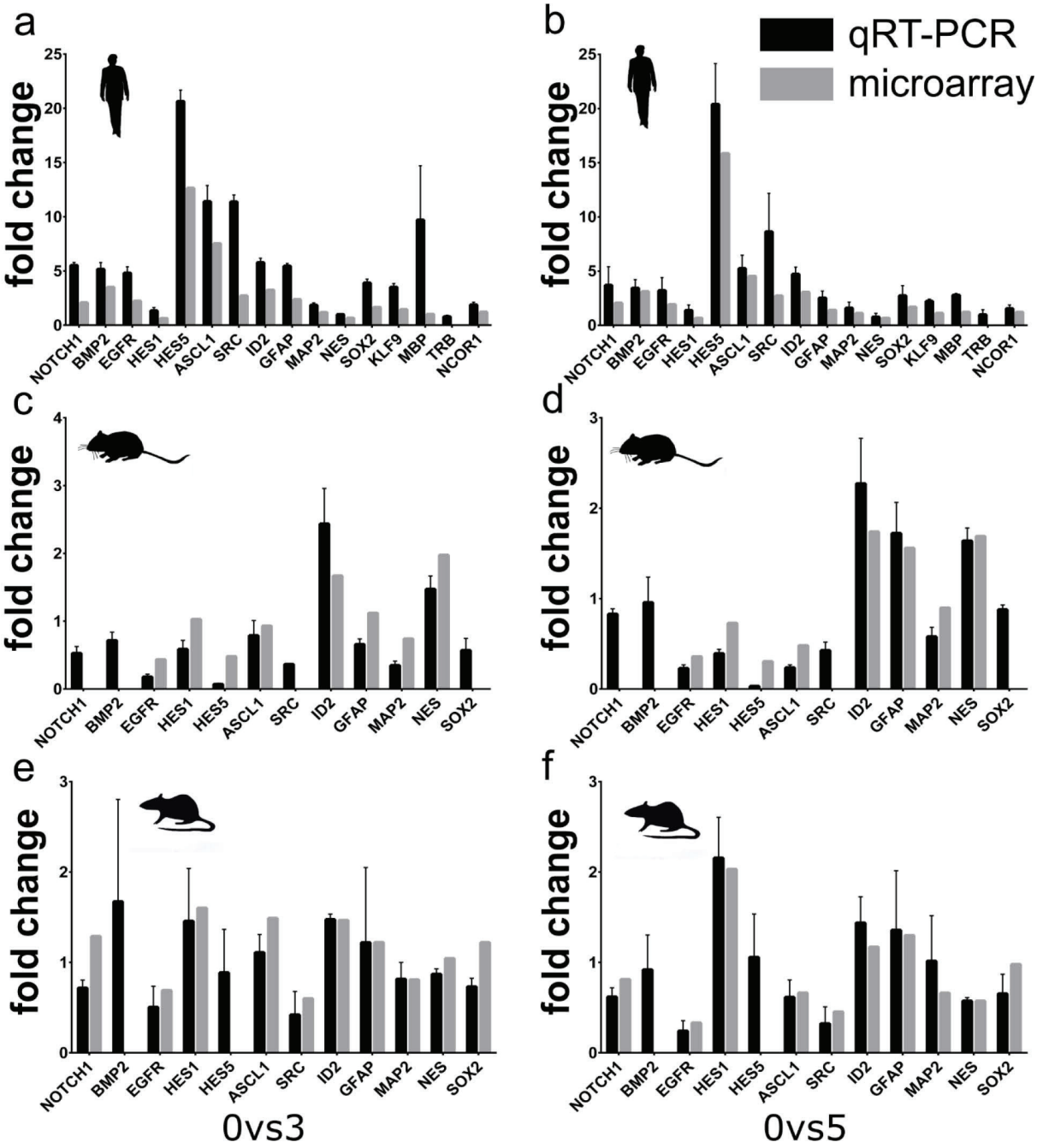
Suppl. Figure 4. Relevant GO term cluster for neurodevelopmental processes in hNPC

GO term clusters for biological processes related to cell migration (a), neurogenesis (b) and gliogenesis (c) and extracted from GO term clustering in Fig. 4. (c) demonstrates the selection of GO terms related to neurogenesis that were extracted for enrichment with information on gene-gene/protein-protein interactions (binding, activation, expression and inhibition) based on the STRING database as presented in Fig. 5. For information on node size and related significance see Fig. 4.



Suppl. Figure 5. Pharmacological modulation of the BMP2, NOTCH and EGF pathway.

NPCs from human (blue), mouse (green) and rat (red) were treated with increasing concentrations of BMP2 (a, b, c, d), DAPT (NOTCH inhibitor; e, f) and PD1530353 (EGFR inhibitor; g, h, I, j) and analyzed for viability and nuclei count (after 72h and 120h). Viability was assessed as mitochondrial activity by Alamar-Blue assay. Nuclei were counterstained with Hoechst33258 and automatically quantified. (k) Percentage of apoptotic compared to non-apoptotic O4 positive cells in mNPC after 120h DAPT treatment. (l) Representative pictures of immunocytochemically stained oligodendrocytes with and without DAPT treatment. Scale bar represent 100 µm. (m) hNPCs cultured in 1% FCS during differentiation were treated with 10 µM PD1530353 and analyzed for neuronal differentiation after 72h. Data of m represents one independent experiment normalized to the solvent control. Data of all other experiments was normalized to the solvent control and is displayed as concentration response relationship as mean ± SEM of at least three independent experiments. *indicates a significant difference from solvent control based on one-way ANOVA ($p<0.05$) followed by Dunnett's multiple comparison test.



Suppl. Figure 6. Validation of microarray experiments by qRT-PCR.

Gene expression of mRNA samples from human, mouse and rat NPCs that were used for microarray analysis (three replicates) was analyzed by qRT-PCR and compared to results of microarray analysis. Data is presented as fold change from qRT-PCR (black) and microarray analysis (grey) between 0 and 3 days (a, c, e) and 0 and 5 days (b, d, f) of human (a, b), mouse (c, d) and rat (e, f) NPCs.

A transcriptome comparison of time-matched developing human, mouse and rat neural progenitor cells reveals human uniqueness

Stefan Masjosthusmann, Daniel Becker, Barbara Petzuch, Jördis Klose, Clara Siebert, Rene Deenen, Marta Barenys, Jenny Baumann, Simon Schliesky, Julia Tigges, Ulrike Hübenthal, Karl Köhrer, Ellen Fritsche

Journal:	Nucleic Acids Research (NAR)
Impact factor:	10,16 (2017)
Contribution to the publication:	70 % Planning and performance of most of the main experiments, supervision of planning, performance and evaluation of many experiments of the pharmacological modulation. Evaluation of array data, writing of the manuscript
Type of authorship:	first-authorship
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2.2 Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

J. Baumann, K. Gassmann, **S. Masjosthusmann**, D. DeBoer, F. Bendt, S. Giersiefer, E. Fritsche

Archives of Toxicology

Das sich entwickelnde Gehirn ist besonders anfällig gegenüber adversen Effekten von Chemikalien, was im Menschen zu Entwicklungsstörungen des Nervensystems führen kann. Derzeit bilden Tierversuchsstudien den Goldstandard für die toxikologische Testung auf Entwicklungsneurotoxizität, allerdings sind diese richtlinien-konformen Studien unzureichend bezüglich ihres Tierverbrauchs, sowie Zeit- und Kostenbedarfs. Des Weiteren bergen Speziesunterschiede das Problem der Extrapolation. Aus diesem Grund besteht internationaler Konsensus über den Bedarf für die Entwicklung von Alternativmethoden, die das entwicklungsneurotoxische Potential von Chemikalien schneller, kostengünstiger und mit einer hohen Prädiktivität für den Menschen ermitteln können. In diesem Zusammenhang hat die Arbeitsgruppe um Prof. Fritsche ein in vitro Model für die Entwicklungsneurotoxizitätstestung entwickelt, welches auf primären neuralen Progenitorzellen von Mensch und Ratte basiert die als Neurosphären kultiviert werden. Diese sind in der Lage, basale Prozesse der frühen fetalen Phase der Gehirnentwicklung nachzustellen und ermöglichen eine Untersuchung von Speziesunterschieden zwischen Mensch und Nager. Ziel dieser Studie war es zu untersuchen, in wie weit humane und Rattenneurosphären das entwicklungsneurotoxische Potential eines gut charakterisierten Trainingssets von neun Chemikalien richtig vorhersagen können, indem Effekte auf die Endpunkte Progenitorzell-Proliferation, Migration und neuronale Differenzierung parallel zu Effekten auf die Viabilität untersucht wurden. Unsere Ergebnisse zeigen, dass (i) eine Korrelation unserer humanen und Rattendaten in vitro mit existierenden in vivo Daten für die meisten Chemikalien eine korrekte Vorhersage des entwicklungsneurotoxischen Potentials ermöglichte und humane und Rattenneurosphären eine wertvolle Komponente in einer modularen Testbatterie für Entwicklungsneurotoxizität bilden können, und, (ii) humane und Rattenneurosphären sich in ihrer Empfindlichkeit gegenüber den meisten Chemikalien unterscheiden und somit toxikodynamische Speziesunterschiede von Chemikalien widerspiegeln.

Arch Toxicol
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IN VITRO SYSTEMS

Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

Jenny Baumann[1] · Kathrin Gassmann[1] · Stefan Masjosthusmann[1] · Denise DeBoer[1] · Farina Bendt[1] · Susanne Giersiefer[1] · Ellen Fritsche[1]

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Abstract The developing brain is highly vulnerable to the adverse effects of chemicals, resulting in neurodevelopmental disorders in humans. Currently, animal experiments in the rat are the gold standard for developmental neurotoxicity (DNT) testing; however, these guideline studies are insufficient in terms of animal use, time and costs and bear the issue of species extrapolation. Therefore, the necessity for alternative methods that predict DNT of chemicals faster, cheaper and with a high predictivity for humans is internationally agreed on. In this respect, we developed an in vitro model for DNT key event screening, which is based on primary human and rat neural progenitor cells grown as neurospheres. They are able to mimic basic processes of early fetal brain development and enable an investigation of species differences between humans and rodents in corresponding cellular models. The goal of this study was to investigate to what extent human and rat neurospheres were able to correctly predict the DNT potential of a well-characterized training set of nine chemicals by investigating effects on progenitor cell proliferation, migration and neuronal differentiation in parallel to cell viability, and to compare these chemical responses between human and rat neurospheres. We demonstrate that (1) by correlating these human and rat in vitro results to existing in vivo data, human and rat neurospheres classified most compounds correctly and thus may serve as a valuable component of a modular DNT testing strategy and (2) human and rat neurospheres differed in their sensitivity to most chemicals, reflecting toxicodynamic species differences of chemicals.

Keywords Neurosphere · Human · Rat · Developmental Neurotoxicity · In vitro · Species difference

Introduction

The socioeconomic potential of a population is substantially determined by the intelligence of its individuals (Bellanger et al. 2013). Therefore, it is of utmost importance to ensure individual development of maximum intellectual potential. Poisoning disasters with, e.g., polychlorinated biphenyls or mercury have strikingly demonstrated that the developing brain is highly vulnerable to the adverse effects of chemicals (Rodier 1995), resulting in neurodevelopmental disorders in humans (Grandjean and Landrigan 2006). Not only poisoning incidences but also low-dose exposures toward environmental chemicals are thought to interfere with human brain development (Grandjean and Landrigan 2014), thus entailing a serious threat to society (Goldman and Koduru 2000). Currently, the rat bioassay is the gold standard for developmental neurotoxicity (DNT) testing (testing guidelines OECD TG426 and US-EPA 870.6300: OECD 2007; USEPA 1998). However, these guideline studies are resource intensive (animals, time, money), bear the issue of species extrapolation and do not necessarily produce satisfying results (Coecke et al. 2007; Lein et al. 2005, 2007). Considering that the majority of chemicals

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on the market has not been studied for their DNT potential (Grandjean and Landrigan 2006), necessity for alternative methods, which predict DNT of chemicals faster, cheaper and with a high predictivity for humans, was recently agreed on by different stakeholders from regulatory agencies, industry and academia on both sides of the Atlantic (Bal-Price et al. 2015a). Such alternative methods might also be used to assess DNT hazard in a mechanistic context of human relevance (Crofton et al. 2011).

To date, there are no validated alternative in vitro DNT assays available, but within the last years significant effort has been made to develop cell-based testing strategies for DNT hazard characterization of toxicants (Bal-Price et al. 2012; Breier et al. 2010; Coecke et al. 2007; Crofton et al. 2011; Lein et al. 2005, 2007). In parallel, toxicological testing principles have been subjected to a paradigm shift, proposing that chemical testing should move toward higher-throughput, mechanism-oriented, preferably human-based methods to circumvent species-specific effects in responses to compound exposure (Krewski et al. 2010; NRC 2007; Seidle and Stephens 2009). Emphasis on the human nature of cell-based assays is a result of mainly pharmacological research with poor translation of drug candidates from highly cited animal research into clinical application (Leist and Hartung 2013). A prerequisite for human in vitro assay validation is knowledge on human toxicants. For human DNT, however, such knowledge is restricted to 12 compounds (Grandjean and Landrigan 2006, 2014). In contrast, there are large amount of rodent in vivo DNT data available (Crofton et al. 2011), which are useful for validating rodent in vitro systems. Thus, rodent in vitro testing systems currently provide valuable tools for studying assay performance (in vivo–in vitro correlation), which can then be translated to human systems.

In this respect, we previously developed in vitro models for DNT key event screening, which are based on primary human and rat neural progenitor cells grown as neurospheres (Baumann et al. 2014). They are able to mimic basic processes of early fetal brain development such as proliferation, migration and differentiation to neural effector cells (Fig. 1) and enable an investigation of species differences between humans and rodents in corresponding cellular models (Gassmann et al. 2010; Moors et al. 2007, 2009). In the current study, we tested a well-characterized training set of six DNT-positive and three negative compounds (Suppl. Table 1) in these in vitro assays to assess their effects on neurodevelopmental key events. With these data, we investigated to what extent the tests correctly predicted the DNT potential of those chemicals to determine the predictive value as well as the application domain of the neurosphere assay. Such prediction was not achieved by pure hazard evaluation but by comparing effective in vitro concentrations (EC_{50} values) determined in this study to effective internal exposures in vivo previously published in the literature according to a parallelogram approach. These analyses revealed that—depending on the biological application domain—the neurosphere assay serves as a valuable component of a modular DNT testing strategy.

Materials and methods

Cell culture

Normal human neural progenitor cells (hNPCs, male, GW 16–19) were purchased from Lonza Verviers SPRL (Verviers, Belgium). Rat neural progenitor cells [rNPCs, postnatal day (PND) 5] were prepared time-matched to hNPCs (Clancy et al. 2007) as described previously (Baumann et al. 2014).

Both human and rat NPCs were cultured in proliferation medium. Differentiation was initiated by growth factor withdrawal in differentiation medium and plating onto poly-D-lysine (PDL)/laminin-coated chamber slides as described previously (Baumann et al. 2014). For details, see Supplementary Material.

Cell viability assay

In every experiment, mitochondrial reductase activity was assessed in the same wells than the specific endpoint evaluations as previously described (Baumann et al. 2014). For details, see Supplementary Material.

Cytotoxicity assay

For the cytotoxicity measurement the lactate dehydrogenase (LDH) assay (CytoTox-One; Promega, Mannheim, Germany) was used as described previously (Baumann et al. 2014). For details, see Supplementary Material.

Proliferation analysis

NPC proliferation was measured by the Cell Proliferation ELISA, BrdU (chemiluminescent) from Roche (Mannheim, Germany) as described previously (Baumann et al. 2014). Spheres cultivated in proliferation medium without growth factors served as endpoint-specific control, and for correction of unspecific binding of the BrdU antibody, some spheres were cultured without BrdU.

Migration analysis

Migration analyses were performed as previously described (Baumann et al. 2014). Ten µM PP2 (Sigma-Aldrich, Taufkirchen, Germany), a selective inhibitor for Src family

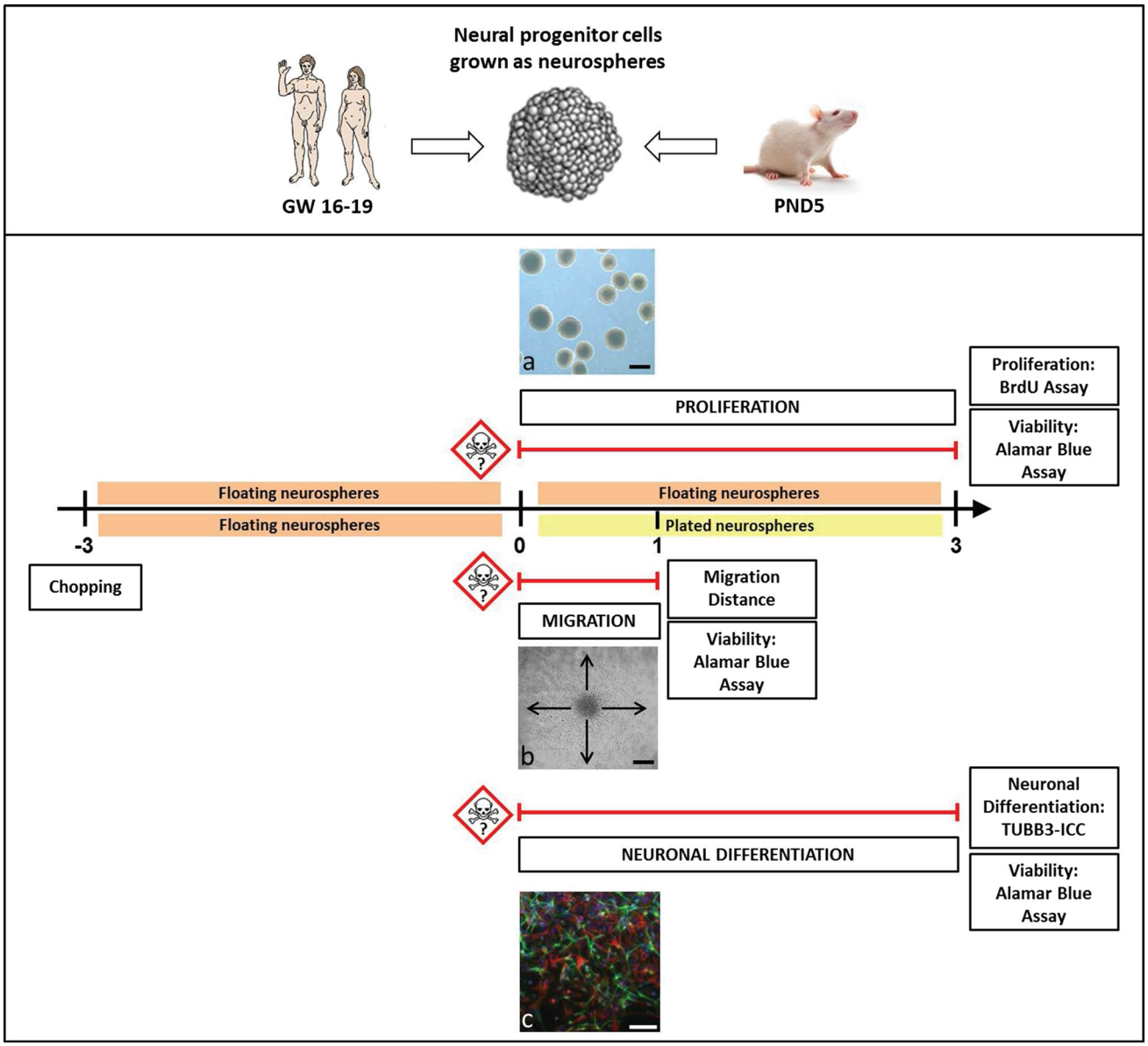


Fig. 1 Schematic overview of the experimental setup and chemical treatment periods of human and rat neurospheres. Human and rat neurospheres are exposed to test compounds (indicated in *red*) as floating neurospheres for assessing proliferation (days 0–3) or as plated neurospheres to assess either migration (days 0–1) or neuronal differentiation (days 0–3). For all endpoints, viability is investigated in parallel. Timeline is in days. *Scale bars* **a** and **b** 300 µm, **c** 100 µm. **c** *Red* GFAP-positive cells, *green* βIII-tubulin-positive cells, *blue* cell nuclei (color figure online)

kinases, was used as endpoint-specific control (Moors et al. 2007).

Differentiation analysis

Differentiated spheres were fixed in 4 % paraformaldehyde for 30 min at 37° C. Neurons were identified by immunocytochemical staining against β(III)-tubulin and quantified as previously described (Baumann et al. 2014). As endpoint-specific control spheres were cultured in differentiation medium with 20 ng/ml epidermal growth factor (EGF; Ayuso-Sacido et al. 2010).

Chemical preparation and exposure

A set of nine commercially available test chemicals was chosen to develop a protocol for screening chemicals over a wide concentration range (Suppl. Table 1). Six chemicals were selected based on data demonstrating adverse effects on the developing nervous system (positive substances).

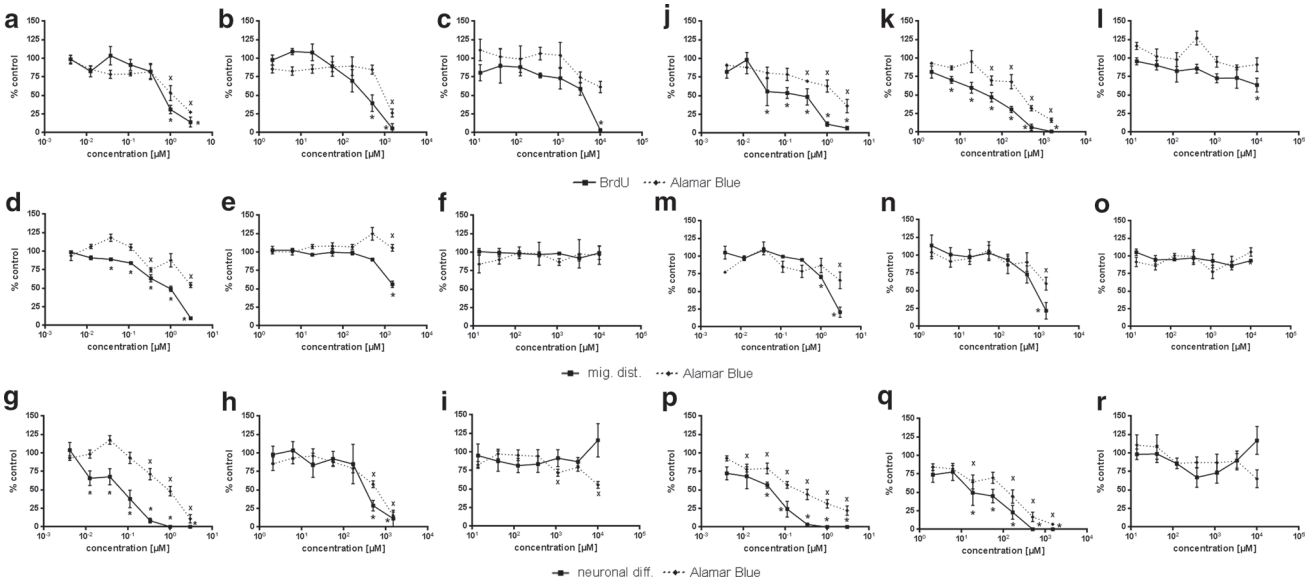


Fig. 2 Representative concentration–response curves for the endpoints proliferation, migration and neuronal differentiation. Concentration–response curves for three representative testing compounds in human (**a**–**i**) and rat neurospheres (**j**–**r**) are shown**. a**–**c**, **j**–**l** Proliferation, **d**–**f**, **m**–**o** migration, **g**–**i**, **p**–**r** neuronal differentiation. **a**, **d**, **g**, **j**, **m**, **p** MeHgCl; **b**, **e**, **h**, **k**, **n**, **q** MAM; **c**, **f**, **i**, **l**, **o**, **r** PenG. Values are given as average percentages of solvent control for the endpoints proliferation (BrdU), migration (mig. dist.) and neuronal differentiation (neuronal diff.) and the respective viability data (Alamar Blue) ± SEM (n = 3–8 independent experiments). Asterisks denote significance respect to solvent control for the endpoint proliferation/migration/neuronal differentiation, and crosses denote significance respect to solvent control for the endpoint viability ($p < 0.05$). For curves of the remaining six testing compounds and experimental details, see Supplementary Fig. 2–4

Another three chemicals were selected based on the presumed absence of data indicating effects on the developing nervous system (negative substances). For further information on chemicals, see Supplementary Material. For each experiment, stock solutions were diluted according to their starting concentration in medium (Suppl. Table 1) and serial 1:3 dilutions were prepared from this starting concentration in medium with the respective solvent concentration.

Under proliferative conditions, human and rat neurospheres were plated one sphere per well into 96-well plates in 100 µl of exposure media (proliferation medium + test compound). Four wells per exposure condition were used to assess proliferation by BrdU incorporation as well as viability. To measure cell migration or differentiation in combination with viability, five neurospheres were plated in one well of a PDL/laminin-coated eight-chamber slide under differentiating conditions. For assessment of migration, cells were exposed to chemicals for 24 h and for proliferation or differentiation analyses, the exposure duration was 72 h (Fig. 1). Each experiment was repeated at least three times on separate days and with different preparations of rat neurospheres or in case of the human neurospheres with cells of 2–3 different donors.

Statistics

Data analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). In concentration–response experiments, all data were normalized to the respective solvent control and are presented as mean percent of solvent control ± standard error of the mean (SEM). Chemical effects were determined using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Data obtained at each chemical concentration were compared to respective vehicle control, and $p \leq 0.05$ was considered significant. For the sigmoidal dose–response curve fitting and the calculation of the EC_{50} values and 95 % confidence intervals, a four-parameter logistic nonlinear regression model with the top set to 100 % and the bottom set to 0 % was used. However, in case of lacking effects of a compound on an endpoint, it was not possible to obtain curve fits with these settings. Therefore, we did not set the top and/or bottom to fixed values in those cases. Data were collected across 3–15 independent experiments with four to five neurospheres each. For pairwise comparisons, Student's t test was performed with $p \leq 0.05$ considered as significant.

Table 1 EC_{50} values and 95 % confidence intervals for the nine compounds tested in human and rat neurospheres

	BrdU		Alamar Blue (PROL)		Migration distance		Alamar Blue (MIG)		Neuronal differentiation		Alamar Blue (DIFF)	
	Human	Rat	Human	Rat	Human	Rat	Human	Rat	Human	Rat	Human	Rat
MeHgCl												
EC_{50}	0.7046	0.1301	1.134	1.678	0.6466	1.543	>3	>3	*0.05622*	*0.02955*	0.8157	0.2346
95 % CI	0.5973–0.8310	0.09460–0.1789	0.7434–1.730	1.200–2.345	0.5070–0.8245	1.404–1.696			0.04673–0.06762	0.02271–0.03847	0.7149–0.9307	0.2039–0.2701
AsNaO2												
EC_{50}	*1.728*	4.067	4.574	>10	5.749	>10	>10	>10	3.203	*0.4061*	7.360	>10
95 % CI	1.167–2.560	3.453–4.791	3.469–6.032		4.480–7.377				2.515–4.079	0.2860–0.5766	6.942–7.803	
CPF												
EC_{50}	>213.9	*28.54*	>213.9	>213.9	>213.9	>213.9	>213.9	>213.9	*140.5*	48.56	>213.9	>213.9
95 % CI		20.44–39.83							112.7–175.2	30.49–77.32		
Parathion												
EC_{50}	>257.5	>257.5	>257.5	>257.5	>257.5	>257.5	>257.5	>257.5	*252.5*	>257.5	>257.5	*251.2*
95 % CI												
MAM												
EC_{50}	*325.7*	*31.82*	1245	247	>1500	803.6	>1500	>1500	*344.5*	*23.47*	648.9	82.75
95 % CI	277.3–382.5	26.93–37.61	1145–1353	192.7–316.5		706.5–914.2			297.7–398.7	19.11–28.82	606.5–694.3	66.61–102.8
NaVPA												
EC_{50}	*756.3*	*379.5*	>11,250	4019	>11,250	7625	>11,250	9729	3177	*321.1*	2399	1903
95 % CI	547.5–1045	356.7–403.8		3811–4238		5911–9836		6597–14,349	2009–5026	252.2–408.8	1911–3011	1390–2606
Glutamate												
EC_{50}	*1938*	5122	>10,000	>10,000	>10,000	7663	>10,000	>10,000	>10,000	*374.6*	>10,000	8655
95 % CI	1514–2479	3894–6737				4883–12,025				222.3–631.3		5535–13,532
Paracetamol												
EC_{50}	*2219*	790.9	3884	4113	>5000	>5000	3693	>5000	>5000	*399.1*	3617	1538
95 % CI	1859–2648	686.4–911.2	3370–4476	3516–4812			3118–4373			285.1–558.6	2708–4832	1073–2205
PenG												
EC_{50}	*2512*	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
95 % CI	1598–3948											

Values are given in micromolar concentrations. The lowest obtained EC_{50} value for each species and compound is marked in italic

Results

For chemical testing in human and rat NPCs, we developed a testing scheme in which neurospheres were mechanically dissociated by chopping 3 days prior to plating in order to obtain a defined and uniform sphere population. Under proliferative conditions, floating neurospheres were exposed to testing chemicals for 3 days and afterward assessed for changes in proliferation and viability. Under differentiating conditions, neurospheres plated on laminin-coated surfaces were exposed to testing chemicals for 24 h to assess migration by measuring migration distances and viability, and for evaluating neuronal differentiation, spheres were exposed for 3 days to analyze the neuronal marker βIII-tubulin and viability (Fig. 1). This experimental setup allows: (1) a distinction of specific chemical effects on neurodevelopmental endpoints and viability and (2) a direct comparison of such between human and rat NPCs.

The usage of endpoint-specific controls is one important criterion for the development of alternative methods for chemical screening (Crofton et al. 2011). Therefore, we established control chemicals that reliably change the respective endpoint to a certain amount without reducing viability. Proliferation was inhibited by growth factor withdrawal, which reduced BrdU luminescence from 190,238 ± 20,102 RLU to 50,673 ± 18,312 RLU in hNPCs, and from 64,534 ± 13,155 RLU to 23,307 ± 6242 RLU in rNPCs (Suppl. Fig. 1a), respectively, whereas no cytotoxicity was detected by LDH assay (Suppl. Fig. 1b). The Src kinase inhibitor PP2 reduced migration distances (Moors et al. 2007) in hNPCs from 404 ± 12 to 247 ± 12 µm 24 h after plating, and from 456 ± 39 to 73 ± 18 µm in rNPCs, respectively (Suppl. Fig. 1c). Again, viability was not reduced (hNPCs) or reduced to a lesser extent than migration (rNPCs, Suppl. Fig. 1d). EGF was used to inhibit neuronal differentiation (hNPCs: 9.6 ± 0.6–1.9 ± 0.3 % neurons; rNPCs: 15.2 ± 1.6–0.6 ± 0.3 % neurons) without being cytotoxic (Suppl. Fig. 1e and f).

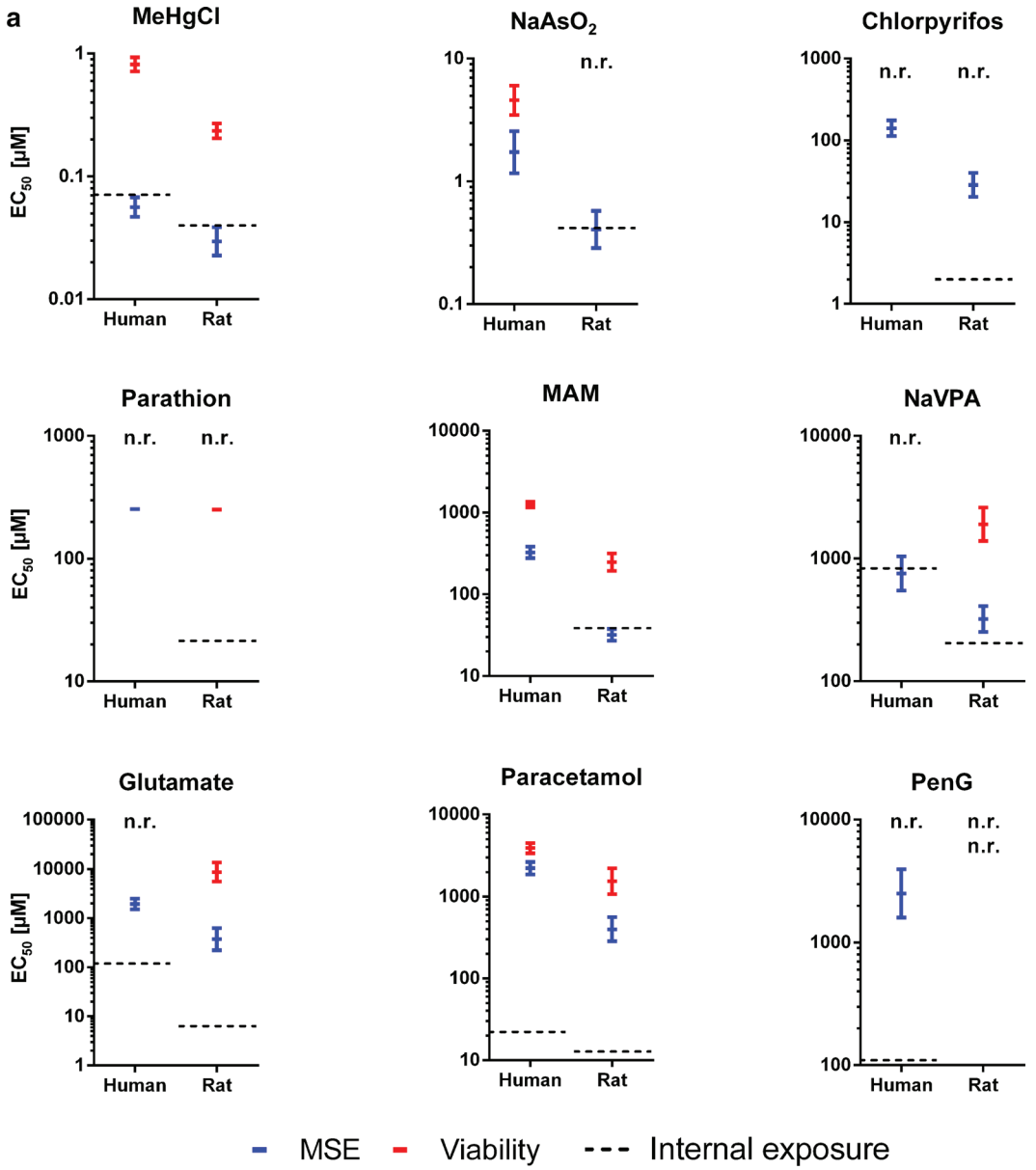
Next, we tested a training set of six positive and three negative compounds (Suppl. Table 1) for their effects on proliferation, migration and neuronal differentiation in human and rat NPCs (Fig. 2; Suppl. Fig. 2–4). For every endpoint and chemical, concentration–response curves were recorded and EC_{50} values with their corresponding 95 % confidence intervals were calculated after performing a sigmoidal dose–response curve fitting (Table 1; Suppl. Fig. 5–7). Because we assume that disturbance of any neurodevelopmental key event will cause an adverse neurodevelopmental outcome, the most sensitive endpoint (MSE) for every chemical and species was determined and compared to its corresponding EC_{50} value for viability (Fig. 3a) to decide whether specific effects on proliferation, migration or neuronal differentiation can be distinguished from general cytotoxicity (Crofton et al. 2011). Moreover, the EC_{50} values for the MSE for each compound within each species regardless of the nature of the endpoint determined the more sensitive species.

Fig. 3 Pairwise comparison of the most sensitive endpoint and viability between human and rat neurospheres. **a** EC_{50} values of the most sensitive endpoint (MSE) and viability in human and rat neurospheres for each testing compound are shown with its 95 % confidence intervals and, if available, internal exposure levels of humans and rats. MSEs and estimated or measured internal exposures are as follows: MeHgCl—neuronal differentiation (hNPCs and rNPCs), brain concentration (hNPCs and rNPCs); $NaAsO_2$—proliferation (hNPCs) and neuronal differentiation (rNPCs), estimated brain concentration (rNPCs); chlorpyrifos—neuronal differentiation (hNPCs) and proliferation (rNPCs), brain concentration (rNPCs); parathion—neuronal differentiation (hNPCs), brain concentration (rNPCs); MAM—proliferation (hNPCs and rNPCs), brain concentration (rNPCs); NaVPA—proliferation (hNPCs) and neuronal differentiation (rNPCs), brain concentration (hNPCs and rNPCs); glutamate—proliferation (hNPCs) and neuronal differentiation (rNPCs), plasma level (hNPCs) and brain concentration (rNPCs); paracetamol—proliferation (hNPCs) and neuronal differentiation (rNPCs), CSF concentration (hNPCs and rNPCs); PenG—proliferation (hNPCs) and CSF concentration (hNPCs). n.r. = EC_{50} not reached within the tested concentration range. **b** EC_{50} values of MeHgCl for MSEs in human and rat neurospheres are applied in a parallelogram approach. Therefore, existing rat in vivo data are compared to rat in vitro data to illustrate in vivo–in vitro similarities/differences. Rat in vitro data are compared with human in vitro data to obtain information regarding interspecies differences. All these data will then allow an extrapolation of possible effects in humans in vivo. *Green* experimental data, *red* extrapolation (color figure online)

The MSE after NPC exposure toward MeHgCl was neuronal differentiation (hNPCs: 56.22 nM; rNPCs: 29.55 nM), with viability affected in both species at a higher order of magnitude (hNPCs: 815.7 nM; rNPCs: 234.6 nM; Fig. 2; Table 1). Confidence intervals (95 %) of EC_{50} values for the MSE and viability did not overlap in either rat or human NPCs, showing that MeHgCl specifically inhibited neuronal differentiation. Moreover, 95 % confidence intervals for the MSE in human and rat NPCs did not overlap either, demonstrating the higher sensitivity of rat versus human NPCs toward MeHgCl exposure.

Upon $NaAsO_2$ treatment, hNPC proliferation was the MSE (EC_{50} = 1.728 µM; Suppl. Fig. 2; Table 1), whereas neuronal differentiation was inhibited most potently in rNPC (EC_{50} = 0.4061 µM, Suppl. Fig. 4; Table 1). EC_{50} values for viability were either higher than the MSE (human: 4.574 µM) or not reached at all (rat; Suppl. Fig. 2 and 4; Table 1), supporting specific DNT effects of $NaAsO_2$. However, with regards to the respective MSE, rNPCs were more sensitive than hNPCs.

The EC_{50} value for chlorpyrifos was only reached for the endpoint neuronal differentiation in hNPCs, although the curve for viability was mostly overlapping (140.5 µM;


a
MeHgCl
NaAsO2
Chlorpyrifos
Parathion
MAM
NaVPA
Glutamate
Paracetamol
PenG
EC50 [µM]
n.r.
Human
Rat
MSE
Viability
Internal exposure
b
Rat in vitro
0.03 µM
Human in vitro
0.06 µM
MeHgCl
Rat in vivo
0.03 µM
Human in vivo
0.07 µM

Suppl. Fig. 4; Table 1). In contrast, proliferation was the MSE in rNPCs (28.54 µM; Suppl. Fig. 2; Table 1) with the EC_{50} value for viability not reached. Thus, within the endpoints studied, rNPCs were the more sensitive species toward chlorpyrifos.

Parathion only impaired the endpoint neuronal differentiation in hNPCs (252.5 µM) and viability under differentiating conditions in rNPCs (251.2 µM; Suppl. Fig. 4; Table 1). Looking at the concentration–response curves for hNPCs, it is likely that parathion did specifically impair neuronal differentiation and although the EC_{50} value for the endpoint migration was not reached, the highest concentration tested (257 µM) significantly reduced migration (Suppl. Fig. 3).

MAM inhibited both proliferation and neuronal differentiation in human and rat NPCs at similar potencies. However, proliferation was chosen as MSE (human EC_{50} = 325.7 µM, rat EC_{50} = 31.82 µM) as effects between proliferation and viability deviated most for both species (human EC_{50} = 1245 µM, rat EC_{50} = 247 µM; Fig. 2; Table 1). Rat NPCs were found to be more vulnerable toward MAM-induced reduction in proliferation than hNPCs.

hNPC proliferation was specifically inhibited by NaVPA (EC_{50} = 756.3 µM, Suppl. Fig. 2; Table 1) without affecting viability (EC_{50} not reached within tested concentration range). In contrast, NaVPA reduced proliferation and neuronal differentiation in rNPCs at similar concentrations (EC_{50} = 379.5 µM and EC_{50} = 321.1 µM, respectively) distinguishable from effects on viability (EC_{50} = 4019 µM and EC_{50} = 1903 µM, respectively; Suppl. Fig. 2 and 4; Table 1). The MSE of rNPCs was more sensitive than hNPCs.

Exposure to sodium glutamate revealed an inhibition of proliferation as only specifically affected endpoint in hNPCs with an EC_{50} value of 1938 µM (Suppl. Fig. 2; Table 1). In rNPCs, neuronal differentiation was specifically inhibited at lower concentrations (EC_{50} = 374.6 µM, viability: EC_{50} = 8655 µM; Suppl. Fig. 4; Table 1), making the rat again the more sensitive species.

Paracetamol specifically inhibited proliferation in hNPCs (EC_{50} = 2219 µM, viability: EC_{50} = 3884 µM; Suppl. Fig. 2; Table 1). rNPCs were more sensitive than human ones, and the endpoint neuronal differentiation was most sensitive and specifically inhibited in the rat (EC_{50} = 399.1 µM, viability: EC_{50} = 1538 µM; Suppl. Fig. 4; Table 1).

Last, penicillin G only had a specific effect on proliferation in hNPCs (EC_{50} = 2512 µM), whereas in rNPCs the EC_{50} value was not reached for any of the endpoints (Fig. 2; Table 1) although the highest concentration (10,000 µM) significantly reduced proliferation as well.

Discussion

During the last decade, when the toxicological paradigm shift toward more mechanism- and pathway-driven approaches for human hazard and risk assessment has been evolving, also alternative assay development for DNT testing has gained priority within the regulatory environment (Bal-Price et al. 2015a). This is mainly due to the enormous resource intensity of the DNT guideline studies and their high variability supported by the overall dissatisfactory prediction of animals to humans (Leist and Hartung 2013). As one approach to DNT in vitro testing, we developed a 3D cell culture model based on primary human and rat NPCs grown as neurospheres (Baumann et al. 2014; Moors et al. 2009). According to general recommendations for alternative methods development (Crofton et al. 2011), here we demonstrate that: (1) The neurosphere assay can be used to determine concentration–response effects of a training set of chemicals on key events of neurodevelopment (proliferation, migration and neuronal differentiation) in a species-specific manner (Fig. 2), (2) by using endpoint-specific controls, key events can reliably and consistently be modulated (Suppl. Fig. 1), (3) this experimental setup enables a determination of the respective endpoint multiplexed with viability to distinguish specific chemical actions on neurodevelopmental key events from secondary effects due to cell death (Figs. 2, 3), and (4) data cannot be interpreted on a pure hazard basis but need exposure data for correct chemical classification (Fig. 3).

Species differences entail an important issue for regulators in pharmacology and toxicology as the predictive value of animal experiments for effects in humans is often poor (Leist and Hartung 2013). By directly comparing chemical effects on neurodevelopmental key events of rat and human neurospheres generated from equivalent developmental time points (Clancy et al. 2007), species differences based on cellular toxicodynamics can be tackled. Our study shows that rat and human NPCs differ in their susceptibility to almost all of the chemicals tested. For this set of compounds, rNPCs respond overall at lower concentrations than hNPCs (Table 1; Fig. 3a). As this compound set is rather small, no general conclusion can be drawn from these data on general species-specific sensitivity of NPCs from humans and rats. Testing of more compounds with different modes of action (MOA) is rather needed to get a more detailed view on pathway-specific sensitivities across these species. Moreover, this data set suggests that neuronal differentiation might be the MSE in rNPCs, while this seems to be NPC proliferation for hNPCs. This conclusion would also be premature due to the small number of compounds in this training set and more compound testing will reveal if at all such a general assumption can be made.

Species differences in sensitivity toward DNT chemicals have sparsely been evaluated so far. Differences were found for compound-compromised neurite outgrowth in human ESC-derived neural cultures and rat cortical cultures (Harrill et al. 2011) as well as for chemically induced reduction in NPC proliferation and migration in primary human versus mouse cultures (Gassmann et al. 2010). Given the fact that molecular equipment of the human developing brain seems to contain unique features in the animal kingdom (Somel et al. 2011; Zhang et al. 2011), it seems necessary to understand human-specific developmental toxicity of compounds to this sensitive organ. Such information combined with MOA analyses of chemicals can provide information on molecular and functional differences between rodents and humans which can be applied in a quantitative way to determine whether the animal data have any relevance to humans and whether interspecies uncertainty factors need to be adjusted (Burgess-Herbert and Euling 2013).

One way of determining whether these hazards are at all relevant to human health is implementation of exposure. Such an approach was already proposed for in vitro developmental toxicity testing (Daston et al. 2010) and successfully applied for in vitro testing for endocrine disruption (Rotroff et al. 2014). Moreover, Rotroff et al. (2010) combined human oral exposure levels with in vitro AC_{50} values of the ToxCast assays for a subset of 35 ToxCast chemicals to incorporate human dosimetry and exposure into high-throughput in vitro toxicity testing. Accordingly, we compared the experimentally assessed human and rat EC_{50} values from this study to in vivo internal exposure levels of the nine testing chemicals in humans and rats for a comparative risk assessment according to the parallelogram approach (Fig. 3b). Due to the lack of information on precise MOA of DNT compounds, this comparative in vitro–in vivo approach is imperfect; for example, the key event neurogenesis is hardly studied in vivo. Because neurogenesis was the most sensitive endpoint for many of the compounds tested in this training set in rat neurospheres, we chose data on cognitive in vivo endpoints as the adverse outcome (AO) if no other data were available and correlated AO LOAELs with EC_{50} values for functional endpoints studied in vitro. This instance highlights the need for more mechanistic data on DNT compounds for a comprehensive correlation of in vivo and in vitro effects.

Prenatal MeHgCl exposure causes mental retardation and developmental delays in children (Grandjean and Landrigan 2006). Neuropathological examinations showed microcephaly and global brain disorganization due to disturbances in cell migration and division (Schettler 2001). Likewise, hNPC proliferation and migration were specifically inhibited by MeHgCl in vitro, but the most sensitive endpoint was neuronal differentiation with an EC_{50} value of 56 nM. In vivo studies revealed that a maternal hair concentration of 4.5 ppm MeHgCl as the lowest observed adverse effect level (LOAEL) found in the literature results in neuropsychological deficits in children (Castoldi et al. 2001). According to toxicokinetic calculations (Burbacher et al. 1990; Lewandowski et al. 2003), this hair concentration should resemble an infant brain concentration of approximately 72 nM. In rats, prenatal low-dose administrations of 0.01 mg/kg MeHgCl from gestational day (GD) 6 to 9, which are estimated to result in maximal fetal brain concentrations of 30 nM (Burbacher et al. 1990; Lewandowski et al. 2003), affected learning behavior in the progeny (Bornhausen et al. 1980). Similarly, rNPC proliferation, migration and neuronal differentiation were affected at subcytotoxic concentrations, whereas neuronal differentiation was most sensitive ($EC_{50} = 30$ nM). Arranging experimentally obtained in vitro and calculated internal in vivo concentrations in a parallelogram demonstrates a good correlation between in vitro and in vivo concentrations for both species (Fig. 3b). A similar approach was carried out by Lewandowski et al. (2003) who summarized that rat neuroblast proliferation in vitro and in vivo was inhibited at similar orders of magnitude (approx. 1 µM (Ponce et al. 1994) and 3 µM (Chen et al. 1979) MeHgCl, respectively). Our data for rNPC proliferation are in good agreement with these historical in vitro data (Table 1). However, proliferation was not the MSE for MeHgCl in this study and to the best of our knowledge effects of MeHgCl on neuronal differentiation in vivo has not been studied so far.

The pesticide chlorpyrifos was recently added to the group of human developmental neurotoxicants based on evidence from epidemiological studies (Grandjean and Landrigan 2014). In hNPCs, chlorpyrifos affected neuronal differentiation with an EC_{50} value of 141 µM in a rather nonspecific way as concentration–response curves for neuronal differentiation and viability overlapped. In a prospective cohort study examining early childhood development after prenatal exposure to chlorpyrifos, altered attention was detected in highly exposed children. Cord blood concentrations with a LOAEL of 6.17 pg/g were measured (Rauh et al. 2006), translating to a concentration of 18 pM. Although children's brain concentrations were not calculated, it is obvious that the experimentally derived results from hNPCs in vitro are far from any in vivo relevance. In rNPCs, proliferation was specifically inhibited with an EC_{50} value of 29 µM. Similarly, an administration of 1 mg/kg chlorpyrifos between PND 1 and 4 decreased DNA synthesis in the brain (Dam et al. 1998). According to pharmacokinetic modeling, this dose would result in a brain concentration of 2.1 µM (Timchalk et al. 2006), which is around 10 times lower than the effective in vitro concentration inhibiting rat NPC proliferation. Thus, human and rat NPCs failed to predict the DNT potential of chlorpyrifos

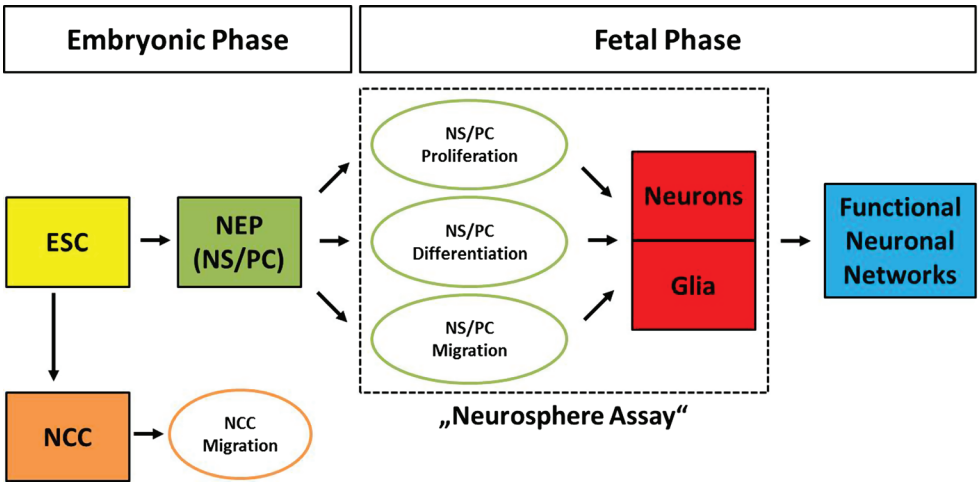


Fig. 4 Testing strategy for in vitro DNT testing. The assessment of different early and late neurodevelopmental key events provides a comprehensive approach for developmental neurotoxicity testing. Thereby, the endpoints evaluated within the neurosphere assay integrate into early fetal development. *ESC* embryonic stem cell, *NCC* neural crest cell, *NEP* neuroepthelial precursor cell, *NS/PC* neural stem/progenitor cell

correctly as effects were not seen unless toxicologically irrelevant concentrations were applied. This could be due to lack of cytochrome P450 metabolism in developing brain cells (Gassmann et al. 2010; Jiang et al. 2010), chlorpyrifos acting on earlier phases of brain development, on later neurodevelopmental endpoints such as axon and dendrite formation and synaptogenesis (Howard et al. 2005; Yang et al. 2008) or in an indirect way, e.g., involving neuroinflammation, which cannot be assessed with this assay.

MAM disturbs central nervous system development during the fetal and neonatal period (Cattabeni and Di Luca 1997). It mainly acts through an inhibition of proliferation and affects developing neurons through DNA alkylation (Kisby et al. 2009). In line with this, the endpoints proliferation and neuronal differentiation were specifically inhibited in both human and rat NPCs at concentrations of 326–345 µM (human) and 23–32 µM (rat). Although developmental MAM exposure through contaminated cycad flour is strongly linked to neurological disorders in the Western Pacific (Spencer et al. 1991), there are no reliable data available on human exposure levels. However, in rats an administration of 7.5 mg/kg between GD 13 and 15 caused substantial changes in brain morphology (De Groot et al. 2005). According to a study of Bassanini et al. (2007), such a dose probably results in a fetal brain concentration of 30 µM, which is very similar to the effective concentrations in our rat in vitro results (EC_{50} = 31.82 µM; Table 1), demonstrating that rNPCs were able to predict the actual risk of MAM properly.

The antibiotic penicillin G was used as a negative DNT compound in this study. Penicillin inhibited proliferation of only hNPCs at high concentrations (2512 µM). Therapeutic plasma and CSF concentrations are several orders of magnitude lower than the effective concentration measured in the hNPC in vitro system (111 µM and 2.4 µM, respectively; Karlsson et al. 1996). Thus, this compound is classified correctly as a negative substance with regard to health risk. For further discussion of the remaining five test chemicals, see Supplementary Discussion.

Taking species-specific human and rat internal exposure levels into account, four out of six DNT-positive compounds and all three negative compounds were classified correctly by assessing the four endpoints viability, NPC proliferation, migration and neuronal differentiation using human and rat NPCs (Fig. 3a). For the data-rich compounds MeHgCl and NaVPA, a comprehensive risk assessment according to the parallelogram approach was possible and revealed that for both species in vivo and in vitro concentrations correlated well with disturbance of neurodevelopmental endpoints in vivo (Supplementary discussion, Suppl. Fig. 8). This supports the hypothesis articulated earlier that neurodevelopmental processes as key events of brain development can be mimicked in vitro and might serve as the basis for alternative DNT testing strategies in vitro (Lein et al. 2005). For arsenic and MAM, only rat internal exposure concentrations were available so that a conclusive assessment of hNPC data was not feasible. Due to the good correlations of the available rat in vivo and in vitro data on those two compounds, a correct classification of arsenic and MAM based on human NPC data is thus likely. This example demonstrates that human toxicokinetic modeling to estimate internal exposure levels has utmost importance for a comprehensive decision-making process if in vitro results are implemented (Croom et al. 2015; Patlewicz et al. 2015).

In contrast to the correctly identified DNT compounds, the two pesticides chlorpyrifos and parathion were not correctly classified as DNT-positive compounds in the human and rat neurosphere assay as EC_{50} values exceeded their estimated effective internal exposure levels. This might be due to the reasons discussed above. Specifically, chlorpyrifos seems to inhibit axonal growth and induce dendritic growth in primary rat neuronal cultures at nanomolar concentrations or below (Howard et al. 2005). This clearly

indicates that it is very important to define the biological application domain of each in vitro system to determine which MOA it is able to assess and especially where its limitations lie. This knowledge is necessary to gain certainty about its use in a regulatory context (Bal-Price et al. 2015a).

All three DNT-negative compounds affected NPC development at toxicologically absolutely irrelevant concentrations, demonstrating that human and rat NPCs were able to detect negative compounds correctly.

One reason why appropriate test concentrations are of high importance in such physiologically relevant organoids consisting of primary cells might be the correct homeostasis of cellular components in these cells resembling the in vivo situation. Two notions support this assumption. For one, ex vivo NPCs seem to maintain their properties after taking them out of the whole organism, which was shown by compound effects in in vivo–ex vivo comparisons (Foti et al. 2013; Go et al. 2012; L'Episcopo et al. 2013). Secondly, the 3D format of cultures with cell–cell communication and interaction supports physiological cellular functions and thus in vivo-relevant responses toward xenobiotics (Alépée et al. 2014; Yamada and Cukierman 2007). Thus, we expected neurospheres to react only at compound concentrations relevant for interfering with signaling pathways necessary for the tested endpoints. That such a physiological context of primary cells has a strong implication on in vitro testing has recently also been shown by Kleinstreuer et al. (2014). In this very elegant work, the authors did not identify VPA as an HDAC inhibitor and they discussed that this is probably due to insufficient test concentrations of this drug (40 μM), which is pharmacologically active in the mM range.

In summary, results of a training set of nine chemicals in human and rat NPCs revealed that species differed in their sensitivity to most chemicals. A comparison of rat and human in vivo internal exposure levels and in vitro results seem to correlate well for compounds where data are available. Due to insufficient information, however, such a comparison could not be made for all compounds. In combination with assays that have the ability to assess chemical effects on early neurodevelopment and methods evaluating further key events needed for proper neuronal network formation (e.g., axon, dendrite, spine, synapse formation, neuronal network activity), the neurosphere assay is a valuable tool for DNT testing (Fig. 4). Because we have previously shown that the throughput of our assay can be increased by automation of neurosphere sorting and plating (Gassmann et al. 2012), this method renders useful for medium-throughput applications. High-content image analysis methods are on the way to further facilitate evaluation of such complex, multi-cellular structures. Data from such testing strategies can then be integrated into the 'Adverse Outcome Pathway' (AOP) framework (Ankley et al. 2010; Bal-Price et al. 2015b) and will help to develop so called 'Integrated Approaches to Testing Assessment' (IATA), which gather and weigh any existing relevant information—in vivo, in vitro, in silico and in chemico—to support regulatory or safety decisions (Tollefsen et al. 2014).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The animals used for NPC preparation were maintained in an accredited on-site testing facility according to the guideline provided by the Society for Laboratory Animals Science (GV-SOLAS). They were treated humanely and with regard for alleviation of suffering. NPC preparation was approved by the North Rhine-Westphalia State Environment Agency.

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Electronic supplementary material

Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

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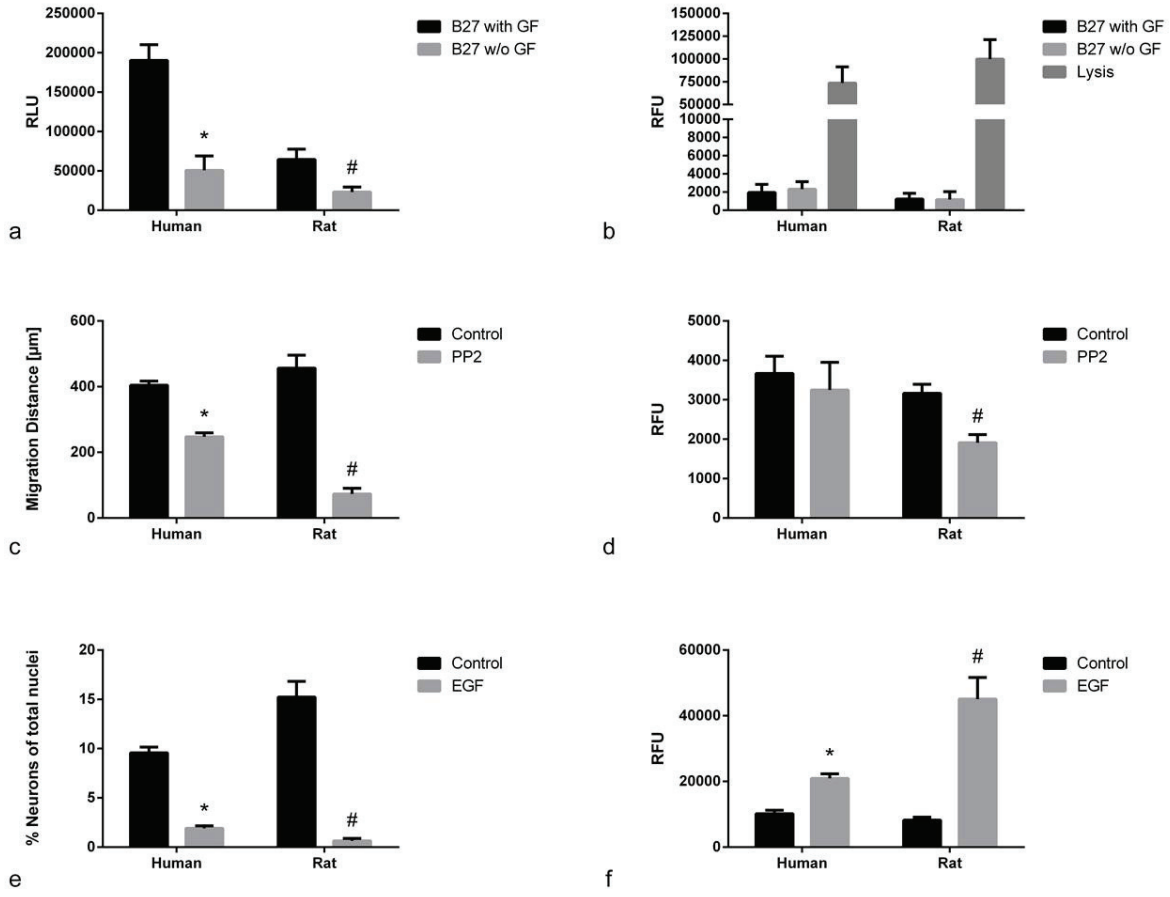
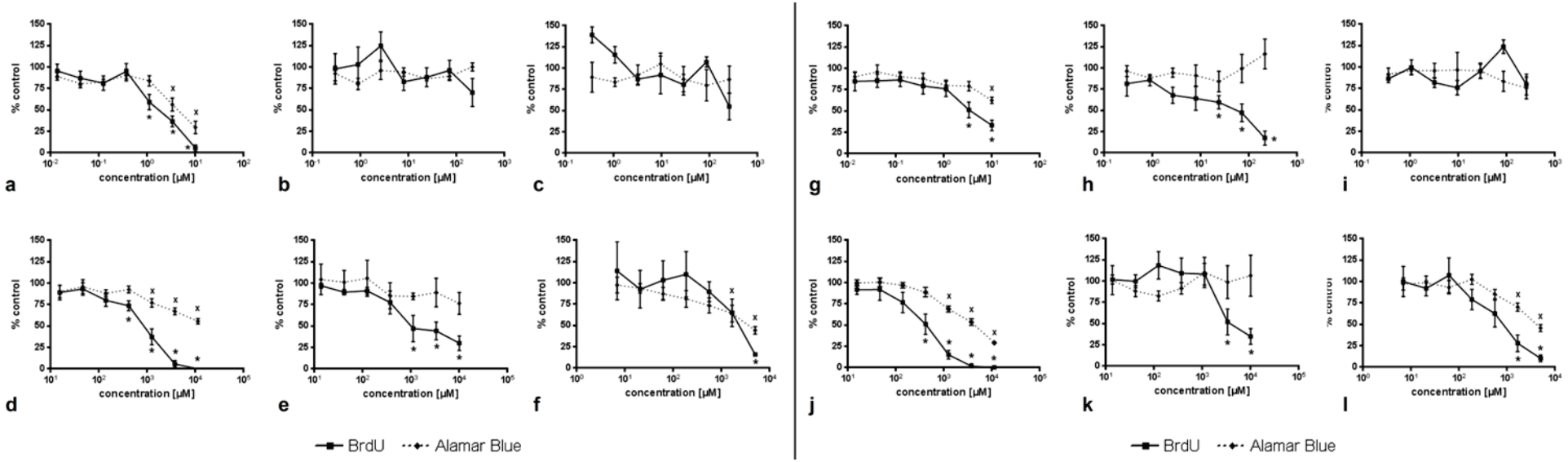
This file contains Supplementary Figures for human and rat endpoint specific controls (Fig. S1), concentration response curves for the endpoints proliferation (Fig. S2), migration (Fig. S3) and neuronal differentiation (Fig. S4) of the chemicals $NaAsO_2$, Chlorpyrifos, Parathion, Sodium Valproate, Monosodium Glutamate and Paracetamol. Additionally, Fig. S5-S7 show sigmoidal dose-response curve fits for the endpoints proliferation (Fig. S5), migration (Fig. S6) and neuronal differentiation (Fig. S7) for all chemicals. Moreover, a parallelogram approach which was applied to the data of sodium valproate is shown in Fig. S8. Supplementary Table 1 lists all chemical compounds of the training set used in this study. In addition, a Supplementary Materials and Methods section as well as a Supplementary Discussion is provided. References used in the Supplementary Discussion are listed at the end of this document as Supplementary References.

Fig. S1:



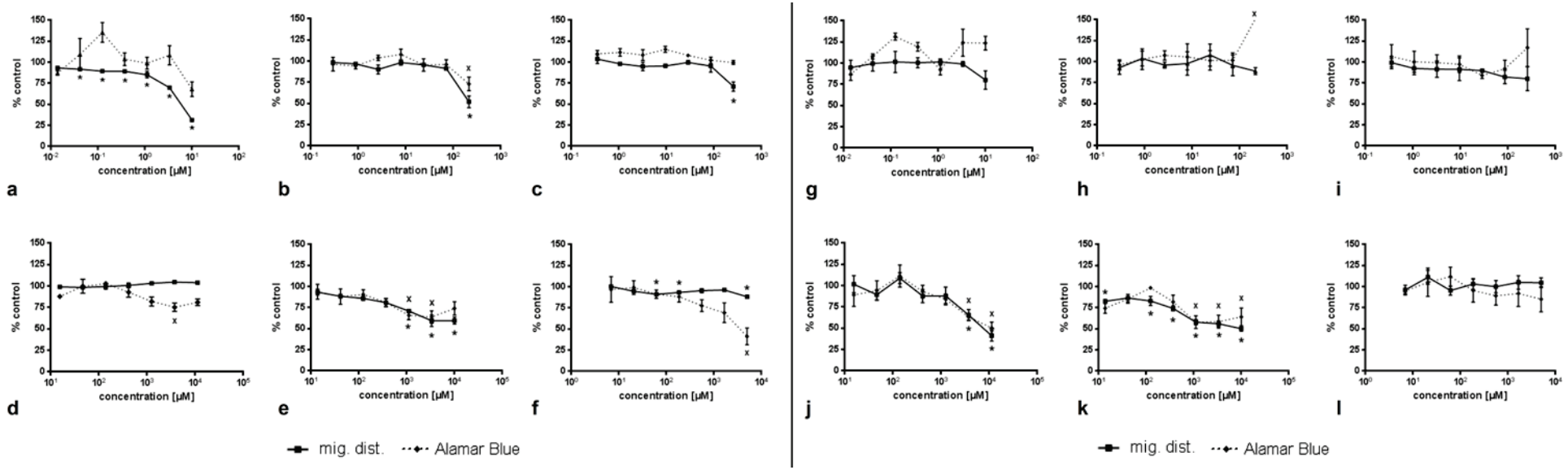
Supplementary Fig. 1 Control of general assay performance in the "Neurosphere Assay" with endpoint-specific controls. **a. and b.** Four human or rat neurospheres were plated with one floating sphere per 96-well in presence (B27 with GF) or absence of growth factors (B27 w/o GF). After 3 days **a.** proliferation was assessed by BrdU Assay and **b.** cytotoxicity was assessed by LDH Assay. Complete cell lysis by Triton X was used as a cytotoxicity control. Values are given in average of relative luminescence (RLU) or fluorescence (RFU) values ± SEM (n = 4 independent experiments). **c. and d.** Five human or rat neurospheres were plated in a PDL/Laminin coated chamber of an 8-well chamber slide in control media or in presence of 10 µM PP2. After 24 hrs phase contrast pictures of migrated spheres were taken. **c.** Migration distance was measured and **d.** viability was assessed by Alamar Blue Assay. Values are given in average migration distances in µm (**c.**) or RFU (**d.**) values ± SEM (n = 4 independent experiments), respectively. **e. and f.** Five human or rat neurospheres were plated in a PDL/Laminin coated 8-well chamber in control media or in presence of 20 ng/mL EGF. After 3 days **e.** cells were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst and **f.** viability was assessed by Alamar Blue Assay. Immunofluorescent pictures were taken and neurons were quantified by manual counting. Values are given in percentages of neurons to total nuclei (**e.**) or RFU (**f.**) values ± SEM (n = 4 independent experiments). Asterisks and hashs denote significance in respect to controls in hNPCs and rNPCs, respectively ($p < 0.05$)

Fig. S2:



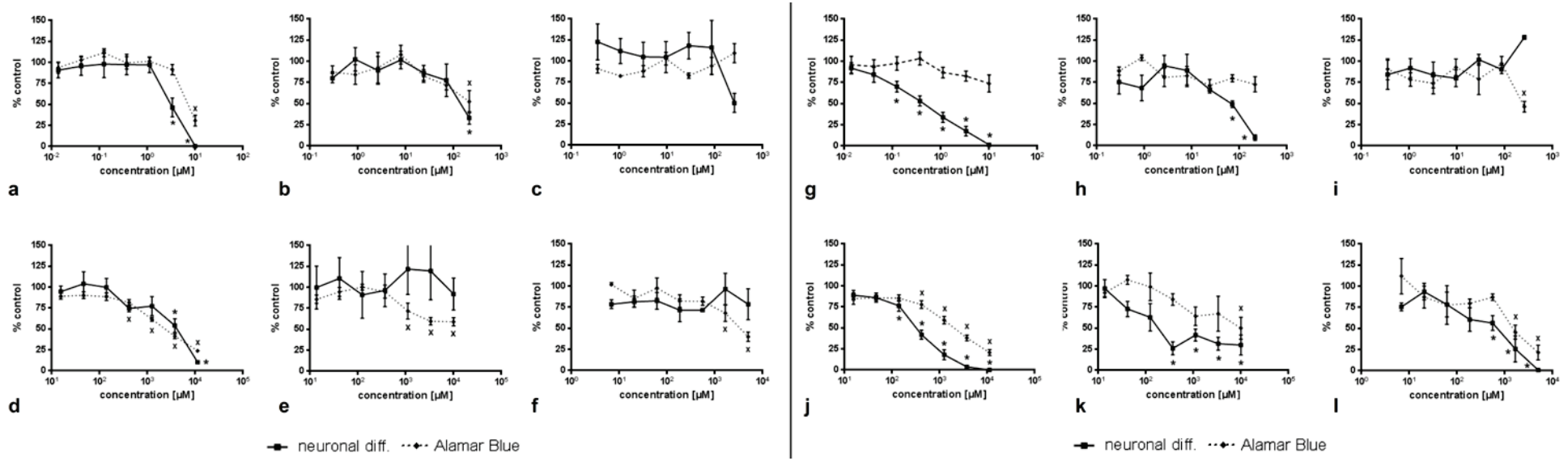
Supplementary Fig. 2 Proliferation assay. Human (**a-f**) and rat neurospheres (**g-l**) were exposed to six testing compounds (**a and g**: $NaAsO_2$; **b and h**: CPF; **c and i**: parathion; **d and j**: NaVPA; **e and k**: glutamate; **f und l**: paracetamol) with 7 concentrations per compound. Therefore, 4 floating spheres per exposure group were plated one sphere per 96-well for 3 days. At the end of exposure time, proliferation and viability were assessed by the BrdU and the Alamar Blue Assay, respectively. Concentration response curves for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints proliferation (BrdU) and viability (Alamar Blue) ± SEM (n = 3-13 independent experiments). Asterisks denote significance respect to solvent control for the endpoint proliferation and crosses denote significance respect to solvent control for the endpoint viability ($p < 0.05$)

Fig. S3:



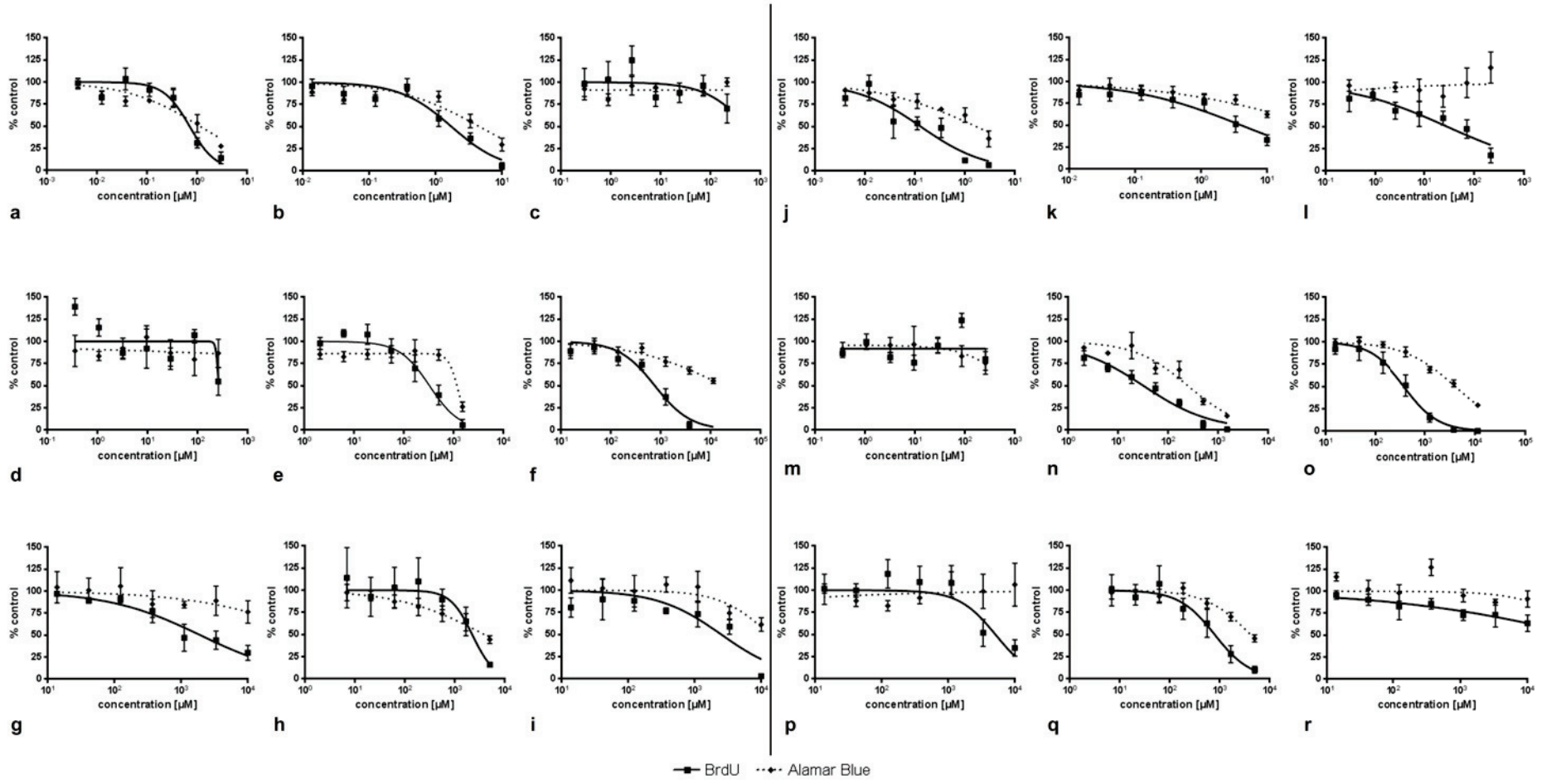
Supplementary Fig. 3 Migration assay. Human (**a-f**) and rat neurospheres (**g-l**) were exposed to six testing compounds (**a and g**: $NaAsO_2$; **b and h**: CPF; **c and i**: parathion; **d and j**: NaVPA; **e and k**: glutamate; **f und l**: paracetamol) with 7 concentrations per compound. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide 96-well for 24 hrs. At the end of exposure time, phase contrast pictures were taken for measuring migration distances and viability was monitored by the Alamar Blue Assay. Concentration response curves for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints migration (mig. dist.) and viability (Alamar Blue) ± SEM (n = 3 independent experiments). Asterisks denote significance respect to solvent control for the endpoint migration and crosses denote significance respect to solvent control for the endpoint viability ($p < 0.05$)

Fig. S4:



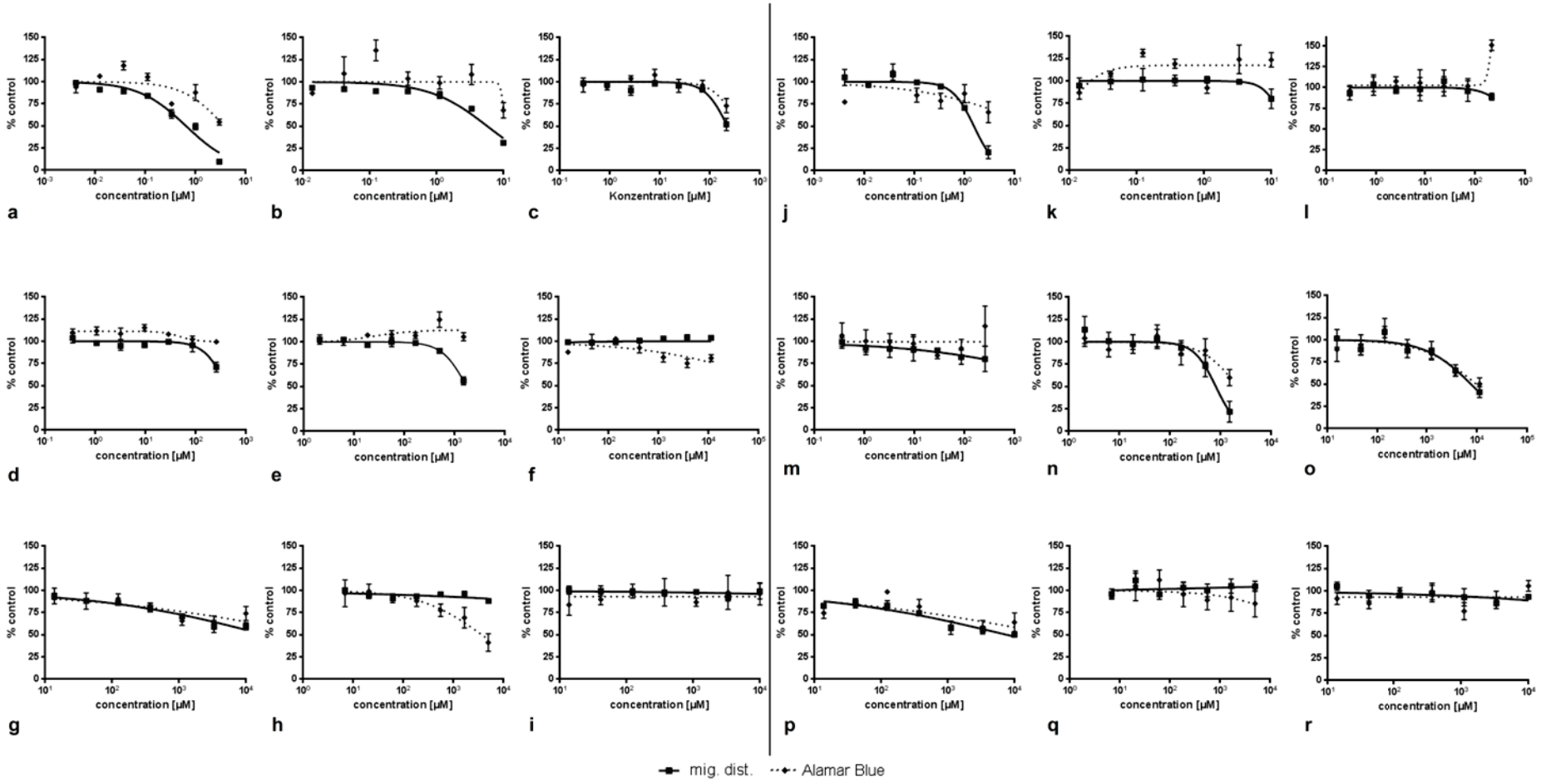
Supplementary Fig. 4 Neuronal differentiation assay. Human (**a-f**) and rat neurospheres (**g-l**) were exposed to six testing compounds (**a and g**: $NaAsO_2$; **b and h**: CPF; **c and i**: parathion; **d and j**: NaVPA; **e and k**: glutamate; **f und l**: paracetamol) with 7 concentrations per compound. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide for 3 days. At the end of exposure time, viability was monitored by the Alamar Blue Assay and neurospheres were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst. Immunoflourescent pictures were taken and neurons were quantified by manual counting. Concentration response curves for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints neuronal differentiation (neuronal diff.) and viability (Alamar Blue) ± SEM (n = 3-15 independent experiments). Asterisks denote significance respect to solvent control for the endpoint neuronal differentiation and crosses denote significance respect to solvent control for the endpoint viability ($p < 0.05$)

Fig. S5:



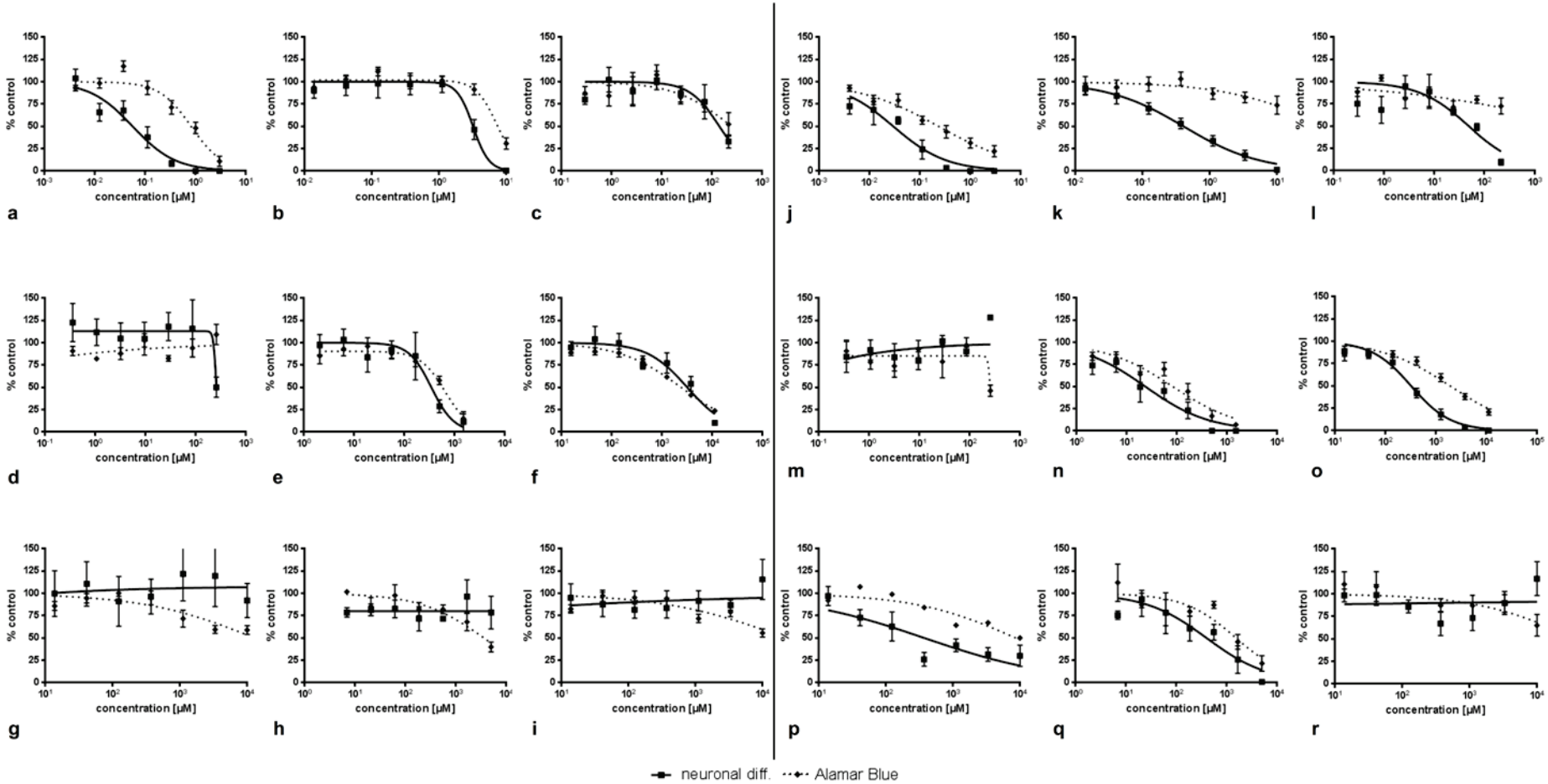
Supplementary Fig. 5 Curve Fits of proliferation assay. Human (**a-i**) and rat neurospheres (**j-r**) were exposed to nine testing compounds (**a and j**: MeHgCl; **b and k**: $NaAsO_2$; **c and l**: CPF; **d and m**: parathion; **e and n:** MAM; **f and o**: NaVPA; **g and p**: glutamate; **h und q**: paracetamol; **i and r:** PenG) with 7 concentrations per compound. Therefore, 4 floating spheres per exposure group were plated one sphere per 96-well for 3 days. At the end of exposure time, proliferation and viability were assessed by the BrdU and the Alamar Blue Assay, respectively. Sigmoidal dose response curve fits for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints proliferation (BrdU) and viability (Alamar Blue) ± SEM (n = 3-13 independent experiments)

Fig. S6:



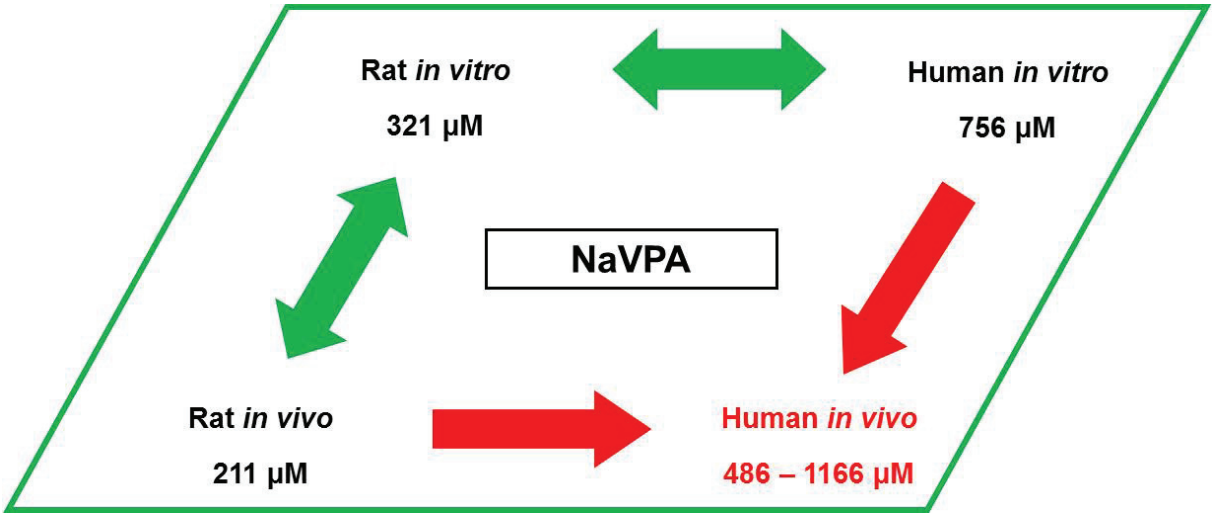
Supplementary Fig. 6 Curve Fits of migration assay. Human (**a-i**) and rat neurospheres (**j-r**) were exposed to nine testing compounds (**a and j**: MeHgCl; **b and k**: $NaAsO_2$; **c and l**: CPF; **d and m**: parathion; **e and n:** MAM; **f and o**: NaVPA; **g and p**: glutamate; **h und q**: paracetamol; **i and r:** PenG) with 7 concentrations per compound. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide 96-well for 24 hrs. At the end of exposure time, phase contrast pictures were taken for measuring migration distances and viability was monitored by the Alamar Blue Assay. Sigmoidal dose response curve fits for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints migration (mig. dist.) and viability (Alamar Blue) ± SEM (n = 3 independent experiments)

Fig. S7:



Supplementary Fig. 7 Curve Fits of neuronal differentiation assay. Human (**a-i**) and rat neurospheres (**j-r**) were exposed to nine testing compounds (**a and j**: MeHgCl; **b and k**: $NaAsO_2$; **c and l**: CPF; **d and m**: parathion; **e and n:** MAM; **f and o**: NaVPA; **g and p**: glutamate; **h und q**: paracetamol; **i and r:** PenG) with 7 concentrations per compound. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide for 3 days. At the end of exposure time, viability was monitored by the Alamar Blue Assay and neurospheres were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst. Immunoflourescent pictures were taken and neurons were quantified by manual counting. Sigmoidal dose response curve fits for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints neuronal differentiation (neuronal diff.) and viability (Alamar Blue) ± SEM (n = 3-15 independent experiments)

Fig. S8:



Supplementary Figure 8: Parallelogram Approach for NaVPA. EC_{50} values for the most sensitive endpoints (MSEs) of NaVPA in human and rat neurospheres are applied in a parallelogram approach. Therefore, existing rat *in vivo* data are compared to rat *in vitro* data to illustrate *in vivo* - *in vitro* similarities/differences. Rat *in vitro* data are compared with human *in vitro* data to obtain information regarding interspecies differences. All these data will then allow an extrapolation of possible effects in humans *in vivo*. Green = experimental data, red = extrapolation

Supplementary Tables

Supplementary Table 1: Testing compound set used in this study. The set of chemicals comprised of six compounds with known developmental neurotoxic potential (positive substances) and three negative compounds without proven developmental neurotoxic potential (negative substances)

compound	CAS-No.	vehicle	source	stock concentration	concentration range tested
positive substances:					
methylmercurychloride (MeHgCl)	**115-09-3**	**DMSO**	**Sigma Aldrich**	**2 mM**	**3 µM-0.004 µM**
sodium (meta)arsenite ($NaAsO_2$)	**7784-46-5**	**DMSO**	**Sigma Aldrich**	**10 mM**	**10 µM-0.014 µM**
methylazoxy methanol acetate (MAM)	**592-62-1**	**H2O**	**NCI**	**1500 mM**	**1500 µM-2.058 µM**
valproic acid sodium salt (NaVPA)	**1069-66-5**	**H2O**	**Sigma Aldrich**	**1125 mM**	**11250 µM-15.432 µM**
chlorpyrifos ethyl (CPF)	**39475-55-3**	**DMSO**	**LGC Standards**	**285.2 mM**	**213.9 µM-0.293 µM**
parathion ethyl (parathion)	**56-38-2**	**DMSO**	**LGC Standards**	**343.3 mM**	**257.5 µM-0.353 µM**
negative substances:					
L(+)-Monosodium glutamate monohydrate (glutamate)	**6106-04-3**	**H2O**	**Sigma Aldrich**	**1000 mM**	**10000 µM-13.717 µM**
4-Acetamidophenol (paracetamol)	**103-90-2**	**DMSO**	**Sigma Aldrich**	**5000 mM**	**5000 µM-6.859 µM**
Penicillin G sodium salt (PenG)	**69-57-8**	**PBS**	**Sigma Aldrich**	**1000 mM**	**1000 µM-13.717 µM**

Supplementary Material and Methods

Neurosphere culture. Rat neurospheres were prepared as described previously (Baumann et al. 2014). Briefly, brains of wild-type Wistar rats (Charles River, Sulzfeld, Germany) were removed at postnatal day (PND) 5, dissected and digested in Papain/DNase solution (Worthington Biochemical Corporation, Troisdorf, Germany). Afterwards, the tissue suspension was triturated to obtain a single-cell suspension and ovomucoid solution was added to stop the tissue digestion. The cell suspension was centrifuged, pellets were resuspended and plated in 10-cm petri-dishes. The animals were treated humanely and with regard for alleviation of suffering.
Both human and rat NPCs were cultured in proliferation medium [DMEM and Hams F12 (3:1) supplemented with B27 (Life Technologies, Darmstadt, Germany), 20 ng/mL epidermal growth factor (EGF; Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin (PAN Biotech, Aidenbach, Germany)] in a humidified 95% air/5% CO_2 incubator at 37° C in suspension culture.
Differentiation was initiated by growth factor withdrawal in differentiation medium [DMEM and Hams F12 (3:1) supplemented with N2 (Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin] and plating onto poly-D-lysine (PDL)/laminin–coated chamber slides.

Cell viability assay. In every experiment cell viability was assessed in the same wells used for the more specific DNT endpoints like progenitor cell proliferation and differentiation as previously described (Baumann et al. 2014). Therefore we measured cell viability using an Alamar Blue assay (CellTiter-Blue assay Promega, Mannheim, Germany). Cells completely lysed by 0.36% Triton X-100 (Sigma Aldrich) serve as endpoint specific control. As background control wells with the respective medium but without cells were used.

Cytotoxicity assay. For the cytotoxicity measurement the lactate dehydrogenase (LDH) assay (CytoTox-One; Promega, Mannheim, Germany) was used as described previously (Baumann et al. 2014). Cells completely lysed by 0.36% Triton X-100 (Sigma Aldrich) serve as endpoint specific control. As background control wells with the respective medium but without cells were used.

Chemicals. Chemicals were of the highest purity available and dissolved in either 100% DMSO (Carl Roth GmbH, Karlsruhe, Germany; maximum solvent concentration 0.1%), sterile distilled H_2O or sterile PBS (Life Technologies, Darmstadt, Germany; maximum solvent concentration 1%) based on the solubility of each chemical and stock solutions of 1.125 M – 2 mM were prepared.

Supplementary Discussion

Arsenic is an environmental toxicant with known DNT potential as pre- or postnatal exposure through contaminated water or dried milk is associated with mental retardation in humans (Grandjean and Landrigan 2006). One of its most prominent modes of action is the generation of reactive oxygen species in the brain (Flora 2011). In hNPCs, proliferation was the most affected endpoint with an EC_{50} value of 1.7 µM. Epidemiological findings showed that arsenic concentrations of 50 µg/L in drinking water, which might result in a blood concentration of 2.25 µg/L (Concha et al. 1998), already decreased intellectual abilities in children (Wasserman et al. 2004). However, arsenic brain concentrations in humans are not known. In rNPCs arsenic inhibited neuronal differentiation with an EC_{50} value of 0.4 µM and was thus the most sensitive endpoint (MSE). An administration of 36,7 mg/L sodium arsenite in drinking water from GD 15 on for 4 months showed increased locomotor activity and learning deficits in rats. This exposure produced arsenic brain concentrations of 4,4 µg/g (35 µM) in the offspring (Rodrıguez et al. 2002). A study with much lower doses administered (0,3 mg/L in drinking water) during gestation observed altered spontaneous behavior in neonatal rats (Chattopadhyay et al. 2002) suggesting, in support of the here presented neurosphere data, adverse neurodevelopmental effects at much lower concentrations than 35 µM. Arsenic brain concentrations were not measured in these animals and exposure time differed from the Rodriguez et al. (2002) study, but may be estimated to approximately 0,3 µM (Rodrıguez et al. 2002). However, this has to be handled with caution and measured arsenic brain concentrations are needed to perform a quantitative evaluation of the sensitivity of the rat neurosphere assay. Moreover, for a comprehensive human risk assessment, an estimation of arsenic concentrations in human fetal or child brain is necessary.

For the organophosphate pesticide parathion there is evidence from rat studies that it impairs brain development at concentrations below those inhibiting acetylcholine esterase (Slotkin et al. 2006). Parathion only inhibited neuronal differentiation in hNPCs at a concentration of 253 µM. Although exposure to parathion is related to neurodevelopmental disturbances (Ruckart et al. 2004), there is – to the best of our knowledge - no data for internal exposure levels available which precludes us from a conclusive assessment of hNPC data for the actual human risk. Nevertheless, effective parathion concentrations *in vitro* seem to exceed internal exposure levels. In rNPCs parathion did not specifically inhibit any neurodevelopment-related endpoint. However, administration of 0.1 mg/kg parathion from PND 1 to 4 in rats, which might produce a brain concentration of 23 µM (Gearhart et al. 1994), resulted in learning deficits (Slotkin et al. 2009). This indicates that the rat neurosphere assay did not classify parathion as hazardous to rat neurodevelopment. The endpoints measured with the neurosphere assay are not the only key events relevant for brain development. Therefore, it is highly likely that parathion disturbs neurodevelopment via different key events than the ones assessed here. This important negative result thus supports the concept of a modulatory approach to neurodevelopmental toxicity testing taking developmental timing and a

comprehensive selection of necessary key events into consideration (Fig. 4). It also underlines the importance of knowing the biological application domain for an *in vitro* assay.

NaVPA is a prominent antiepileptic drug with proven teratogenic and DNT potential if taken during pregnancy (Ornoy 2009). In hNPCs, proliferation was inhibited at subcytotoxic concentrations (EC_{50} = 756 µM). Clinical relevant plasma concentrations lie between 50 and 120 µg/mL (347 to 833 µM; Warner et al. 1998) and accumulate to estimated fetal brain concentrations of 486 to 1166 µM (Künig et al. 1998; Ornoy 2009). Therefore, NaVPA inhibited proliferation of hNPCs within a clinically relevant concentration range. In rNPCs, both proliferation and neuronal differentiation were specifically inhibited with EC_{50} values between 300 and 400 µM. *In vivo*, a low dose of 50 mg/kg in PND 7 rats, which caused apoptotic neurodegeneration (Bittigau et al. 2002), results in an approximate brain concentration of 211 µM (Eskandari et al. 2011), which is close to the EC_{50} values found for inhibition of rNPC proliferation and differentiation. Data on VPA-induced neurogenesis *in vivo* is sparse. An elegant *in vivo* study in mice, however, supports our notion as a daily VPA dose of 250 µg/g between PND 7 and 14 reduced proliferation and neurogenesis in postnatal mouse brains (Foti et al. 2013). To the best of our knowledge, there has no behavioral study or study investigating proliferation or neurogenesis in rats been performed with equivalent low doses and an appropriate (postnatal) timing of administration. Thus, although prediction of human and rat neurospheres for DNT risk of NaVPA with the available data looks promising, one has to take into consideration that data used in this approach compares a functional endpoint (intelligence quotient) in humans with a histopathological endpoint (apoptotic neurodegeneration) in rats (Suppl. Fig. 8). It also has to be pointed out here, that effects assessed with the neurosphere assay do not relate to the VPA-induced disruption of neural tube closure. Therefore, one needs a different *in vitro* assay (Fig. 4).

The two negative compounds glutamate and paracetamol affected DNT endpoints in human or rat neurospheres, and those effects were also separated from general cytotoxicity. However, EC_{50} values were consistently higher than those of positive compounds and dramatically exceeded therapeutic internal doses as discussed below. hNPC proliferation was inhibited by the excitotoxic neurotransmitter glutamate with an EC_{50} value of 1938 µM. However, normal plasma levels of glutamate in humans lie between 40 and 70 µM and may be elevated up to 110 µM after glutamate-rich food intake (Stegink et al. 1979). Moreover, the blood brain barrier is relatively impermeable for glutamate (Hawkins 2009), showing that concentrations which inhibited proliferation in hNPCs were probably far from any human relevance. In rNPCs neuronal differentiation was inhibited with an EC_{50} value of 375 µM. *In vivo*, 4g/kg glutamate in rats from PND 1 to 10 affected learning behavior in subadult animals. However, glutamate brain concentrations did not exceed 6 µM in the animals, showing that the EC_{50} values causing decreased neuronal differentiation in rat neurospheres are of no physiological relevance. Paracetamol, which is hepatotoxic at high concentrations, inhibited proliferation in hNPCs at millimolar concentrations (2219 µM) and neuronal differentiation in rNPCs at micromolar

concentrations (399 µM). A therapeutic administration of 15 mg/kg in either humans or rats resulted in a plasma concentration of 66 µM and in CSF concentrations of 26 µM (human) and 13 µM (rat), respectively (Westerhout et al. 2012), which is again much lower than EC_{50} values determined in the neurosphere assays.

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Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

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2.3 Arsenite Interrupts Neurodevelopmental Processes of Human and Rat Neural Progenitor Cells: the Role of Reactive Oxygen Species and Species-Specific Antioxidative Defense

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Archives of Toxicology

Die Exposition gegenüber Arsen stört die Gehirnentwicklung des Menschen. Obwohl die Entwicklungsneurotoxizität (ENT) von Arsen bereits in vielen in vivo und in vitro Studien untersucht wurde, ist der Wirkmechanismus in sich entwickelnden Gehirnzellen bisher nicht vollständig verstanden. In dieser Studie wurden die adversen Effekte von Arsen auf die neurale Entwicklung in neuralen Progenitorzellen (NPC) von Mensch und Ratte charakterisiert. Weiterhin wurde Spezies-vergleichend, die Beteiligung von Reaktiven Sauerstoff Spezies (ROS) und die Rolle der Glutathion (GSH)-abhängigen antioxidativen Abwehr in der ENT von Arsen untersucht. Dazu wurden die EC_{50}-Werte für eine Arsenit-abhängige Inhibition der Migration, neuronalen und Oligodendrozyten Differenzierung durch die Generierung von Konzentrations-Wirkungskurven bestimmt. Die Beteiligung von ROS wurde durch die Quantifizierung der Expression von ROS-regulierten Genen, die Messung der Glutathion (GSH) Level, die Inhibition der GSH Synthese und die Co-Behandlung mit dem Antioxidans n-Acetylcystein untersucht. Bezogen auf die gesamte Kultur reduziert Arsenit die Neurogenese und Oligodendrogenese von differenzierenden hNPC und rNPC bereits in subzytotoxischen Konzentrationen. Spezies-spezifische Arsenit Zytotoxizität und Induktion der Expression antioxidativer Gene korreliert invers mit dem GSH Level von NPCs. Dabei besitzen rNPC im Vergleich zu hNPC eine mehr als 3-fach höhere Menge an GSH. Eine pharmakologische Reduktion der GSH Synthese erhöht die Sensitivität gegenüber Arsenit in rNPC im Vergleich zu hNPC. N-Acetylcystein antagonisiert die Arsenit-vermittelte Induktion der *HMOX1* Expression sowie die neuronale und Oligodendrozyten Differenzierung. Humane NPC sind im Vergleich zu rNPC sensitiver gegenüber einer Arsenit-induzierten ENT. Dies liegt wahrscheinlich an der geringeren Kapazität ihrer antioxidativen Abwehr. Dieser Spezies-spezifische Wirkmechanismus kann für die Generierung von sogenannten *Adverse Outcome Pathways* und zukünftige Risikobewertungsstrategien von Nutzen sein.

Archives of Toxicology

Arsenite Interrupts Neurodevelopmental Processes of Human and Rat Neural Progenitor Cells: the Role of Reactive Oxygen Species and Species-Specific Antioxidative Defense

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Abstract:	Arsenic exposure disturbs brain development in humans. Although developmental neurotoxicity (DNT) of Arsenic has been studied in vivo and in vitro, its mode-of-action (MoA) is not completely understood. Here, we characterize the adverse neurodevelopmental effects of arsenite on developing human and rat neural progenitor cells (hNPC and rNPC). Moreover, we analyze the involvement of reactive oxygen species (ROS) and the role of the glutathione (GSH)-dependent antioxidative defense for arsenite-induced DNT in a species-specific manner. We determined IC50 values (NaAsO2 range: 0.1-10 µM) for arsenite-dependent inhibition of hNPC and rNPC migration, neuronal and oligodendrocyte differentiation. ROS involvement was studied by quantifying the expression of ROS-regulated gene products, measuring glutathione (GSH) levels, inhibiting GSH synthesis and co-exposing cells to the antioxidant N-acetylcystein. Arsenite reduces neurogenesis and oligodendrogenesis of differentiating hNPC and rNPC at sub-cytotoxic concentrations with regards to the whole culture. Species-specific arsenite cytotoxicity and induction of antioxidative gene expression is inversely related to GSH levels with rNPC possessing >3-fold the amount of GSH than hNPC. Depletion of GSH synthesis increased the sensitivity towards arsenite in rNPC>hNPC. N-acetylcysteine antagonized arsenite-mediated induction of HMOX1 expression as well as neuronal and oligodendrocyte differentiation. Human NPC are more sensitive towards arsenite-induced neurodevelopmental toxicity than rNPC, probably due to their lower antioxidative defense capacities. This species-specific MoA data might be useful for adverse outcome pathway generation and future integrated

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Title Page

Arsenite Interrupts Neurodevelopmental Processes of Human and Rat Neural Progenitor Cells: the Role of Reactive Oxygen Species and Species-Specific Antioxidative Defense

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Abstract

Arsenic exposure disturbs brain development in humans. Although developmental neurotoxicity (DNT) of Arsenic has been studied in vivo and in vitro, its mode-of-action (MoA) is not completely understood. Here, we characterize the adverse neurodevelopmental effects of arsenite on developing human and rat neural progenitor cells (hNPC and rNPC). Moreover, we analyze the involvement of reactive oxygen species (ROS) and the role of the glutathione (GSH)-dependent antioxidative defense for arsenite-induced DNT in a species-specific manner. We determined IC_{50} values ($NaAsO_2$ range: 0.1-10 µM) for arsenite-dependent inhibition of hNPC and rNPC migration, neuronal and oligodendrocyte differentiation. ROS involvement was studied by quantifying the expression of ROS-regulated gene products, measuring glutathione (GSH) levels, inhibiting GSH synthesis and co-exposing cells to the antioxidant N-acetylcystein. Arsenite reduces neurogenesis and oligodendrogenesis of differentiating hNPC and rNPC at sub-cytotoxic concentrations with regards to the whole culture. Species-specific arsenite cytotoxicity and induction of antioxidative gene expression is inversely related to GSH levels with rNPC possessing >3-fold the amount of GSH than hNPC. Depletion of GSH synthesis increased the sensitivity towards arsenite in rNPC>hNPC. N-acetylcysteine antagonized arsenite-mediated induction of *HMOX1* expression as well as neuronal and oligodendrocyte differentiation. Human NPC are more sensitive towards arsenite-induced neurodevelopmental toxicity than rNPC, probably due to their lower antioxidative defense capacities. This species-specific MoA data might be useful for adverse outcome pathway generation and future integrated risk assessment strategies concerning DNT.

Keywords: Developmental neurotoxicity, testing battery, neural progenitor cells, mode-of-action, ROS, alternative method

Introduction

Arsenic is a widely distributed environmental toxicant and, according to the WHO, belongs to the top 10 chemicals that pose major global health concerns (WHO 2016). In more than 24 countries around the world there are regions where natural or anthropogenic groundwater contamination exceeds the provisional safety level of 10 µg As/L explaining the major route of arsenic exposure by consumption of contaminated water (WHO 2011). It is estimated that worldwide 140-300 million people are at risk of Arsenic-induced health effects (Prakash et al. 2016; States *et al.*, 2011; Quansah *et al.*, 2015). This might be an underestimation due to the uncertainty of adverse health consequences occurring at exposure levels below 10 µg As/L water (WHO 2011). The exposure to arsenic happens mainly by the inorganic pentavalent arsenate, which is reduced to the trivalent arsenite. In general, the inorganic arsenic species are more toxic than organic arsenicals (WHO 2011; Prakash et al. 2015).

Chronic exposure to arsenicals is associated with skin lesions, cardiovascular disease, respiratory system disease, diabetes, effects on the reproductive system (Kapaj et al. 2006; WHO 2011; Quansah et al. 2015) and cancer (IARC 2012). Furthermore, arsenic is neurotoxic with a high susceptibility of the developing nervous system (Grandjean and Landrigan 2014). Epidemiological studies show an association of human early life arsenic exposure and impaired neurocognitive outcomes (Tolins et al. 2014; Tsuji et al. 2015). Arsenic toxicity is transported through a plethora of different mode-of-actions (MoAs), yet the molecular mechanisms by which arsenic disturbs brain development are not well understood. The most favored theory is that arsenic exerts developmental neurotoxicity (DNT) through the generation of ROS (Flora 2011; Jomova et al. 2011; Prakash et al. 2016).

To study how arsenic interferes with neurodevelopmental key events (KE), i.e. cell biological processes of brain development, we used an *in vitro* model based on human and rat NPC growing as three-dimensional neurospheres (Moors et al. 2009; Baumann et al. 2015a). We previously showed that arsenic reduces human and rat NPC cell proliferation, migration and neuronal differentiation (Baumann, et al., 2015b). In this study we extended these data for the endpoint oligodendrogenesis and tested whether ROS are involved in arsenic effects on developing NPCs.

Material and Methods

For all methods, detailed descriptions are provided in the Supplementary Material and Method section.

Chemicals

Sodium arsenite ($NaAsO_2$) and n-Acetylcysteine (NAC; both from Sigma-Aldrich, Taufkirchen, Germany), Buthionine Sulfoximine (BSO; Cayman Chemicals, Ann Arbor, USA).

Cell Culture

hNPC (GW16-19), 2 different individuals, were purchased from Lonza Verviers SPRL (Verviers, Belgium) and time-matched rNPC (Workman et al. 2013) from postnatal day (PND)1 were prepared as described previously (Baumann et al. 2014, 2015a). NPCs were cultured as neurospheres in proliferation medium. To initiate differentiation, NPCs were plated on an extracellular matrix in differentiation medium as previously described (Baumann et al. 2014, 2015a).

Neurosphere Assay

We quantified the endpoints migration, neuronal and oligodendrocyte differentiation along with general viability after treatment with either arsenite alone or in combination with BSO or NAC and the respective solvents. In co-treatments, cells were pre-exposed 2h before arsenite was added. Endpoint assessment was performed according to the description in Baumann et al. (2014, 2015a).

qRT-PCR analysis

mRNA expression analyses were performed for 24h pre-differentiated NPC treated with arsenite for 8h or NAC for 4h and human (GW15-33) or rat (PND1) mRNA samples from brains *in vivo*. Absolute copy numbers were determined using product-specific standards. The copy number of

the respective gene was normalized to 10000 copies of β-actin. Primer sequences for HMOX1, GPX1, SOD1, CAT, GCLC, GCLM and ACTB are presented in Suppl. Tab. 1.

Glutathione Assay

For GSH determination, cells were differentiated for 24h and treated either with arsenite for 4, 8 or 24h or with BSO for 24h. GSH levels were determined using the GSH assay kit from Sigma Aldrich. The manufacturer's protocol was adapted according to (Rahman et al. 2006).

Data analysis

Data analysis was performed using GraphPad Prism 6.00 (www.graphpad.com). Data from at least three independent experiments was normalized to the respective solvent control (except for cell migration which is presented as migration distance). Concentration-response curves, IC_{50} values and 95 % confidence intervals were calculated using a non-linear regression model with sigmoidal dose–response curve fit. For all concentration-response curves, the data from the highest concentration (10 µM arsenite) was generated in separate experiments. Statistical significance analysis of single exposure was performed by one-way ANOVA followed by Dunnett's multiple comparisons test. For co-treatments, statistical analysis was performed by two way ANOVA followed by Sidak multiple comparison test. For comparison of transcript copy numbers from NPCs and brain samples, statistical analysis was performed by multiple t test followed by Holm-Sidak multiple testing correction. The significance cutoff was set to $p \leq 0.05$.

Results

Arsenite affects neurodevelopmental key events.

In this study, we re-evaluated arsenite toxicity on hNPC and rNPC migration and neuronal differentiation and extended our previous data set by studying the effects of arsenite on oligodendrogenesis followed by a MoA analysis. A comparison of IC_{50} values for all analyzed endpoints indicates that for both species the most sensitive endpoint (MSE) is oligodendrocyte differentiation after 5 days (IC_{50} = 1.1 µM in hNPC; 2.0 µM in rNPC; Fig. 1, Tab. 1) followed by neuronal differentiation after 3 days (2.7 µM in hNPC; 4.4 µM in rNPC; Suppl. Fig. 1, Tab. 1). Migration after 24 h is only affected in hNPC (IC_{50} = 6 µM). In both species, arsenite affects DNT endpoints at lower concentrations than general viability (Tab. 1). However, rNPC are less sensitive towards arsenite-induced cell death as evident by the concentration-response curves and related IC_{50} values for viability after 3d and 5d of differentiation (Tab. 1).

Species-specific basal and Arsenite-induced antioxidative enzyme gene expression and GSH content.

To study if the observed cellular effects of arsenite on NPC development are due to ROS formation (Flora 2011; Jomova et al. 2011; Prakash et al. 2016), we measured the oxidative status of 24h pre-differentiated NPCs indirectly by analyzing the mRNA expression of the antioxidative enzymes *superoxide dismutase 1* (*SOD1*), *glutathione peroxidase* 1 (*GPX1*), *catalase* (*CAT*) and the ROS-responsive enzyme *heme oxygenase 1* (*HMOX1*) in response to 8 h arsenite exposure. The concentrations used cover a range up to the approximate IC_{50} values for arsenite effects on viability in the respective species (see Tab. 1). In hNPCs, 2.5 µM arsenite significantly induces the expression of *SOD1* and *HMOX1* to 1.5 ± 0.1- and 38.9 ± 17.8-fold of control, respectively and in rNPC 10 µM arsenite induces the *HMOX1* gene expression to 10.8 ± 1.8-fold of control (Fig. 2a,b) suggesting a stronger ROS-stress in human than rNPC. Induction of *HMOX1* expression at concentrations interfering with DNT-specific endpoints (2.5 µM) in hNPC, but not in rNPC, indicates an involvement of ROS in arsenite-induced DNT solely in human NPC and thus suggests a species-specific antioxidative defense. Comparison of basal 24h differentiated NPC gene expression of antioxidative enzymes supports this suggestion as rNPC express higher copy numbers of basal *GPX1* (5.5 ± 1.6 copies/10.000 copies *β-actin* in hNPCs vs. 894.9 copies/10.000 copies *β-actin* in rNPCs) and *CAT* (69.5 ± 2.3 copies/10.000 copies *β-actin* in hNPCs vs. 224.8 ± 12.4 copies/10.000 copies *β-actin* in rNPCs; Fig. 2a,b).

Gene expression analyses of *GPX1* and *CAT* determined in mRNA samples derived from human and rat fetal brains *in vivo* revealed 5.8 ± 0.7 copies of *GPX1*/10.000 copies *β-actin* in human and 257.3 ± 13.8 copies/10.000 copies *β-actin* in rat brain and 37.7 ± 8.8 copies/10.000 copies *β-actin* of *CAT* in human and 265.7 ± 44.5 copies/10.000 copies *β-actin* in rat brain (Fig. 2c) supporting the *in vivo* relevance of the *in vitro* findings. Due to the higher rat than human *GPX1* expression, one might also expect higher GSH content and thus higher GSH-dependent antioxidative defense capacity in rat than in hNPC.

To test this supposition, we determined the cellular GSH content and NPC mRNA expression of the GSH synthesis rate-limiting enzyme, *glutamate cysteine ligase* (*GCL*). Indeed, rNPC contain 4-times more GSH than hNPC (6.0 ± 1.3 and 1.4 ± 0.2 nmol/10^6 cells, respectively; Fig. 3a). Arsenite (2.5 µM) significantly induces human and rat GSH levels 2.1 ± 0.2 and 1.9 ± 0.2 -fold over control levels after 24h, respectively, while only hNPC up-regulate GSH levels (to 2.2 ± 0.4-fold of control) and expression of the modifier subunit of *GCL* (GCLM; to 4.1 ± 0.3-fold of control) after 8h of exposure (Fig. 3b-d). The earlier response of hNPC compared to rNPC is probably due to their lower basal GSH-dependent antioxidative defense.

Reduction of GSH increases the sensitivity of NPC.

To determine if there is a causal relationship between arsenite toxicity and NPC GSH levels, we inhibited GSH synthesis with the *GCL* inhibitor BSO (10 µM) and co-exposed NPCs with arsenite (0.1 – 10 µM). BSO treatment significantly reduces NPC GSH levels (from 6.3 ± 2 to 1.4 ± 0.4 nmol/10^6 cells in rNPC and from 1.8 ± 0.3 to 0.4 ± 0.1 nmol/10^6 cells in hNPC; Fig. 4a). In rNPC BSO treatment resulted in GSH levels similar to basal GSH levels of hNPC. BSO alone did not affect NPC viability, migration, neuronal or oligodendrocyte differentiation (Fig. 4b). In presence of BSO, arsenite became highly cytotoxic in NPC from both species demonstrating their dependence on GSH as an antioxidative defense (Fig. 4c-f). Although in presence of BSO arsenite-dependent IC_{50} values of DNT-specific endpoints cannot be distinguished from cytotoxicity (Fig. 4e,f), slopes of the concentration-response curves for differentiation suggest a higher sensitivity of rat neurons and oligodendrocytes towards arsenite compared to astroglial cells, which represent the majority of cells in the neurosphere migration area (Baumann et al., 2015), and a lower sensitivity of human neurons compared to astrocytes (Fig. 4c-f). Yet, hNPC are 10-times less sensitive towards arsenite-induced toxicity than rNPC when compared at similar GSH levels (Fig. 4a): IC_{50} values for viability after 3d: $IC_{50\ rNPC\ with\ BSO}$ = 0.8 vs $IC_{50\ hNPC\ w/o}$

$_{BSO}$ = 8.2 (Fig. 4e, f). These data indicate that ROS defense of hNPC does not depend as much on GSH as ROS defense of rNPC.

N-acetylcysteine protects hNPC against arsenite toxicity.

To confirm that arsenite toxicity on hNPC is ROS dependent, we co-treated 24h pre-differentiated hNPC with arsenite (2.5 µM) and NAC (10 mM) for 4h. NAC significantly antagonizes arsenite-dependent induction of *HMOX1* gene expression (from 32.6 ± 7.1 to 5.0 ± 0.7 fold of control), reduction of migration (73.2 ± 2.6 to 94.9 ± 1.2% of control), neuronal- (39.7 ± 10.0 to 81.9 ± 6.0% of control) and oligodendrocyte differentiation (13.9 ± 4.3 to 60.1 ± 0.8% of control; Fig. 5a-d). These data indicate that arsenite-induced effects on DNT-specific endpoints is ROS dependent.

Despite these protective effects, NAC alone inhibits migration, neuronal- and oligodendrocyte differentiation (76.1 ± 5.6%, 58.9 ± 11.2% and 31.7 ± 2.6% of control, respectively), without affecting *HMOX1* expression or cell viability (Suppl. Fig. 2).

Discussion

Arsenic is one of the few environmental chemicals with sufficient human evidence for its neurodevelopmental toxicity. Early life exposure towards arsenite is associated with decrements in intellectual function involving effects on verbal IQ, performance IQ, long-term memory, problem solving and language or attention in children between 5 and 15 years of age (reviewed in Tolins et al. 2014). Pre- and perinatal exposure of rodents to arsenite causes comparable neurocognitive effects such as impaired spatial and working memory, reduced locomotor activity, deficits in learning and the reaction to novelty (Nagaraja and Desiraju 1994; Chattopadhyay et al. 2002; Rodríguez et al. 2002; Luo et al. 2009; Martinez-Finley et al. 2009; Gumilar et al. 2015; Aung et al. 2016). On the cellular level, rodent developmental arsenite exposure reduces migration, inhibits neuronal differentiation (Liu et al. 2012) and causes loss in myelin (Zarazúa et al. 2010; Rios et al. 2012).

In this study we demonstrate in an *in vitro* approach using neurospheres that arsenite interferes with migration, neuron and oligodendrocyte differentiation of human and rat NPC. Oligodendrocyte differentiation is the MSE and to the best of our knowledge we are the first showing that arsenite interferes with the generation of O4+ oligodendrocytes from hNPC. *In vivo*, exposure of young rats from beginning of pregnancy until 3 to 4 months after birth towards arsenite in drinking water (3 ppm; resulting in an arsenite offspring brain concentration of 74 to 155 ng/g tissue which corresponds to 1 to 2.1 µM) caused up to a 75% reduction of brain MBP (Zarazúa et al. 2010; Rios et al. 2012). Physiological consequences of a disturbed myelination are characterized by dysfunctions in cognitive development and learning (Fields 2008) as well as in motor functions (Choi et al. 2016). Besides arsenic's destructive effects on myelin fibers (Rios et al. 2012), another underlying reason for MBP reduction in arsenic exposed offspring rat brains could be a decrease in oligodendrocyte numbers. This was recently also suggested for the effects of the polybrominated diphenyl ether BDE99 (Dach et al. 2017). Because we see a reduction in O4+ cells in NPC after arsenite treatment at similar concentrations that reduced MBP in rat offspring brains, there might be a causal link between the cellular and organ effects of arsenic that needs further confirmation.

Two prominently discussed MoA of arsenic are interference with cellular methylation pathways (Bailey and Fry 2014) and oxidative stress (Flora 2011; Jomova et al. 2011; Prakash et al. 2016; Rodrıguez et al. 2003). While Rios et al. (2012) ruled out that arsenic-dependent reduction in offspring rat brain MBP is caused by disturbed brain methylation pathways, the hypothesis that ROS formation is the initial factor for reduced MBP has not been studied yet. The developing

brain is especially sensitive towards the production of ROS because it has a high metabolic demand with considerable oxygen consumption (Kety and Schmidt 1948; Kennedy and Sokoloff 1957) and contains a high amount of polyunsaturated fatty acids providing targets for lipid peroxidation (Coyle and Puttfarcken 1993; Rodrıguez et al. 2003). Arsenite exposure (50 µM) caused hydroxyl radical formation in adult rat brain (García-Chávez et al. 2003). In developing rodent brains it hast been demonstrated that Arsenite initiates lipid peroxidation, reduces the expression of antioxidative genes *Sod1*, Gpx1, *Cat*, *glutathione reductase* (*Gr*) or *glutathione-s-transferase* (*Gst*) and reduces cellular GSH content (reviewed in Prakash et al. 2016). Some of these effects where observed at concentrations calculating to 6.5 µM internal arsenite exposure on PND21 (Sannadi et al. 2013; Kadeyala et al. 2013). ROS production by arsenite in *in vitro* cultures at similar concentrations than used in this study were measured earlier in different cell systems (reviewed in Flora 2011), e.g. in a primary culture of human fetal midbrain cells (GW16) 2.3 µM arsenite exposure for 18d produced significant amounts of ROS (Chattopadhyay et al. 2002). Given the *in vivo* relevance of arsenite-dependent ROS formation, our findings that arsenite disturbs neurodevelopmental processes of developing NPC in a ROS-dependent manner adds crucial information to the MoA of arsenite as a developmental neurotoxicant.

Our results indicate that human NPC are more sensitive towards arsenite-induced oxidative stress than rat NPC due to a lower basal expression of antioxidative defense strategies, especially GSH. That higher glutathione-based detoxification capacities protect against ROS-mediated toxicity was observed earlier in arsenite-treated embryonic mouse brains (Allan et al. 2015) as well as in thalidomide-mediated toxicity in rat and rabbit whole embryo cultures (Hansen et al. 1999). Higher antioxidative capacities in rodents compared to humans were also reported for embryonic fibroblasts in vitro and adult heart tissue in vivo yielding in a better protection of rodents towards oxidative stress (Knobloch et al. 2008, Janssen et al. 1993). That myocardial cells of smaller animals like rodents are better protected against ROS might be due to their higher heart rate that requires increased energy production accompanied by higher intrinsic ROS production (Barth et al. 1992). If rodent brain cells possess higher mitochondrial activity than those of larger species is not known.

To study if indeed GSH is protecting rNPC against ROS toxicity, we depleted human and rat NPC for GSH by treating them with BSO. The data suggest that for rNPC GSH is one of the key detoxification mechanisms for arsenite, while hNPC seem to possess additional protection strategies. GSH-mediated detoxification mechanisms for arsenite include (i) reduction of reactive peroxides (Dringen et al. 2015), (ii) methylation of arsenite to less toxic methylated arsenic species (Thomas et al. 2007) or (iii) direct arsenite binding due to the thiol affinity of GSH for

heavy metals (Scott et al. 1993) that is associated with increased cellular metal export through MRP1 & 2 (Dallas et al. 2006; Watanabe et al. 2009; Keppler 2011). However, arsenic can also be detoxified by GSH-independent metabolism (Thomas et al. 2007) and cellular export in its non-conjugated form or conjugated with a methyl group through a different subset of transporters (aquaporins, OATP, MDR1 and GLUTs; Maciaszczyk-Dziubinska et al. 2012). It is possible that hNPCs might detoxify arsenite more efficiently by methylation and methylated/parent export than rNPCs as previously reported for erythrocytes (Shiobara et al. 2001).

Although rNPC have a higher GSH-dependent antioxidative defense and are thus more protected against arsenite-induced cell death than human NPC, they display similar sensitivities towards arsenite-induced reduction of neuronal and oligodendrocyte differentiation at concentrations not interfering with the viability of the whole culture. Here, the question evolves if arsenite interferes with neuron and oligodendrocyte differentiation by specific MoAs or if these cell types, which represent the minority of cells in the neurosphere migration area (Baumann et al. 2015), are just more sensitive towards arsenite-induced cell death than the majority of astrocytes in the culture, which will consequently not be visible by measuring culture viability. There are a couple of arguments supporting the latter hypothesis. Rai et al. (2010) and Jin et al. (2004) both show that arsenite affects viability of primary rat astrocytes at concentrations between 10 and 40 µM whereas rat neuronal cultures are affected at lower concentrations (5-10 µM; Namgung & Xia 2001; Namgung & Xia 2000). Oligodendrocytes and their precursors are known to be highly sensitive towards ROS because in comparison to astrocytes they contain low GSH levels and have at the same time a high iron content (Husain and Juurlink 1995; Thorburne and Juurlink 1996; Juurlink 1997; Juurlink et al. 1998; Back et al. 1998). In the presence of hydrogen peroxide and superoxide anions, iron catalyzes the production of the highly reactive hydroxyl radical through the Haber-Weiss reaction (Haber and Weiss 1934). Hence, low primary ROS defense and high iron content, a combination observed in oligodendrocytes, makes cells prone to oxidative cell damage (Ahmad et al. 2000). Co-treating the cells with arsenite and the antioxidant NAC supports this hypothesis as NAC antagonizes the arsenite-mediated toxicities. NAC by itself also reduces NPC development. This instance is discussed in the supplementary discussion. The higher GSH content of neural rat compared to human cultures thus might be due to astrocyte GSH as these cells constitute the majority of cells in the neurosphere migration area (Baumann et al. 2015) leaving the less protected neurons and oligodendrocytes of both species with similar sensitivities towards arsenite.

Inorganic arsenic exposure from food and water across 19 European countries and United States high exposure populations (Tribal, Asian and Pacific) was estimated to range from 0.1 to 1.22 µg/kg bw/d (EFSA 2010; Mantha et al. 2017). In developing rodents, exposure towards 0.3 – 7 mg/kg bw/d (3 – 70 ppm in drinking water; approximated pup brain concentrations of 1 – 50 µM) caused adverse neurodevelopmental outcomes (Luo et al. 2009; Zarazúa et al. 2010). Assuming similar kinetics of arsenic between humans and rodents, which poses a high level of uncertainty, estimated arsenic exposure of aforementioned populations lead to internal fetal brain concentrations below the LOAECs observed in vivo and in this study. However, there are regions with high arsenic exposure due to water levels of up to 2 ppm (Farías et al. 2008), which is 200-times higher than the WHO provisional standard for drinking water (10 ppb; WHO 2011). Internal arsenic exposure of these higher exposed populations can be estimated to be >0.5 µM, which is similar to the effective concentrations in this study.

In summary, our data contributes to the species-specific MoA of arsenite on developing brain cells and suggests that human developing brains are more sensitive towards compounds with a ROS-dependent MoA than evolving rat brains. These results can be used as early key event (KE) data to a hypothetical adverse outcome pathway (AOP; Patlewicz et al. 2015) that might be entitled ‘ROS generation in developing brain cells leading to impaired cognitive function in children’. Here, the previously well-described molecular initiating event (MIE) is ROS formation, exemplified by arsenic in this study. Cellular KE determined for human and rat cells in mixed-culture scenarios are new and include oligodendrocyte > neuronal > astrocyte toxicity. Astrocyte toxicity is much stronger in human than rat NPC possibly due to a significantly lower GSH content. GSH plays a major role in the detoxification of many toxicants and in the regulation of the cellular redox balance (Zhang and Forman 2012). Therefore, ‘Depletion of reduced GSH in neuronal and glial cells’ is a cellular KE in the AOP ‘Impairment of learning and memory induced by binding of electrophilic chemicals to the SH(thiol)-group of proteins and non-protein molecules in neuronal and glial cells during development’ (AOP VI in Bal-Price et al. 2015). This KE could thus be a common KE of these two AOPs and hence an intersection of a future AOP network. More studies are needed addressing arsenite kinetics as well as cell type- and species-specific MIE and MoA to define an AOP-informed Integrated Approach for Testing and Assessment (IATA; Ockleford et al. 2017; OECD 2014) for ROS-induced DNT in the future.

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

Experiments with human NPCs were ethically approved by the ethics committee of the Heinrich-Heine-University, Düsseldorf. Preparation of NPCs from rat brain tissue are in accordance with German regulations and the experimental guidelines of the State Agency for Nature, Environment and Consumer Protection in North Rhine-Westphalia in Germany (LANUV) and were approved by the LANUV.

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Tables

Table 1 IC_{50} values and 95 % confidence intervals for concentrations-response curves of arsenite treatment of human and rat neurospheres.

Figure legends

Figure 1 Effects of Arsenite on oligodendrocyte formation

Human (a, c) and rat (b, d) neurospheres were differentiated with increasing concentrations of arsenite (0.1, 0.5, 0.75, 1, 2.5, 5 and 10 µM) for 120h. Prior to fixation cell viability (dotted line) was analyzed by Alamar-Blue assay. Oligodendrocytes were immunocytochemically stained for O4 and nuclei were counterstained with Hoechst33258. Viability and percentage of oligodendrocytes/nuclei in the migration area were normalized to solvent control and are displayed as concentration response relationship with mean ± SEM of at least three independent experiments. * indicates a significant difference from solvent control based on one-way ANOVA ($p<0.05$). (c, d) representative pictures of immunocytochemically stained oligodendrocytes after 120h arsenite treatment of human (c) and rat (d). Scale bar represent 100 µm.

Figure 2 Expression changes in antioxidative genes after arsenite treatment

Gene expression of *SOD1*, *GPX1*, *CAT* and *HMOX1* was analyzed by qRT-PCR in hNPC (a) and rNPC (b) that were pre-differentiated for 24h and treated with increasing concentrations of arsenite for 8h or analyzed in untreated human (GW15-33) and rat (PND1) whole brain samples (c). Copy number of each gene was normalized to 10.000 copies of *β-actin*. Mean ± SEM of 3 independent experiments/brain samples. * indicates a significant difference from solvent control based on one-way ANOVA ($p<0.05$). # indicates a significant difference between species based on multiple t-test ($p<0.05$).

Figure 3 Effect of arsenite on Glutathione synthesis

Human and rat neurospheres were treated with arsenite (2.5 µM) for 4, 8 and 24h during a 24h differentiation period. (a) Total GSH level in nmol normalized to 10^6 cells after 24h differentiation without arsenite treatment. (b) Normalized GSH level in fold of control after 4, 8 and 24h arsenite treatment. (c, d) Gene expression of *GCLC* and *GCLM* was analyzed by qRT-PCR after 24h pre-differentiation following 8h arsenite treatment in differentiating human (c) and rat (d) NPC. Copy number of each gene was normalized to 10 000 copies of β-actin. Mean ± SEM of at least 3 independent experiments. * indicates a significant difference from solvent control based on one-

way ANOVA ($p<0.05$), # indicates a significant difference between species based on unpaired t test ($p<0.05$).

Figure 4 Effects of arsenite on differentiating NPCs after inhibition of GSH synthesis

hNPC and rNPC were treated with increasing concentrations of arsenite (0.1-10µM) with or without BSO (1 and 10 µM) and analyzed for the effect on migration, neuronal and oligodendrocyte differentiation and viability. (a) Total GSH was measured in h and rNPC after 24h treatment with BSO (1, 10 µM). Mean ± SEM of at least three independent experiments. * indicates a significant difference from solvent control based on one-way ANOVA ($p<0.05$). (b) Effect of BSO treatment on migration, differentiation and viability of hNPC (blue) and rNPC (pink) shown as mean ± SEM of raw data of at least 3 independent experiments. RFU is relative fluorescence unit. (c, d) Concentration-response curves for the effect of arsenite (dotted line) and arsenite with BSO (solid line) on migration, neuronal differentiation and viability after 72h and oligodendrocyte differentiation and viability after 120h in hNPC (blue) and rNPC (pink). Curve fit was calculated based on control-normalized data from at least 3 independent experiments. (e) IC_{50} values calculated from concentration-response curves (c, d) ± 95% confidence interval (CI) of all endpoints for hNPC (blue) and rNPC (pink) with (not filled) and without (filled) BSO co-treatment. (f) IC_{50} values calculated from concentration-response curves (c, d) as heat map from low (yellow) to high (green) values.

Figure 5 Protective effects of n-Acetylcysteine on arsenite mediated effects

(a) hNPC were pre-differentiated for 24h and exposed to NAC (10 mM), arsenite (2.5 mM) or a combination of both for 4h. *HMOX1* expression was analyzed by qRT-PCR. Copy nr. was normalized to 10.000 copies of *β-actin*. (b-e) hNPC were treated with arsenite (2.5 or 2 µM) with or without NAC (1 and 10 mM) and analyzed for the effect on migration, neuronal and oligodendrocyte differentiation and number of nuclei. Mean ± SEM normalized to respective control of at least 3 independent experiments. * indicates a significant difference from respective control, # indicates a significant difference from w/o based on two-way ANOVA ($p<0.05$).

Table 1

		Specific endpoint		Viability	
		Human	Rat	Human	Rat
Migration distance (72h)	IC_{50} 95% CI	6.0 4.8 to 7.4	> 10	6.0 0.4 to 95	> 10
Neuronal differentiation (72h)	IC_{50} 95% CI	2.7 1.5 to 4.7	4.4 2.9 to 6.7	6.0 0.4 to 95	> 10
Oligodendrocyte differentiation (120h)	IC_{50} 95% CI	*1.1* 0.8 to 1.4	*2.0* 1.5 to 2.8	4.8 3.5 to 6.6	9.2 7.5 to 11.0

Values are in µM. The lowest IC_{50} for each species is marked in italic. CI is confidence interval.

Figure 1

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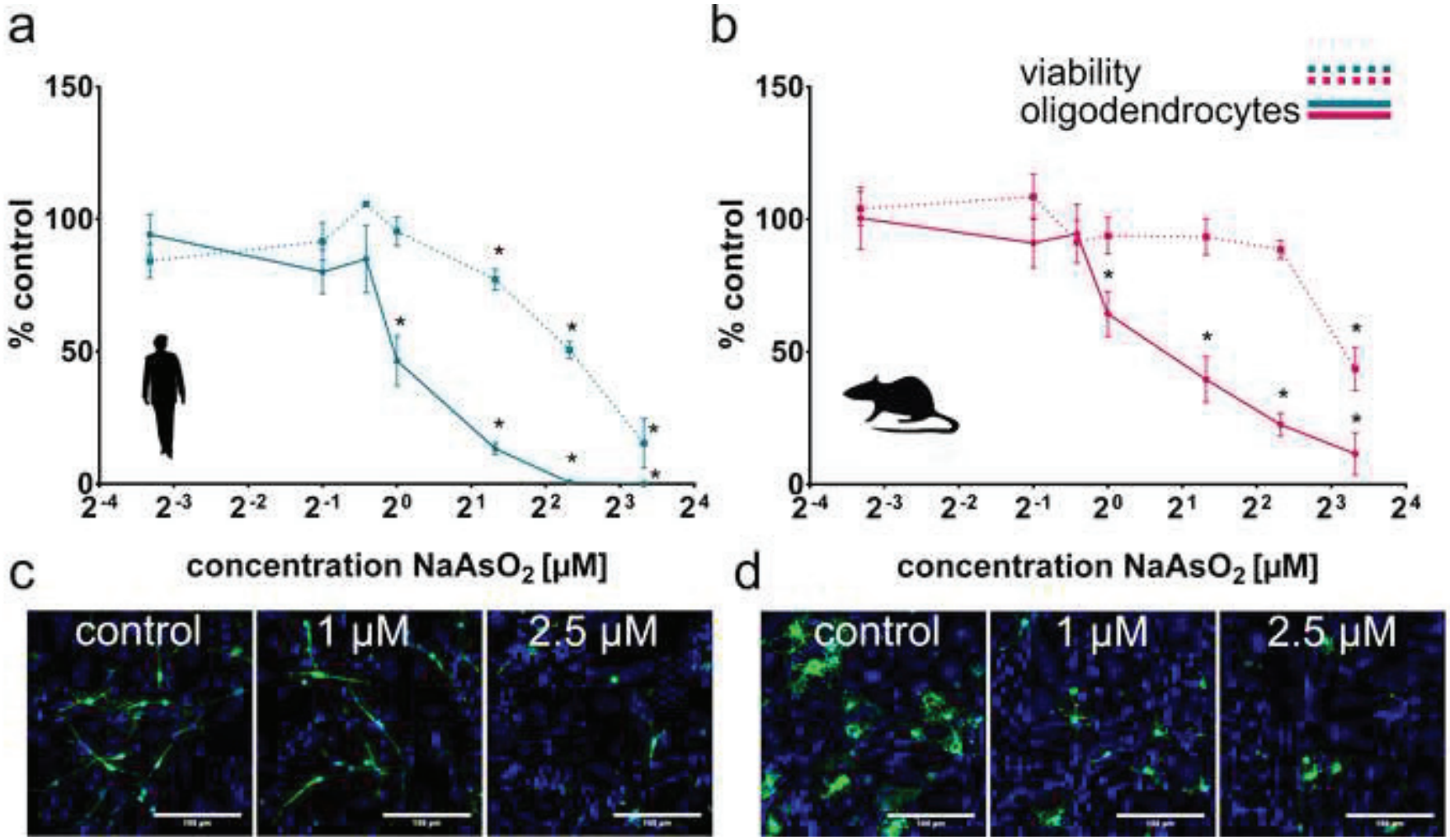

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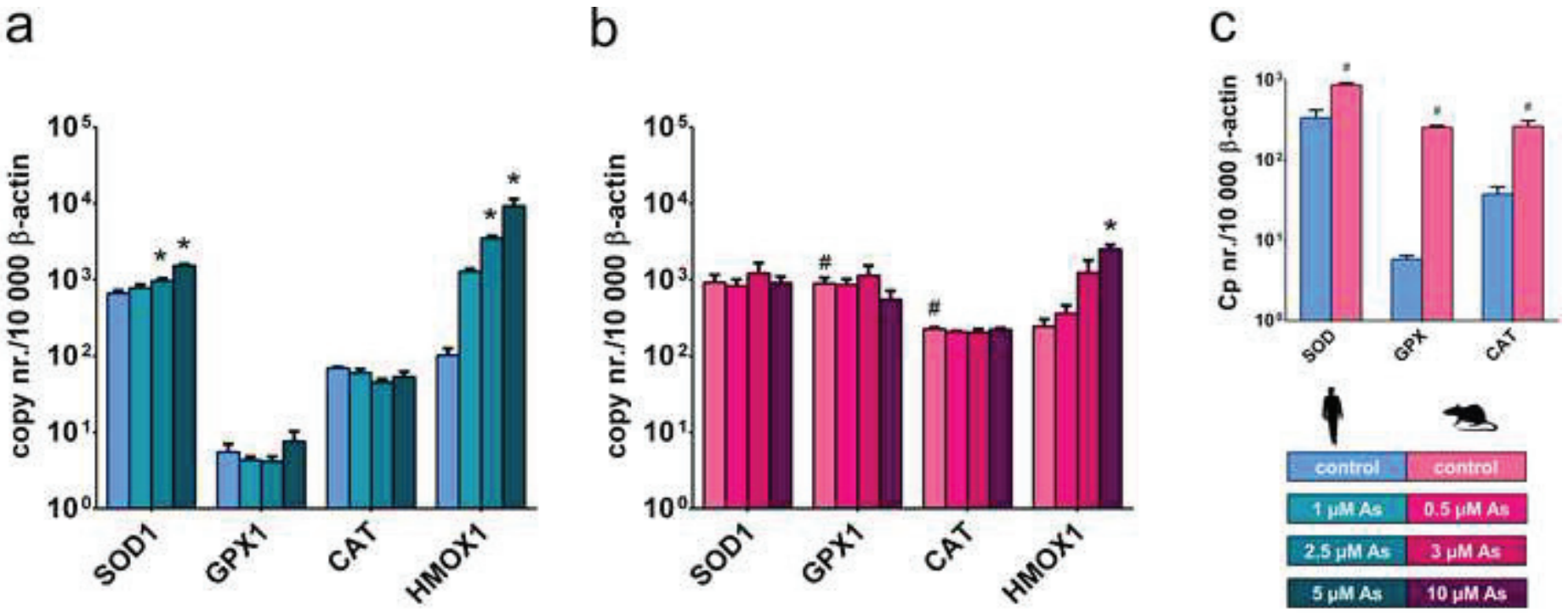

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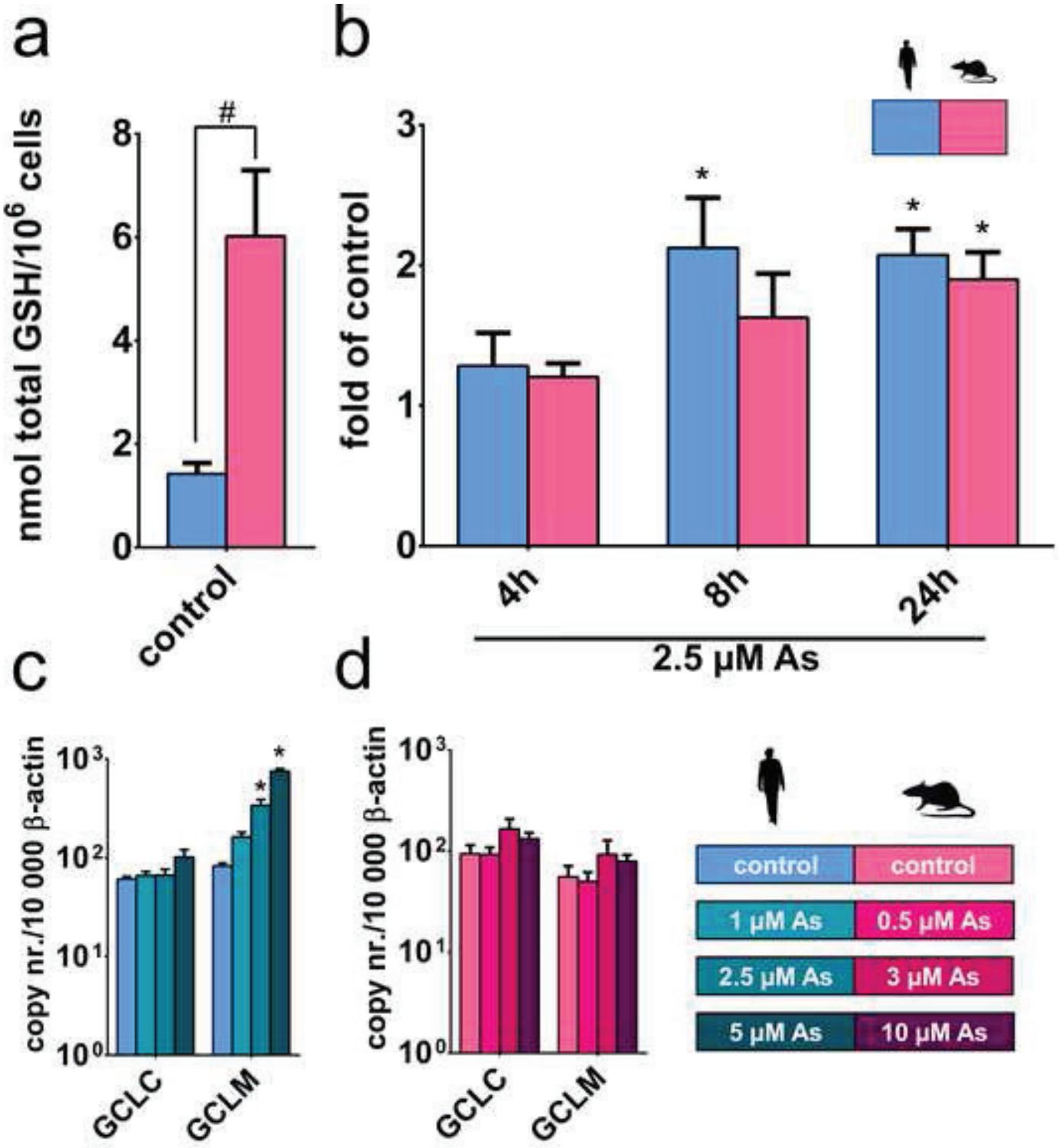

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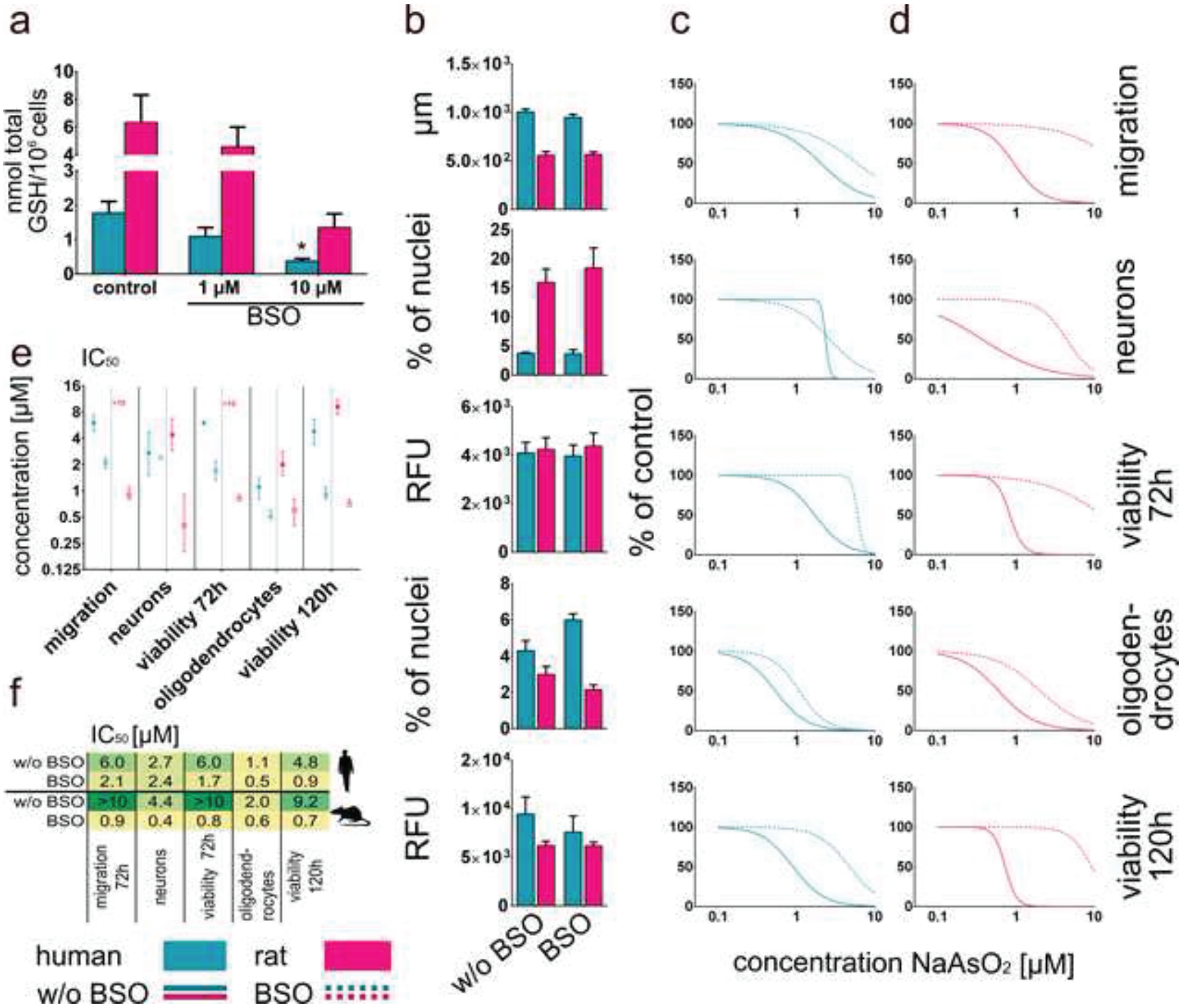

Figure 5

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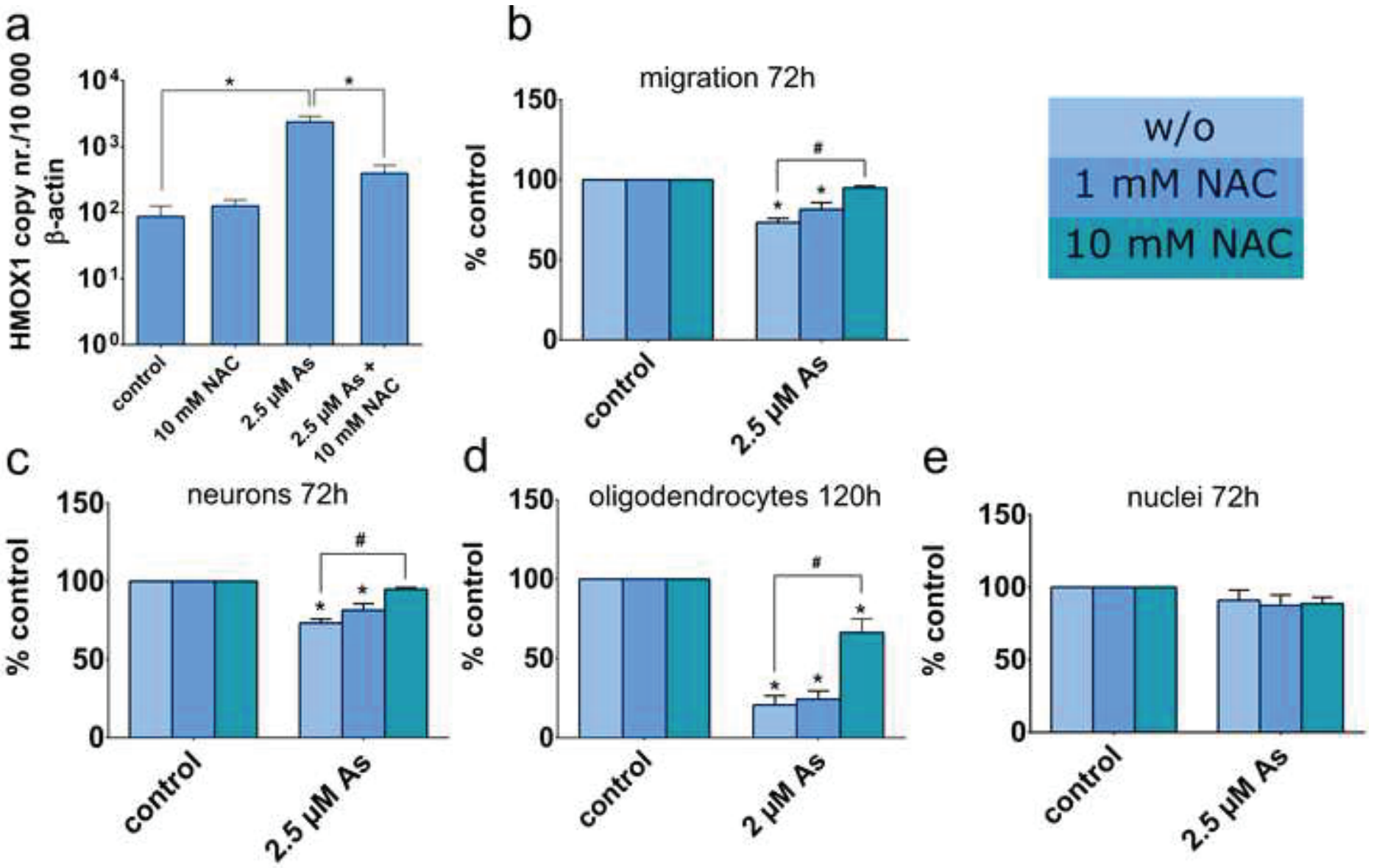

Supplementary Material

Arsenite Interrupts Neurodevelopmental Processes of Human and Rat Neural Progenitor Cells: the Role of Reactive Oxygen Species and Species-Specific Antioxidative Defense

Archives of Toxicology

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Supplementary Material and Methods

Chemicals

Sodium arsenite ($NaAsO_2$) was purchased from Sigma-Aldrich (#71287; Taufkirchen, Germany). A stock solution (100 mM) was prepared in deionized water. Working solutions were 0.1 – 10 µM diluted in N2. Concentration range for Arsenite exposure was selected on the basis of cytotoxic response with two non-cytotoxic concentration and one concentration with at least 50% response. Buthionine Sulfoximine (BSO) was purchased from Cayman Chemicals (#14484; (BSO; Ann Arbor, USA). n-Acetylcysteine (NAC) was purchased from Sigma-Aldrich (#A7250). Stock solution (1M) was prepared in PBS. All working solutions were freshly prepared in N2 medium.

Cell Culture

Human neural progenitor cells (hNPCs, two male individuals, GW16-19) were purchased from Lonza Verviers SPRL (Verviers, Belgium). Rat neural progenitor cells [rNPCs, post-natal day (PND)1] were prepared time-matched to hNPCs (Workman et al. 2013) as described previously (Baumann et al. 2014, 2015a). Experiments with human NPCs purchased from Lonza Verviers SPRL (Belgium) were ethically approved by the ethics committee of the Heinrich-Heine-University, Düsseldorf. Preparation of NPCs from rat brain tissue and experiments with rat NPCs are in accordance with German regulations and the experimental guidelines of the State Agency for Nature, Environment and Consumer Protection in North Rhine-Westphalia in Germany (LANUV) and were approved by the LANUV. Human and rat NPCs were cultured as neurospheres in proliferation medium (B27) consisting of DMEM (Life Technologies, Darmstadt, Germany) and Hams F12 (Life Technologies) (3:1) supplemented with 2% B27 (Life Technologies), 1% penicillin and streptomycin (Pan-Biotech, Aidenbach, Germany), 20 ng/ml epidermal growth factor (EGF, Life Technologies), 20 ng/ml recombinant human fibroblast growth factor (FGF, R&D systems, Wiesbaden, Germany) for hNPC, and 10 ng/ml recombinant rat FGF (R&D systems) for rNPC. The culture was maintained at 37°C with 5% CO_2. The cells were fed every two to three days by replacing half the medium, and passaged every week by mechanical chopping of the Spheres with a tissues chopper (McIlwain Tissue Chopper, Vibratome). To initiate differentiation, NPCs were plated on poly-D-lysine/laminin (Sigma Aldrich) coated dishes in differentiation medium (N2). The differentiation medium consists of DMEM (Life Technologies) and Ham's F12 (Life Technologies) at a ratio of 3 to 1 supplemented with 1% N2 (Life Technologies) and 1% penicillin and streptomycin (Pan Biotech).

Neurosphere Assay

We quantified the DNT specific endpoints migration, neuronal and oligodendrocyte differentiation along with general viability after treatment with either arsenite alone or in combination with BSO or NAC. For all analysis 5 spheres/chamber with a diameter of 0.3 mm were plated on poly-D-lysine/laminin coated 8 chamber slides in differentiation medium for 3 (neuronal differentiation) and 5 days (oligodendrocyte differentiation; Baumann et al. 2014, 2015a). In case of co-treatment, cells were pre-exposed 2h before arsenite was added.

Cell migration was analyzed by measuring the migration distance of each sphere after 72h. A src kinase inhibitor (PP2) that selectively inhibits cell migration was used as endpoint specific positive control (Baumann et al. 2014, 2015a). Neuronal and oligodendrocyte differentiation were analyzed by immunocytochemistry. Therefore, spheres were fixed after 3 or 5 days of differentiation with 4 % paraformaldehyde for 30 min at 37°C and stained for neurons (βIII-tubulin-positive cells) or oligodendrocytes (O4-positive cells). EGF and BMP7 were used as endpoint specific controls for neuronal differentiation and oligodendrocyte differentiation, respectively. Quantification of neurons and oligodendrocytes was performed in Omnisphero (Schmuck et al. 2016) by automatic (neurons) and manual (oligodendrocytes) counting. With the exception of experiments with NAC exposure cell viability was analyzed by Alamar Blue assay (CellTitier-Blue assay, Promega, Mannheim, Germany) in the same chamber/well that was used to analyze the DNT specific endpoint (Baumann et al. 2014, 2015a). Triton X-100 (0,2 %) was used as a positive control. NAC interferes with the fluorescence-based alamar blue assay (data not shown) which is why cell viability in those experiments was determined by counting cell numbers in the migration area using the Omnisphero software (Schmuck et al. 2016).

qRT-PCR analysis

For expression analysis of differentiating NPC, 100-150 spheres with a diameter of 0.1 mm were plated on poly-D-lysine/laminin (Sigma Aldrich) coated 24 well plates. After 24h of differentiation, spheres were treated with arsenite for 8h or arsenite and NAC for 4h. Total RNA was isolated from proliferating or differentiating NPC from both species. Human brain RNA was purchased from Biochain Institute, Inc (GW22), Clontech (GW20-33) and Cell Applications (GW15). Rat brain RNA was isolated from 3 different rat pubs (PND1) from different litters. Whole brain was shredded in a tissue disruptor. RNA was isolated using the RNeasy Mini Kit (Quiagen, Hilden, Germany)

according to the manufacturer's protocol. For reverse transcription of whole RNA from NPC and 500 ng RNA from brain samples the QuantiTect Rev Transcription Kit (Qiagen) was used according to the manufacturer's protocol. q-RT-PCR was performed using the Rotor Gene Q Cycler (Qiagen) with the QuantiFast SYBR Green PCR Kit (Qiagen) according to manufacturer's protocol. To allow species comparison of expression level absolute copy numbers were determined using product-specific standards. The copy number of the respective gene was normalized to 10 000 copy nr. β-actin. Primer sequences for HMOX1, GPX1, SOD1, CAT, GCLC, GCLM and ACTB are presented in suppl. Tab. 1.

Glutathione Assay

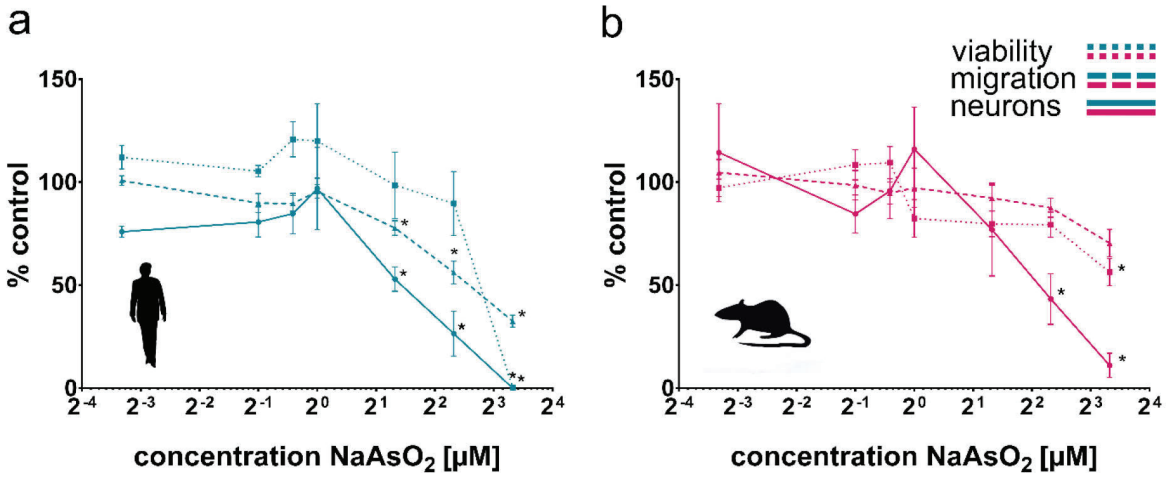
For the analysis of total GSH, spheres were chopped to 0.1 mm and plated on poly-D-lysine/laminin coated 6 well plates in a density of 600 sphere pieces/well. For GSH assay cells were differentiated for 24h and treated either with arsenite for 4, 8 or 24h or with BSO for 24h of the differentiation time. GSH levels were determined using the GSH assay kit from Sigma Aldrich. The manufacturer's protocol was adapted according to (Rahman et al. 2006). Briefly, cells were washed with cold PBS (w/o Ca^{2+}/Mg^{2+}) and detached with 500 µL accutase (Life Technologies). Digestion was stopped with cold N2, cell were pelleted at 1000g and again washed with cold PBS. Cells were lysed in 5 % sulfosalicylic acid solution by freezing and thawing in liquid nitrogen. Cell extract was centrifuged at 10.000g and the supernatant was taken for GSH measurement according to the manufacturer's protocol. The amount of GSH was calculated in nM of 10^6 cells.

Supplementary Discussion

NAC was previously used as a ROS scavenger protecting against arsenite neurotoxicity in vitro (Wang et al. 2010) and in vivo (Flora 1999). However, NAC alone also reduces migration as well as neuronal and oligodendrocyte differentiation without affecting *HMOX-1* expression or cell viability (Suppl. Fig 2). Besides its antioxidative properties, NAC has the ability to reduce disulphide bonds in proteins, to undergo autoxidation with H_2O_2 production (summarized in Zafarullah et al. 2003 and Atkuri et al. 2007), to modulate gene expression of numerous genes and to alter several signaling pathways (summarized in De Flora et al. 2001; Samuni et al. 2013). Which of these actions might be responsible for the intrinsic toxicity of NAC on developing NPC in vitro is not known at this point. To the best of our knowledge, so far no adverse effects of NAC on the developing nervous system in vitro or in vivo have been described in the literature. However, NAC is able to revert the hydrogen peroxide mediated activation of c-Src by its cysteine reducing properties in cancer cells in vitro (Krasnowska et al. 2008). c-Src is involved in NPC migration (Moors et al. 2007) and a reduction in its activity might explain NAC-mediated reduction in cell migration. NAC is considered as a safe nutritional supplement (Jenkins et al. 2016; Shahin et al. 2009) and recommended for the treatment of a variety of disorders including the prevention of premature birth and recurrent pregnancy loss (Mokhtari et al. 2017). As exemplified by our data earlier (Baumann et al. 2015b), exposure considerations are crucial not only for risk decisions relying on in vivo data, but also for interpretation of in vitro results (Wetmore et al. 2012, 2015). Here, we provide another example supporting this concept by NAC hazard combined with internal exposure assessment. NAC intake during pregnancy leads to measured cord blood concentrations between 0.1 and 1 mM (Wiest et al. 2014). Although fetal kinetics of NAC are not known, it can be assumed that NAC fetal brain concentrations will even be lower as NAC has limited abilities to cross biological barriers and does not have chemical properties required for bioaccumulation (Samuni et al. 2013). NAC interferes with NPC development at concentrations more than 10- to 100-times higher than its estimated internal exposure, while lower concentrations do not have any effect on NPC performance (data not shown).

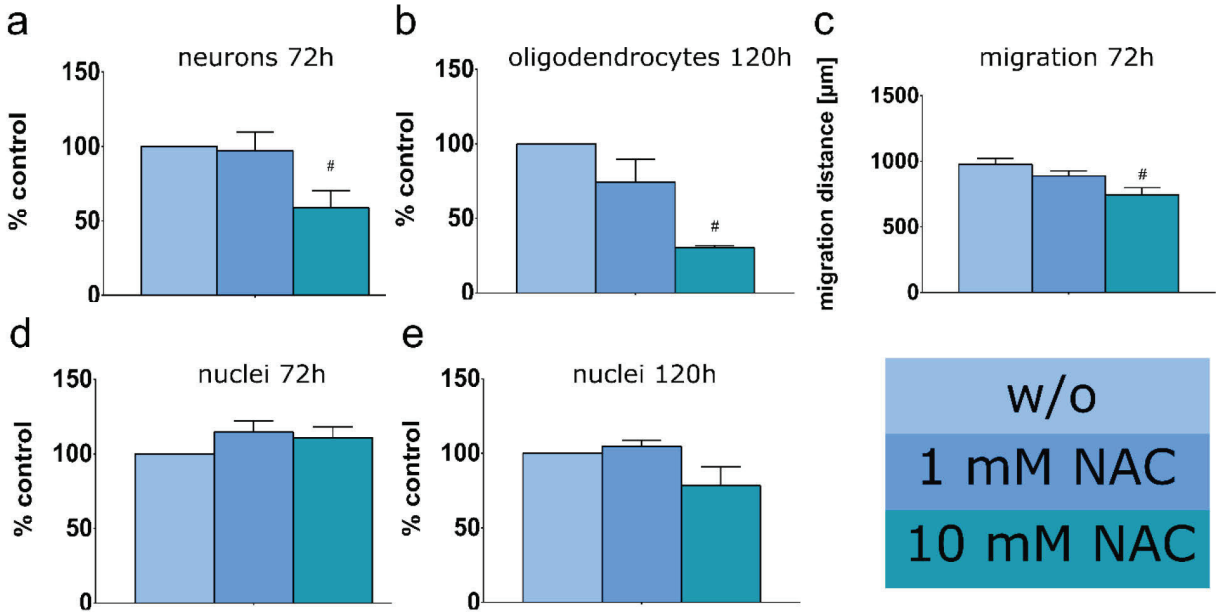
Supplementary Table 1 Primer sequence for RT-qPCR

Species	Gene	Primer 1	Primer 2
Human	ACTB	CAGGAAGTCCCTTGCCATCC	ACCAAAAGCCTTCATACATCTCA
	CAT	CGTGCTGAATGAGGAACAGA	AGTCATGGTGGACCTCAGTG
	GCLC	GCTGTTGCAGGAAGGCATTG	AGTTTGGAGGAGGGGGCTTA
	GCLM	TTCAGTCCTTGGAGTTGCACA	GGTTTTACCTGTGCCCACTGA
	GPX1	TCTGTTGCTCATAGCTGCTG	GGGGTCAAGAGGAGGAGAGA
	HMOX1	GCCATGAACTTTGTCCGGTG	GGATGTGCTTTTCGTTGGGG
	SOD1	GGCCGATGTGTCTATTGAAGA	GGGCCTCAGACTACATCCAA
Rat	ACTB	CCTCTATGCCAACACAGT	AGCCACCAATCCACACAG
	CAT	CACAGTCGCTGGAGAGTCAG	CCCACAAGGTCCCAGTTACC
	GCLC	ACAGCACGTTGCTCATCTCT	TCGTCAACCTTGGACAGTGG
	GCLM	ACATGGCATGCTCAGTCCTT	ATCTGGTGGCATCACACAGC
	GPX1	CCGGGACTACACCGAAATGA	TGCCATTCTCCTGATGTCCG
	HMOX1	CTCTGTCTCATGTAGCCTTCT	GCTTTTGGTGAGGGAAATGTG
	SOD1	GTCCAGCGGATGAAGAGAGG	CTCGTGGACCACCATAGTACG



Suppl. Figure 1 Effects of arsenite on neuronal differentiation and cell migration

Human (a) and rat (b) neurospheres were differentiated with increasing concentrations of arsenite (0.1, 0.5, 0.75, 1, 2.5, 5 and 10 µM) for 72h. Prior to fixation images of each sphere were taken for migration analysis and cell viability was analyzed by Alamar-Blue assay. Neurons were immunocytochemically stained for βIII-tubulin and nuclei were counterstained with Hoechst33258. Viability, migration and percentage of neurons/nuclei in the migration area were normalized to the solvent control and are displayed as concentration response relationship (mean ± SEM) of at least three independent experiments. * indicates a significant difference from solvent control based on one-way ANOVA ($p<0.05$).



Suppl. Figure 2 Protective effects of n-Acetylcysteine on arsenite mediated effects

hNPC were treated with NAC (1 and 10 mM) and analyzed for the effect on migration, neuronal and oligodendrocyte differentiation and number of nuclei after 72h and 120h. Mean ± SEM of at least three independent experiments normalized to control. # indicates a significant difference from respective control (w/o) based on two-way ANOVA ($p<0.05$).

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3 Discussion

We are exposed to a multiplicity of chemicals every day. Most of these chemicals benefit our everyday life. They make our lives easier, healthier or more efficient. However, to make sure that these chemicals do not compromise our health, we need to identify potential hazards and establish a safe dose of chemical exposure. The most vulnerable members of our society are children (National Research Council U.S, 1993), as especially neurodevelopmental processes are susceptible to chemicals' actions (Grandjean and Landrigan 2014). Adverse chemical action during brain development manifests as cognitive or behavioral changes like reduced intellectual abilities or antisocial behavior. In many cases these effects will not be detected until years after the exposure occurred or not be detected at all because of only small changes in the individual (e.g. reduction of only a few IQ points) or the lack of epidemiological data (Grandjean & Landrigan 2006).

For an early identification of these DNT compounds, we need to perform testing which is currently done according to two *in vivo* guideline studies that assess the DNT potential of chemicals in rodent, preferably the rat (Epa 1998; OECD 2007). These studies are only mandatory for pesticides in the United States and required solely for substances that cause neurotoxicity or endocrine disruption in the European Union (Bal-Price et al. 2015) and they have only been conducted approximately 140 times (Fritsche et al. 2017). This leaves a huge data gap on the DNT potential of thousands of chemicals present in industrial, agricultural or consumer products, some of which have shown to be neurotoxic in man (Grandjean and Landrigan 2006). It is widely accepted that this knowledge gap can only be filled by the application of alternative methods that overcome the limitation of current animal studies, such as ethical concerns, resource intensity, limited predictive power due to species differences or methodological limitations (Tsuji and Crofton 2012). In addition these methods offer the possibility to generate more mechanism based data as envisioned by the NRC (Gibb 2008).

In a recent workshop on alternative DNT testing held by the OECD and EFSA, scientists, regulators and representatives of industry agreed that there is: 'a need to develop a standardized in vitro testing battery to generate additional data on the effects of chemicals on the developing nervous system' (Fritsche et al. 2017). A testing battery should thereby consist of alternative models that closely resemble human physiology and by combining different assays represent a variety of processes crucial for human brain development in a correct developmental timing.

3.1 Alternative models for DNT testing

A variety of alternative models have been used to generate DNT readouts. These can be grouped into stem/progenitor cells, primary cells, tumor/immortalized cells and alternative organisms (Fritsche et al. 2015). In vitro cultures are mainly prepared from human, rat and mouse. For historical reasons, rat primary cells represent the most frequently used in vitro model (Fritsche et al. 2015). Rodents are still the gold standard for toxicological testing. Mouse is the preferred species for knock out studies and is therefore mainly applied to answer basic research questions. Most in vivo data on toxicological action or mechanistic understanding is therefore generated from studies in mouse or rat. Primary cells from these species in culture are useful to compare in vitro with in vivo findings within one species. These comparisons demonstrate the general applicability and increase confidence in in vitro models. Rodent primary cell based in vitro models show that many cellular and molecular functions, as well as responses to xenobiotics, present in vivo, are represented in cell culture (Bose et al. 2012; Wang et al. 2013, 2014; Yeo et al. 2013). However, the international consensus for alternative chemical testing is the use of human based models (Krewski et al. 2010; Seidle and Stephens 2009) to avoid false predictions based on species differences (Knight 2007; Leist and Hartung 2013; Schmidt et al. 2016). Tumor and immortalized cells exist of human origin, they are not restricted in material, there are many well-established protocols and they have been used in a high throughput set up in DNT screening assays (Fritsche *et al.*, 2015; Stern *et al.*, 2014). Those cells however are changed in their molecular program, raising the question, how well these cells represent physiological processes in terms of basic cellular signaling and compound sensitivity (Moors *et al.*, 2009; Radio *et al.*, 2008; Drobic *et al.*, 2006; Geerts *et al.*, 2003). Stem and progenitor cell based models are promising alternatives to primary rodent and immortalized cells. Those cells have been intensively studied and characterized over the past decade and several models have been used for compound screening (Fritsche 2016; Fritsche et al. 2015). Stem and progenitor cells can be generated from human, but also from rodent material allowing direct in vitro species comparison and the translation of in vitro findings according to the parallelogram approach (Baumann et al. 2015), which translates human in vitro results to the human in vivo situation based on the direct in vitro – in vivo comparison in rodents. Stem and progenitor cells are expandable and often consist of a physiological composition of the different brain cells which is important for a correct prediction of in vivo effects as was demonstrated by the fact, that neurons in vitro show a different sensitivity to chemical exposure when glial cells are present (Mytilineou *et al.* 1999; Witt *et al.*, 2017). Some of the stem and progenitor cell based methods are

organized in a three dimensional set up (Hoelting et al. 2013; Moors et al. 2009; Theunissen et al. 2012). These cells more closely resemble in vivo physiological conditions and it was demonstrated that 3D cultures show some physiological differences compared to 2D cultures (Yamada and Cukierman 2007). For instance the glial cell guided neuronal migration, which is an important step in brain development, cannot be mimicked in a 2D culture (Alépée et al. 2014). In an alternative testing approach, cellular models can be complemented by alternative organisms that add the advantage of a whole organism approach such as metabolism and organ-crosstalk and build the bridge between molecular and cellular endpoints to apical functional readouts like motor activity (Fritsche et al. 2015). For the formation of a DNT testing battery, a collection of suitable models, based on the current state-of-science, need to be assembled that reliably represents key processes of human brain development. Thereby the collection of stem/progenitor cell-based systems of human origin is favored, based on current scientific knowledge (Krewski et al. 2010; Seidle and Stephens 2009). However, for processes that cannot be represented by these models, tumor/immortalized or primary cells can be used.

The ‘Neurosphere Assay’ (1.4) might be a promising part of a proposed DNT testing battery. This assay utilizes human, rat and mouse NPC that mimic processes of early fetal brain development (Baumann et al. 2014, 2015a; Gassmann et al. 2012; Moors et al. 2009). Key advantages of this assay are (i) that NPCs can be generated from human and rodent origin providing human relevance and the possibility for species comparison according to the parallelogram approach, (ii) that NPCs form physiological more relevant 3D aggregates, (iii) that NPCs differentiate into a co-culture of different brain cells (neurons, oligodendrocytes and astrocytes) considering the interaction of different cell types and (iv) that multiple DNT specific endpoints (proliferation, migration, neuronal and oligodendrocyte differentiation) can be analyzed in parallel to cell viability in a medium throughput/high content setup (Schmuck et al. 2016).

To increase the confidence in the ‘Neurosphere Assay‘ for future applications in compound prioritization or human risk assessment, this model needs to be thoroughly characterized with regard to its biological application domain and its ability to correctly predict human DNT needs to be demonstrated. Therefore, the aims of this thesis were the molecular and functional characterization of the ‘Neurosphere Assay’, as well as the validation of the assay using a set of DNT-positive and negative compounds to analyze the assays´ performance characteristics. In the third part, the assay was applied for a mechanistical investigation of the DNT-positive compound arsenite to demonstrate the general applicability of the assay to perform species-specific mechanism-based analyses.

3.2 Molecular characterization of developing NPCs

NPCs, as an in vitro model for basic research or compound testing, have been characterized on a cellular and molecular level. These cells differentiate into radial glia cells, neurons, oligodendrocytes and astrocytes with a physiological cell composition (Moors et al. 2009). They mimic radial glia guided neuronal cell migration in vivo (Götz et al. 2002) and develop to different maturation stages while losing their progenitor character with advancing differentiation time (Baumann et al. 2015a; Dach et al. 2017; Moors et al. 2009; Schmuck et al. 2016). On the molecular level, several functions have been characterized in the 'Neurosphere Assay'. NPC proliferation for example can be regulated by withdrawal or addition of growth factors (Baumann et al. 2015a). Cell migration of differentiating NPC depends on the interaction with the extracellular matrix through cell-cell interaction proteins such as integrins (Barenys et al. 2016) and is regulated by ERK1/2 dependent and independent pathways (Moors et al. 2007). Oligodendrocyte differentiation and maturation is in part regulated by thyroid hormone signaling (Dach et al. 2017) and can be inhibited by the addition of bone morphogenetic protein 2 or 7 (BMP2/7), which at the same time induces the differentiation and maturation of astroglia (Baumann et al. 2015a). In addition NPCs express some of phase I and phase II enzymes (Fritsche et al. 2005) which means that they have limited metabolic activity and respond to ROS induced cell stress (Manuscript 2.3). It was further demonstrated that the effect of certain xenobiotics during brain development in vivo, could be represented in NPCs within the 'Neurosphere Assay' (see Manuscript 2.2 for more detail). These examples demonstrate that several cellular processes, their molecular regulation or xenobiotic responses, seem to be preserved in vitro. However, many of the complex processes and the molecular program during brain development, have not yet been investigated in developing NPCs in vitro. Subsequently it is necessary to gain a better understanding on the presence and function of major regulating processes and signaling pathways of human brain development in NPCs in vitro. Because of the sheer amount and complexity of regulating pathways during brain development (Silbereis et al. 2016), the identification of single pathways and their function in vitro is too elaborate to be comprehensively characterize by traditional methods like PCR or Western Blot analyses. Omics approaches, such as transcriptomics or proteomics, offer the possibility to study not only single gene or protein expression, but the complete molecular profile of a biological sample. Thereby they provide information on a cellular and molecular basis and allow a more comprehensive characterization of a biological system (Mattes 2006; Singh et al. 2010).

A valuable application for omics approaches in is the cross-species comparison. Numerous species differences in neurodevelopmental processes and brain architecture have been described (Bass et al. 1971; DeFelipe et al. 2002; Herculano-Houzel 2009; Oberheim et al. 2009; Zhang et al. 2016) and there are clear difference in general developmental timing (Clancy et al. 2001; Workman et al. 2013). These species differences lead to uncertainty and the risk of false predictions when animal-based research is translated to the human situation (Leist and Hartung 2013; Silbereis et al. 2016). The molecular signature of biological system determines structural or functional differences between species which is why a comparison of the transcriptional profile across species can give insides into species-specific molecular regulation and provide the basis for a knowledge driven translation from the animal model to humans.

In manuscript 2.1 'A transcriptome comparison of time-matched developing human, mouse and rat neural progenitor cells reveals human uniqueness' we provide a molecular characterization of developing human, mouse and rat NPCs to investigate species specificities in the molecular equipment and identify as well as functionally validate the molecular key regulators of neurodevelopmental processes using a transcriptomic approach and pharmacological modulation. In this manuscript, it is demonstrated that human and rodent NPCs underlie distinct expressional changes over matched differentiation time with only a few of all DEX genes (around 10%) being similar between the species. Although the expression dynamics of differentiating NPC are distinct, major processes that represent the multicellularity of the system, as well as key neurodevelopmental processes like migration, gliogenesis or neurogenesis, are represented on the molecular level and conserved across species (see manuscript 2.1). In gene networks of these neurodevelopmental processes, several key regulators were identified as important for brain development. In hNPCs the genes BMP2, EGFR and NOTCH appear as key regulators in all three processes. Evaluation of the biological function of these genes in several in vivo and in vitro studies demonstrates, that they are involved in the regulation of migration, neurogenesis and gliogenesis (Bond et al. 2012; Ayuso-Sacido et al. 2010; Kuhn et al. 1997; Sun et al. 2005; for more details see manuscript 2.1). A functional validation was performed by pharmacological modulation with inhibitors or activators of the respective pathway. This analysis demonstrated the importance of these regulators for cell migration, neurogenesis and oligodendrocyte formation. It further revealed that although some pathway modulation responses were comparable in all species (e.g BMP activation or EGFR inhibition on oligodendrocyte formation) there were clear species variations in other modulation responses (e.g. BMP activation on migration or Notch inhibition on oligodendrocyte formation; see manuscript

2. In general, these results demonstrate some species-specific functional responses of neurodevelopmental pathway regulation but it needs further elucidation to gain a complete understanding of human-specific neurodevelopmental outcomes. These findings, however, support the concept of human cell-based in vitro analyses for neurodevelopmental toxicity or efficacy testing. Another valuable application of an approach that provides a functional analysis of neurodevelopmental processes is the identification of chemical response signatures (phenotypes). These signatures offer valuable information for a phenotypic screening and subsequent classification of compounds according to possible compound mechanisms. Compounds, that show similar chemical response signatures, can be grouped and by comparison with signatures from specific pathway inhibition or compounds, with a known mode-of-action (MoA), mechanism based information can be generated (Kleinstreuer et al. 2014). If information from sufficient substance screening is collected, computational modeling could result in the prediction of a compounds' MoA based on structure-activity relationship information (Banerjee et al. 2016; Wang et al. 2012).

Knowledge on the molecular and cellular function of neurodevelopmental regulation in vivo is almost exclusively generated from animal models (Silbereis et al. 2016). Species differences as discussed above, however, introduce some level of uncertainty, when information on the molecular function is transferred from animal studies to the human situation. In addition, human brain development is not completely understood and there remain several signaling molecules and regulating processes that are not characterized for their role in neurodevelopment (Silbereis et al. 2016) which makes it difficult to extrapolate from rodent data. A direct comparison of transcriptional profiles between hNPCs in vitro and the developing human brain could overcome those limitations and lead to a more comprehensive characterization of the 'Neurosphere Assay'. However, there are certain technical and biological challenges in an in vitro - in vivo comparison of transcriptional information from microarray data including the variations between different array chip platforms (Affymetrix, Illumina, Agilent), different microarray chip designs or different protocols. These technical challenges do not allow the direct comparison of transcript expression (Parmigiani 2003; Shi et al. 2006) which means that only differential expression from different experiments can be compared. For our dataset the differential expression was determined for hNPC between different differentiation time points (0 vs. 3 days, 0 vs. 5 and 3 vs. 5 days). For the human brain in vivo, several studies generated datasets on the transcription of individual brain cells from different brain regions and across different developmental stages (Florio et al. 2015; Johnson et al. 2015; Kang et al. 2011; Lui et al. 2014; Thomsen et al. 2015). In a study by Kang et al. (2011) it was demonstrated

that the majority of coding genes (86%) in the human brain are regulated during brain development and that of these genes, 90% are differentially expressed across brain regions and/or time points. This observation indicates the high dynamics of the transcriptional regulation which is especially obvious during prenatal and early postnatal development and underlines the challenge of matching brain region and time point for a meaningful in vitro - in vivo comparison. Managing these challenges is part of ongoing studies of the working group and will further increase the understanding on functions, signaling pathways and processes represented in vitro.

3.3 Performance characteristics of the 'Neurosphere Assay'

Besides the characterization and identification of the application domain of an alternative model, it should be demonstrated that the model is robust, generates reproducible data, measures certain relevant endpoints and correctly predicts in vivo compound action (Eskes and Whelan 2016). To determine these key performance characteristics the model has to be challenged with a set of test compounds. Therefore compounds with a known MoA, that specifically affect one endpoint, the so called 'training set' and known DNT-positive and negative compounds that are used to determine the performance characteristics of the assay, the 'test set', are applied to an assay. The training set is used to determine the ability of an assay to correctly measure an endpoint, whereas the test set analyzes the ability of the assay to correctly identify positives (sensitivity) or negatives (specificity; Kadereit et al. 2012; Mundy et al. 2015). The 'Neurosphere Assay' was challenged with several training compounds to demonstrate its ability to measure the endpoint migration and neuronal/oligodendrocyte differentiation. Among these was the SRC kinase inhibitor PP2 which specifically inhibits the SRC kinase dependent migration (Moors et al. 2007), EGF which inhibits neuronal differentiation and activates cell migration and proliferation (Ayuso-Sacido et al. 2010; Baumann et al. 2015) and BMP7 which inhibits oligodendrocyte differentiation and induces the differentiation to astrocytes (Baumann et al. 2015; Gross et al. 1996; Mabie et al. 1997). In the 'Neurosphere Assay' these training compounds are used as endpoint-specific controls that demonstrate a consistent modulation of the respective endpoint and the reproducibility of the assay for this 'training set' (manuscript 2.2).

The selection of compounds for a 'test set' is a crucial step in the identification of the predictive power of an assay. It was mentioned earlier, that not all processes can be

represented by one in vitro assay. Therefore DNT-positive compounds, that affect neurodevelopmental processes not represented by the 'Neurosphere Assay' will not be identified as positive compounds. DNT of lead, for example, is mediated by NMDA-receptors binding and dysregulation of BDNF-Trk signaling during synaptogenesis (Bal-Price et al. 2015b; Stansfield et al. 2012). NPCs in the 'Neurosphere Assay' do not form functional synapsis (Hofrichter et al. 2017) which is why this effect of lead cannot be detected in this assay. Consequently, knowledge on the mechanism of compound action is needed to determine, if the model or a testing battery is able to correctly identify a compound. With an already limited number of 14 compounds, that have been firmly identified to affect the developing human nervous system (Grandjean and Landrigan 2014), the selection of a sufficient number of compounds is complicated and presents a bottle neck in the validation of DNT alternative methods, including the 'Neurosphere Assay'. With only a small number of chemicals, it is unlikely to cover the variety of potential mechanisms causing developmental neurotoxic outcomes (Mundy et al. 2015) and calculate robust assay performance parameters, such as sensitivity or specificity. Therefore, the extension of the 'test set' and more mechanism of action information is important to advance characterization of performance characteristics of DNT in vitro assays. Another challenge is the interpretation of in vitro effects in relation to the concentration. The general concept of toxicology states: *'Sola dosis facit venenum'* (Paracelsus, 1538) meaning that only the dose makes the poison. In case of in vitro toxicity assessment this means that it is always possible to get a positive result if only the concentration is high enough. To demonstrate that an in vitro assay predicts a known DNT positive or negative compound correctly or if it is over sensitive/not sensitive enough to measure a specific endpoint, internal in vivo exposure concentrations, preferably in the species of interest, need to be considered and compared to the in vitro effective concentration.

In the manuscript 'Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events' these condition were considered and it was analyzed if the 'Neurosphere Assay' correctly identifies a 'test set' of six well characterized DNT-positive and three DNT-negative compounds. The Manuscript further compares compound effects between human and rat NPCs. To determine a correct prediction, internal exposure data, causing adverse outcomes from human and rat, were compared to in vitro EC_{50} concentrations. The 'Neurosphere Assay' could correctly identify four out of six DNT-positive compounds and all three negative compounds by analyzing the effect on NPC proliferation, migration and neurogenesis parallel to viability. For sodium valproate and methylmercury internal concentrations causing adverse developmental

effects in human and rat in vivo are in agreement with EC_{50} values generated in the 'Neurosphere Assay'. For arsenite and MAM exposure, internal exposure data from humans was not available, but, because of the good correlation between in vitro and in vivo data in rat, it was assumed that a correlation to human in vivo is likely. Chlorpyrifos (CPF) and Parathion (PT) on the other hand could not be identified correctly as DNT compounds using the 'Neurosphere Assay'. Both compounds belong to the class of Organophosphates (OP), that are known for their cholinesterase inhibiting properties (Sultatos 2007). Thereby, the OP induced delayed neuropathy, mediated through inhibition of the neuropathy target esterase (NTE), is a main mechanism for neurotoxicity (Johnson and Henschler 1975). Consequently, the different function of cholinesterase's, especially the NTE in NPCs or the inability of neurospheres to metabolize both OP's to the more potent oxon metabolites (Das and Barone 1999; Gassmann et al. 2010; Howard et al. 2007) offer explanations why this model might not be suitable to correctly identify compounds with this specific MoA. For CPF and PT there are still some uncertainties on the exact mechanism of their DNT-potential/action which is why it can only be speculated why the 'Neurosphere Assay' was not able to make the right prediction (Prueitt et al. 2011). This example, however, underlines the importance of understanding the application domain of the assay and the mechanism by which a test compound affects brain development.

Comparison of compound action between species indicates, that for all correctly identified positive compounds, there is a difference in susceptibility. Thereby, rNPCs are more sensitive than hNPCs, with the Most Sensitive Endpoints (MSE) being neuronal differentiation. In hNPC the MSE for arsenite, MAM and valproate is NPC proliferation and for methylmercury it is neuronal differentiation (manuscript 2.2). In general, the compound set tested was too small to determine robust performance characteristics of the 'Neurosphere Assay' and draw general conclusion about differences in species sensitivity. It however demonstrates the general capability of the 'Neurosphere Assay' to correctly predict compound action and species-specific sensitivities support species differences in molecular signature and distinct responses to modulation of key signaling molecules as they have been described in manuscript 2.1. In an ongoing project on the cell biological and toxicological validation of the 'Neurosphere Assay' for future application, the working group of Prof. Fritsche is currently extending the 'test set' to 20 positive and 10 negative compounds and analyzes additional endpoints, such as oligodendrocyte formation, neurite length or neuronal density distribution. This data will feed into a more robust evaluation of performance parameters and characterize the chemical-response signature of known DNT compound in the 'Neurosphere Assay'.

3.4 Mechanistical investigations

In the current approach of toxicity testing based on animal studies, only limited information on mechanisms of chemical mediated adverse health effects is collected. Species differences and discrepancies between exposure levels in animals and the actual internal exposure in human limit the ability to predict human health effects correctly, especially if the MoA of a compound is unknown (Krewski et al. 2010). Therefore, the National research council (NRC) states in his report 'Toxicity testing in the 21st Century: A vision and a strategy' that current toxicity testing is not adequate to fully protect human health and that the field of toxicology needs to move away from an apical endpoint measure to mechanism based risk assessment (Gibb 2008). The use of alternative models offers the possibility for more and mechanism based data collection and toxicity testing in relevant species and at relevant exposure levels. Several studies already exist that have generated chemical, biological and toxicological information useful for the understanding of toxicity mechanism in alternative models (Bal-Price et al. 2015b; Fritsche et al. 2015). This information, however, needs to be evaluated and organized towards the generation of mechanisms of action. The AOP concept offers a framework that can facilitate the evaluation and organization of data with weight of evidence considerations geared towards a mechanistic understanding (OECD 2013). Thereby, information from different organizational level is causally linked to form the connection between the MIE through several KEs to the adverse outcome in the individual or a population (Bal-Price et al. 2015b).

The 'Neurosphere Assay' has been used for several mechanistic investigations (Barenys et al. 2016; Dach et al. 2017; Gassmann et al. 2014; Moors et al. 2012). For example Barenys et al. (2016) found, that the epigallocatechin gallate (EGCG) induced disturbance of migration distance and migration pattern is mediated through the binding of EGCG to the extracellular matrix protein laminin, preventing integrin-dependent cell adhesion. The data of this study was incorporated in the putative AOP 'disrupted laminin-β-integrin interaction leading to developmental neurotoxicity' and helps to understand the link between the MIE 'binding of compounds to laminin' and the adverse neurodevelopmental outcome, in this case impairment of learning (Bal-Price et al. 2016). This example demonstrates the general applicability of the 'Neurosphere Assay' to perform mechanistic investigations and the use of the generated data for AOP building.

In this thesis, the Manuscript (2.3) 'Arsenite Interrupts Neurodevelopmental Processes of Human and Rat Neural Progenitor Cells: the Role of Reactive Oxygen Species and Species-Specific Antioxidative Defense' presents another study, that uses NPCs to gain a

mechanistic understanding of the developmental neurotoxic action of the well-known DNT positive compound sodium arsenite. According to the WHO, arsenic compounds are considered a major global health concern and there are still some uncertainties on the adverse neurodevelopmental effects that occur especially at low level arsenic exposure (below provisional safe level of 10 µg As/L water; Tsuji et al. 2015; WHO 2011), as well as the mechanism by which arsenic compounds interfere with neurodevelopmental processes (Tolins et al. 2014). A mechanistic investigation of arsenite-mediated DNT in a human and rodent based cell model could therefore increase the understanding of arsenic mediated DNT and improve human risk assessment.

In this study, we characterized effects of arsenite on differentiating NPCs and tested the hypothesis if arsenite exerts its DNT potential through the generation of reactive oxygen species (ROS). The major findings of this study were (i) that arsenite affects neuronal and oligodendrocyte differentiation in human and rat NPCs independent of an effect on general viability and at concentrations that are relevant for adverse cognitive effects in vivo, (ii) that the two analyzed species demonstrate different sensitivities in the arsenite mediated cell death which is based on substantial differences in the GSH dependent antioxidative defense between human and rat NPCs and (iii) that the generation of ROS is one possible mechanism causing the reduction in cell migration, neuronal and oligodendrocyte differentiation in hNPCs (Manuscript 2.3).

The generation of ROS has been reported for several environmental toxicants and pharmaceuticals (Valko et al. 2005; Deavall et al. 2012). Anti-cancer drugs such as taxol, cisplatin or methotrexate for example cause neurotoxic symptoms like alterations in consciousness, seizures, cerebral infarctions, paralysis and neuropathy that have been associated with an increased generation of ROS (Pereira et al. 2012). Additionally, the generation of ROS is a critical KE in 6 out of 10 putative AOP's for neurotoxicity as discribed in Bal-Price et al. (2015). The developing brain is especially vulnerable towards the production of ROS because of its high metabolic demand and considerable oxygen consumption (Kennedy and Sokoloff 1957; Kety and Schmidt 1948) and the low antioxidative capacity compared to other tissues (Rodrıguez et al. 2003). Although there is no direct link between ROS formation and developmental neurotoxic outcomes in humans, it is likely, that ROS production is a critical key event in DNT related adverse outcomes which underlines the need for mechanistic studies on the role of ROS in DNT. In this study we establish a link between the KE 'formation of ROS by arsenite' and the KEs 'reduced migration, neuronal and oligodendrocyte formation'. These findings can feed into a hypothetical AOP entitled 'ROS generation in developing brain cells leading to impaired cognitive function in children'. However, to fully understand how the formation of

ROS can induce DNT, more information on the MIE and the causal linkage between key events and cognitive outcomes is needed. Therefore, it is important to consider that the production of ROS related mechanisms will not form a linear AOP with a defined MIE and AO. The AO's and KE's along the way are dependent on the ROS species that are generated, the location of ROS generation, including intracellular location, but also different brain region/cell types, and the timing and dose of exposure (Auten and Davis 2009). The role of ROS as second messengers in signal transduction (Patlewicz et al. 2015), and the differing antioxidative capacity of different neuronal cell types further complicate the understanding of ROS related adverse outcomes and underline the need for the generation of quantitative AOPs.

For arsenite, there are several mechanisms reported, that cause an induction of different ROS species (summarized in Flora 2011). Arsenite is reported to cause an opening of the permeability transition pore and binds glutathione, which in turn leads to a reduction of the cellular antioxidative defense. Both KE are linked to the thiol binding properties of inorganic arsenicals as MIE. Other MIE could be the oxidation from arsenite to arsenate producing hydrogen peroxide (Del Razo et al. 2001), or the release of iron from ferritin (Ahmad et al. 2000) causing an increased production of hydroxyl radicals through the Haber Weiss reaction (Haber and Weiss 1934). The Arsenic species, dose and timing of exposure, as well as the affected cell type, determine the MIE or following KE and can cause a slight or substantial difference in the mechanisms and related adverse outcome. An identification of the MIE and characterization of ROS species, however, was beyond the scope of this study, but should certainly be the aim of future research to better understand the hypothetical AOP described here. In summary, our study improves the understanding of a potential DNT mechanism not only for arsenite but also for other ROS producing compounds. However, there is still a data gap concerning a characterization of ROS species and the causal relationship between KE and adverse neurodevelopmental outcomes. The combination of data generated in mechanism based alternative models, animal intervention studies, computational approaches and epidemiological studies organized in the AOP concept will fill these data gaps and will enable informed human hazard identification and risk assessment in the future.

3.5 The ‘Neurosphere Assay’ as part of a DNT testing battery

In the three manuscripts presented in this thesis I provide a biological characterization of the ‘Neurosphere Assay’, analyze the ability of the assay to predict DNT positive and negative compounds correctly and apply the assay for a mechanistic investigation of the mechanisms of arsenite induced DNT, all of which was performed in a species comparative manner. As discussed earlier, the ‘Neurosphere Assay’ is a well-suited alternative model to be used in a DNT testing battery. The data I present here, further supports this view. Knowledge on the biological application domain of the ‘Neurosphere Assay’ was broadened and the general suitability of the assay to correctly predict the DNT potential of a ‘test set’ of positive and negative compounds was demonstrated. These results further increase the confidence in the application of this assay as part of a DNT testing battery. The species differences, that were identified in the molecular signature, the sensitivity towards compound action and the mechanism of arsenite-mediated DNT, support the generally accepted view, that a human based system should be favored to predict human toxicity (Krewski et al. 2010; Seidle and Stephens 2009). They further demonstrate that a translational approach based on the knowledge from in vitro species comparisons can predict human hazard more accurately than the conventional approach that uses safety factors for toxicodynamic and toxicokinetic species differences (Burgess-Herbert and Euling 2013).

To gain regulatory acceptance for human risk assessment, alternative methods need to undergo a complex process of method validation that includes the assessment of intra- and inter laboratory variability, predictive power/capacity, application domain, performance standards and transferability (Hartung et al. 2004). The high effort in terms of time and money, the limited number of test chemicals as discussed above (3.3), as well as the fact that a whole battery of assays needs to be validated before an alternative approach gets regulatory acceptance, are the main reasons hindering the validation of alternative assays for DNT testing. Therefore, it was proposed recently, that the validation procedure should be adapted according to the intended use of the assay. If the assay is for example used for prioritization or read across decisions, the validation could be less intensive and accept a higher level of uncertainty (Judson et al. 2013).

In a recent workshop on the use of non-animal test methods for regulatory purposes, scientists from academia, regulatory agencies and industry agreed, that the assembly of a DNT testing battery and testing of chemicals across different laboratories in alternative

assays marks the next necessary step for the advancement of alternative DNT testing (Fritsche et al. 2017). Sufficient assays have been developed that provide a high enough level of confidence to be used for screening and prioritization of compounds (Fritsche 2016; Fritsche et al. 2015). For human risk management decisions, available assays do not offer sufficient confidence yet and need to undergo a solid scientific validation with the development of standard data requirements (Fritsche et al. 2017). The start of compound screening for prioritization, however, will increase assay confidence and generate data that can be used for AOP building. This will further support the creation of chemical test sets for a solid validation process, but also refine animal studies or inform epidemiological information (Bal-Price et al. 2017).

A proposed testing strategy as a basis for DNT evaluation, is the combination of alternative, in silico and in vivo approaches (Fritsche 2016). Thereby, the first step is the generation of kinetic data in computational models, like physiologically based pharmacokinetic modeling, which can provide information on relevant human internal exposure levels or relevant metabolites. The second and third steps consist of a compound screening across a battery of in vitro and alternative organism based methods to analyze the effect across neurodevelopmental key events and determine the MSEs. Chemicals, that affect any of the endpoints at relevant concentrations should than be considered for further in vivo testing. Before in vivo testing, a translational approach, that uses rodent in vitro models and compares test results with human in vitro findings, should be performed to determine if the effects is species-independent. This testing strategy will reduce the amount of in vivo studies to a necessary and relevant limit and at the same time generate data on compound action for an increased mechanistical understanding. Thereby, it will advance human risk assessment as outlined by the NRC (NRC 2007) and the European roadmap on future risk assessment approaches (Leist et al. 2014).

Despite clear advantages and possibilities for future toxicity testing, it needs to be considered, that as a model system, similar to animal models, alternative models are subjected to certain limitations. A general limitation is the lack of physiological context in a tissue or organ like structure (Haycock 2011). Although some models offer a 3D set up (Baumann et al. 2015a; Hoelting et al. 2013; Monnet-Tschudi et al. 2000; Moors et al. 2009; Theunissen et al. 2012), they are still limited in their representation of the complex non repetitive 3D cellular assembly, that determines the connectivity and function of the human brain (Alépée et al. 2014). The moving field of tissue engineering and application of 3D bio printing will certainly advance model development and allow for the generation of more complex brain like structures (Ji and Guvendiren 2017). Another major limitation is the lack of physiological pharmacokinetics. In vitro models cannot represent processes

such as Absorption, Distribution, Metabolism and Excretion (ADME). Especially the functionality of biological barriers, such as the blood brain barrier and the placental barrier, greatly determine the hazardous potential of a chemical in the developing nervous system and should be mimicked in in vitro or in silico approaches. Physiologically based pharmacokinetic modeling can be used to characterize a chemicals' potential to cross biological barriers, determine relevant in vivo concentrations and identify relevant metabolites (Lipscomb et al. 2012). Such methods can be combined with in vitro models to facilitate testing of relevant concentrations and metabolites. Another promising approach is the use of 'organ on a chip' models that connect different in vitro models in a microfluidics system with artificial blood circulation and thereby mimic some aspects of ADME as well as inter-organ crosstalk (Maschmeyer et al. 2015; Schimek et al. 2013). These chips could even include certain barrier models as demonstrated by Lee *et al.* (2016), who developed a human based placenta-on-a-chip. A limitation of the 'Neurosphere Assay' in particular, is the absence of cell types like microglia and endothelial cells. Models, that do not contain microglia, are for example not able to predict effects associated to neuroinflammation as demonstrated by He et al. (2010). The 'Neurosphere Assay' consists of NPCs that are isolated from whole brain and thus does not represent a specific brain region. Cells can, however, differ in their function depending on brain region (Hewett 2009) and different brain regions differ with regard to compound susceptibility. Methyl mercury for example specifically inhibits proliferation of astrocytes in the hippocampus, while the effect is non-specific in astrocytes of the cerebellum, the cortex or the brain stem (Costa et al. 2007). Certain parts of brain development like the generation of synapses or the formation of functional neuronal networks (Baumann et al. 2015; Hofrichter et al. 2017) are also not represented in the 'Neurosphere Assay' in its present state, which is why these processes need to be represented by an additional assay within a DNT testing battery. The use of neurospheres from iPSC for example offers the possibility to perform microelectrode array recordings for studying compounds' effects on neuronal network formation including synaptogenesis (Hofrichter et al. 2017; Odawara et al. 2014, 2016). In general, cognitive and behavioral aspects, especially on complex human behavior, cannot be represented in alternative models. Therefore, we need thorough understanding of the MoA of compounds causing adverse cognitive and behavioral outcomes in order to translate findings from an in vitro battery to human risk assessment. It will be the challenge of ongoing research to identify these causal connections. Thereby, the combination of data from alternative models, in vivo and epidemiological studies organized in the AOP concept, will elucidate these unknowns in the future. At the same time, future research needs to advance and further characterize alternative models based on the steadily increasing knowledge on human brain

development to overcome current challenges and limitations of alternative testing and ultimately improve human risk assessment without the need of animal experiments.

4 Abstract

Chemical-induced developmental neurotoxicity (DNT) presents a long-underestimated health risk to our society. There is a consensus that more chemicals need to be tested for their potential to induce DNT to fill the current knowledge gap and facilitate a better human risk assessment. Laboratory animals are no suitable model for large scale DNT testing as they consume high amounts of money, time and animals and are subject to uncertainties in their methodology and predictive power due to species differences. Alternative models, assembled in a DNT testing battery, can evaluate chemical effects on many major neurodevelopmental key events and overcome several limitations of in vivo testing. A well-suited model, as part of a DNT testing battery, is the 'Neurosphere Assay', which employs human and rodent neural progenitor cells (NPC) grown as three dimensional neurospheres that mimic several neurodevelopmental key events like cell proliferation, migration and differentiation into the three major brain effector cells.

The three manuscripts included in this thesis, provide a biological characterization and validation of the 'Neurosphere Assay', analyze the ability of the assay to correctly predict DNT positive and negative compounds and apply the assay for a mechanistic investigation of arsenite induced DNT, all of which was performed in a species comparative manner. Analyses of the mRNA profile of developing human, mouse and rat NPCs demonstrate that although the different species are subject to distinct changes of their expression profiles over differentiation time, processes representing the multicellularity of the system as well as key neurodevelopmental processes like migration, neurogenesis and gliogenesis are overrepresented in all species. Based on these profiles, key regulators of neurodevelopmental processes were identified (BMP2, NOTCH1, EGFR). Their pharmacological modulation using specific inhibitors demonstrated the importance of these regulators for developing neural progenitor cells and revealed species-specific cellular responses.

Furthermore this thesis demonstrates that human and rat NPCs are able to correctly identify seven compounds out of a test set of six DNT-positive and three DNT-negative compounds and that most of these compounds again reveal species-specific sensitivities. Finally, by applying the 'Neurosphere Assay' for the molecular investigation of the mechanism of arsenite-induced DNT, I showed that arsenite interferes with the neurodevelopmental key events neuronal and oligodendrocyte differentiation possibly through the generation of reactive oxygen species.

In summary, this thesis increases the confidence in the 'Neurosphere Assay' and supports its application as part of a DNT testing battery. The Assay was used to identify species differences in the molecular signature of transcriptome data and the sensitivity towards DNT compounds and to unravel the underlying molecular mechanism of the DNT compound Arsenite. This work further demonstrates that a human based alternative system should be favored to predict human toxicity and the importance of translational approaches that, based on the knowledge of in vitro species comparisons, predict human hazard more accurately than the conventional in vivo approaches in rodents.

5 Zusammenfassung

Chemikalien-induzierte Entwicklungsneurotoxizität (ENT) stellt ein lange unterschätztes Gesundheitsrisiko für die Gesellschaft dar. Es herrscht Einigkeit darüber, dass mehr Chemikalien auf ihr entwicklungsneurotoxisches Potenzial getestet werden müssen, um existierende Datenlücken zu füllen und eine bessere Risikobewertung zu ermöglichen. Labortiere sind kein geeignetes Model für die Testung einer hohen Anzahl an Chemikalien, da sie zu kosten- und zeitintensiv sind, zu viele Tiere verbraucht werden und es Unsicherheiten in der Methodik und der Vorhersagekraft gibt. Alternative Modelle, die zu einer ENT-Testbatterie zusammengestellt werden, könnten die Effekte einer Vielzahl von Chemikalien auf verschiedene wesentliche Prozesse der Gehirnentwicklung untersuchen und überwinden dabei einige Limitationen der in vivo Testung. Ein geeignetes Model als Teil einer ENT-Testbatterie ist der ‚Neurosphären Assay', welcher neurale Progenitorzellen (NPC) von Mensch und Nager, gewachsen als dreidimensionale Neurosphären, verwendet und verschiedene Schlüsselereignisse, wie Proliferation, Migration und Differenzierung in die drei Hauptzelltypen des Gehirns, abbildet.
Die drei Manuskripte in dieser Dissertation zeigen eine biologische Charakterisierung und Validierung des ‚Neurosphären Assays`, analysieren die Fähigkeit des Assays entwicklungsneurotoxische Positiv- und Negativsubstanzen korrekt vorauszusagen und verwenden den Assay für eine mechanistische Untersuchung der Arsen-induzierten ENT. Dabei wurden all diese Punkte in einer Spezies-vergleichenden Weise durchgeführt. Die Analyse von mRNA Profilen in sich entwickelnden neuralen Progenitorzellen von Mensch, Maus und Ratte hat gezeigt, dass, obwohl die verschiedene Spezies unterschiedlichen Änderungen ihrer Expressionsprofile über die Zeit der Differenzierung unterliegen, wesentliche Prozesse der Gehirnentwicklung wie Migration, Neurogenese oder Gliogenese in allen Spezies überrepräsentiert sind. Basierend auf den mRNA Profilen konnten Schlüsselregulatoren für verschiedene dieser Prozesse identifiziert werden (BMP2, NOTCH1, EGFR). Die pharmakologische Modulation dieser Regulatoren definierte deren Bedeutung für die Entwicklung neuraler Progenitorzellen und deckte zudem Spezies-spezifische zelluläre Antworten auf. Des Weiteren demonstriert diese Dissertation, dass Neurosphären von Mensch und Ratte in der Lage sind, sieben Chemikalien aus einem Testsatz, bestehend aus sechs ENT-Positiv- und drei ENT-Negativsubstanzen, korrekt zu identifizieren und dass es auch hier für die meisten dieser Chemikalien Unterschiede in der Sensitivität zwischen den Spezies gibt. Durch die molekulare Untersuchung des Mechanismus der Arsen-induzierten ENT im ‚Neurosphären Assays', zeigt diese Dissertation, dass Arsen, wahrscheinlich über die Generierung reaktiver Sauerstoff Spezies, die Prozesse neuronale- und Oligodendrocyten-Differenzierung stört.
Zusammenfassend, steigern diese Ergebnisse das Vertrauen in den ‚Neurosphären Assay' und unterstützen dessen Anwendung als Teil einer ENT-Testbatterie. Diese Methode ist in der Lage Speziesunterschiede in der molekularen Signatur von Transkriptomdaten und in der Sensitivität gegenüber ENT-Substanzen aufzudecken und ist zudem geeignet die molekularen Mechanismen der ENT aufzuklären. Diese Arbeit zeigt weiter, dass auf menschlichen Zellen basierende Modellsysteme für die Vorhersage humaner Toxizität bevorzugt werden sollten und das ein translationaler Ansatz, basierend auf dem Wissen von in vitro Speziesvergleichen, eine bessere Risikobewertung im Menschen ermöglicht als derzeit übliche in vivo Verfahren im Nager.

Abbreviations

ADHD	attention deficit hyperactivity disorder
ADME	absorption, distribution, metabolism and excretion
ANOVA	analysis of variance
ANXA1	annexin A1
AOP	adverse outcome pathway
ARHGAP11B	rho GTPase activating protein 11B
BBB	blood-brain barrier
BDE	polybrominated diphenyl ether
BDNF	brain derived neurotrophic factor
BMBF	German Ministry of Education and Research
BMP	Bone morphogenetic protein
BP	biological process
BSO	buthionine sulfoximine
CALB2	calbindin 2
CAS	chemical abstracts service
CAT	catalase
CI	confidence interval
CM	cardiomyocyte
CNS	central nervous system
CPF	chlorpyrifos
CRYAP	crystallin alpha B
DAPT	n-[n-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DEX	differential gene expression
DMA	Dimethylarsinic
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNT	developmental neurotoxicity
ECM	extracellular matrix
EFSA	European Food Safety Authority
EGCG	epigallocatechin gallate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EHP	Environmental Health Perspectives
ENT	Entwicklungsneurotoxizität
EOMES	eomesodermin
EPA	Environmental Protection Agency
EPHA2	EPH receptor A2
ESC	embryonic stem cell
FAM107A	family with sequence similarity 107 member A
FCS	fetal calf serum
FDR	false discovery rate
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GCLC	glutamate-cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase modifier subunit
GFAP	glial fibrillary acidic protein
GLAST/ SLC1A3	solute carrier family 1 member 3
GLUT	solute carrier family 2 member 1
GO	gene ontology
GPX1	glutathione peroxidase 1
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione-s-transferase
GW	gestational week
HCA	high content image analysis or hierarchical cluster analysis

HES6	hairy and enhancer of split 6
hESC	embryonic stem cell
hiPSC	human induced pluripotent stem cell
HMOX1	heme oxygenase 1
HS6ST2	heparan sulfate 6-O-sulfotransferase 2
IATA	integrated approaches for testing and assessment
IPC	intermediate progenitor cells
IQ	intelligence quotient
JUN	Jun proto-oncogene, AP-1 transcription factor subunit
KE	key event
KR	key regulator
LDH	lactate dehydrogenase
LOAEC	lowest observed adverse effect concentration
LRRC3B	leucine rich repeat containing 3B
LYN	LYN proto-oncogene, Src family tyrosine kinase
MAM	methylazoxymethanol acetate
MAP2	microtubule-associated protein 2
MBP	myelin basic protein
MDR1	multidrug resistance protein 1
MIE	molecular initiating event
MRP1	Multidrug resistance-associated protein 1
MSE	most sensitive endpoint
MYC	MYC proto-oncogene, bHLH transcription factor
NAC	n-acetyl cysteine
NAR	nucleic acid research
NES	nestin
NMDA	N-Methyl-D-aspartic acid
NPC	neural progenitor cell
NRC	National Research Council
NRXN1	neurexin 1
NT	neurotoxicity

NT4	neurotrophin 4
NTE	neuropathy target esterase
OATP	solute carrier organic anion transporter family member 1A2
OECD	Organisation for Economic Co-operation and Development
OP	organophosphates
ORA	overrepresentation analyses
OSVZ	outer subventricular zone
PCA	principal Component Analysis
PCW	post conceptual week
PDGFRB	platelet derived growth factor receptor beta
PND	post-natal day
PT	parathion
PTPRZ1	protein tyrosine phosphatase, receptor type Z1
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
RFU	relative fluorescence unit
RMA	robus multichip average
RNA	ribonucleic acid
ROS	reactive oxygen species
S100b	S100 calcium binding protein B
SC	solvent control
SEM	standard error of the mean
SOD1	superoxide dismutase 1
SPARCL1	SPARC like 1
SRC	SRC proto-oncogene, non-receptor tyrosine kinase
TG	test guideline
TNC	tenascin C
US	United States
VEGFA	vascular endothelial growth factor A
VIM	vimentin
WHO	World Health Organization

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Albert Einstein

Eidesstattliche Erklärung/Declaration

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit „Molekulare und funktionelle Untersuchung toxischer Signalwege in der Entwicklungsneurotoxizität von Chemikalien in neuralen Progenitor Zellen von Mensch und Nager“ selbständig verfasst und ausschließlich die von mir angegebenen Hilfsmittel verwendet habe. Die Dissertation wurde in der vorgelegten oder einer ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

I declare that I have developed and written the enclosed Thesis “Molecular and functional analysis of toxicity pathways contributing to chemical induced developmental neurotoxicity in neural progenitor cells of human and rodent” completely by myself, and have not used sources or means without declaration in the text. Any thoughts from others or literal quotations are clearly marked. The Thesis was not used in the same or in a similar version to achieve an academic grading elsewhere.

Stefan Masjosthusmann

Düsseldorf, November 2017