

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

Jenny Baumann<sup>1</sup> · Kathrin Gassmann<sup>1</sup> · Stefan Masjosthusmann<sup>1</sup> · Denise DeBoer<sup>1</sup> · Farina Bendt<sup>1</sup> · Susanne Giersiefer<sup>1</sup> · Ellen Fritsche<sup>1</sup>

Received: 1 April 2015 / Accepted: 6 July 2015 / Published online: 28 July 2015  
© Springer-Verlag Berlin Heidelberg 2015

**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

---

Jenny Baumann and Kathrin Gassmann have contributed equally to this work.

---

**Electronic supplementary material** The online version of this article (doi:10.1007/s00204-015-1568-8) contains supplementary material, which is available to authorized users.

---

✉ Ellen Fritsche  
ellen.fritsche@uni-duesseldorf.de;  
Ellen.Fritsche@IUF-Duesseldorf.de

<sup>1</sup> Modern Risk Assessment and Sphere Biology, IUF - Leibniz Research Institute for Environmental Medicine, Auf'm Hennekamp 50, 40225 Duesseldorf, Germany

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, post-natal 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

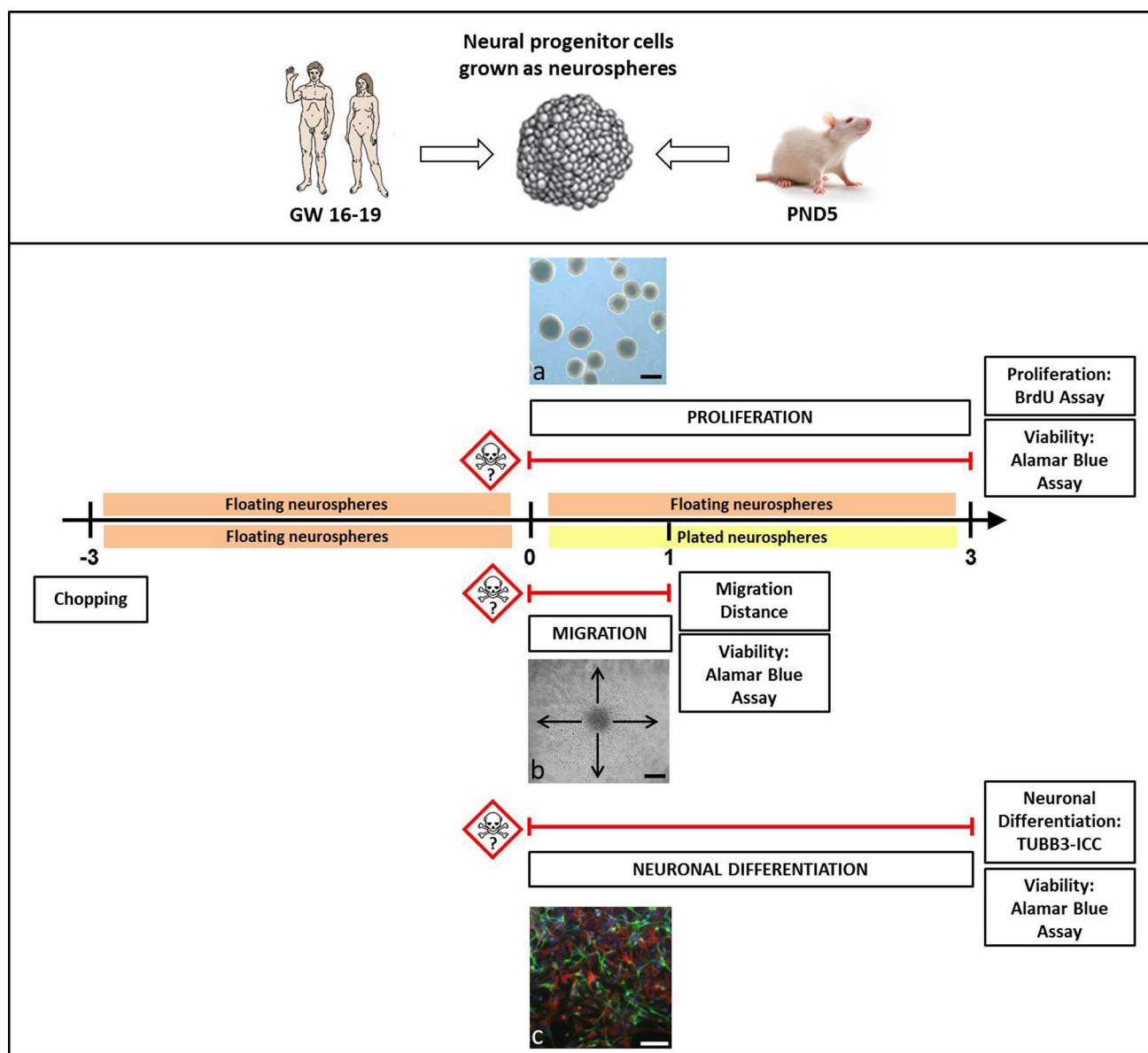
For the cytotoxicity measurement the lactate dehydrogenase (LDH) assay (CytoTox-One; Promega, Mannheim, Germany) was used as described previously (Baumann et al. 2014). For details, see Supplementary Material.

### 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  $\mu$ M 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).

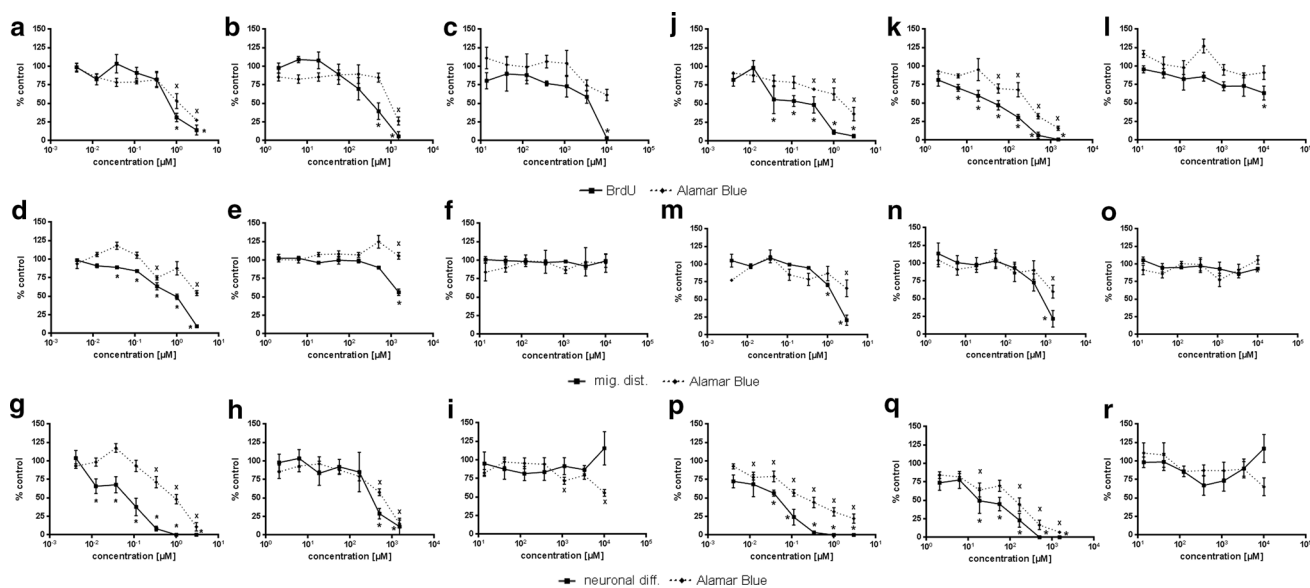
### Differentiation analysis

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 dif-

ferentiation (neuronal diff.) and the respective viability data (Alamar Blue)  $\pm$  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  $\mu$ l 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  $\pm$  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 non-linear 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<sub>50</sub> 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
<b>MeHgCl</b>												
EC <sub>50</sub>	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
<b>AsNaO2</b>												
EC <sub>50</sub>	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	
<b>CPF</b>												
EC <sub>50</sub>	>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		
<b>Parathion</b>												
EC <sub>50</sub>	>257.5	>257.5	>257.5	>257.5	>257.5	>257.5	>257.5	>257.5	252.5	>257.5	>257.5	257.2
95 % CI												
<b>MAM</b>												
EC <sub>50</sub>	325.7	37.82	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
<b>NaVPA</b>												
EC <sub>50</sub>	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
<b>Glutamate</b>												
EC <sub>50</sub>	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
<b>Paracetamol</b>												
EC <sub>50</sub>	2219	790.9	3884	4113	>5000	>5000	>5000	>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
<b>PenG</b>												
EC <sub>50</sub>	2572	>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<sub>50</sub> 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  $\beta$ III-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  $190,238 \pm 20,102$  RLU to  $50,673 \pm 18,312$  RLU in hNPCs, and from  $64,534 \pm 13,155$  RLU to  $23,307 \pm 6242$  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  $404 \pm 12$  to  $247 \pm 12$   $\mu$ m 24 h after plating, and from  $456 \pm 39$  to  $73 \pm 18$   $\mu$ m 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:  $9.6 \pm 0.6$ – $1.9 \pm 0.3$  % neurons; rNPCs:  $15.2 \pm 1.6$ – $0.6 \pm 0.3$  % 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,

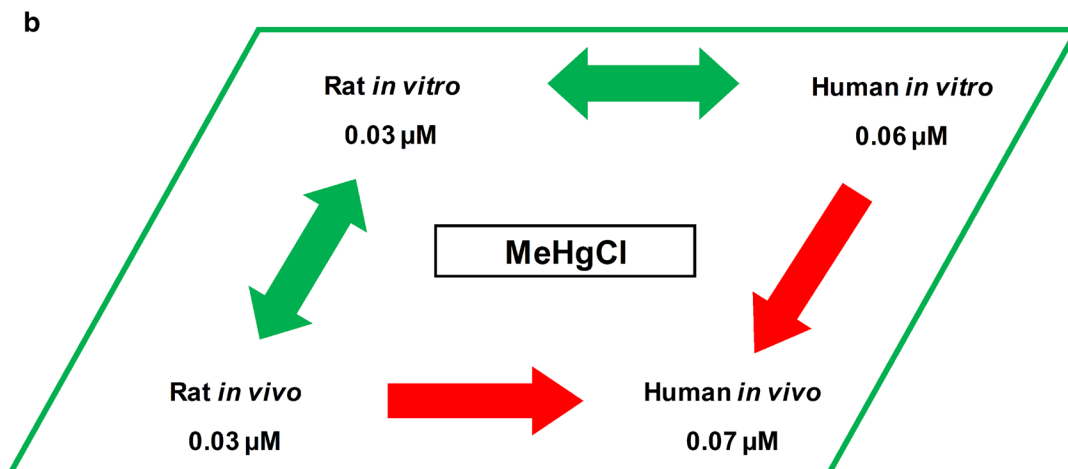
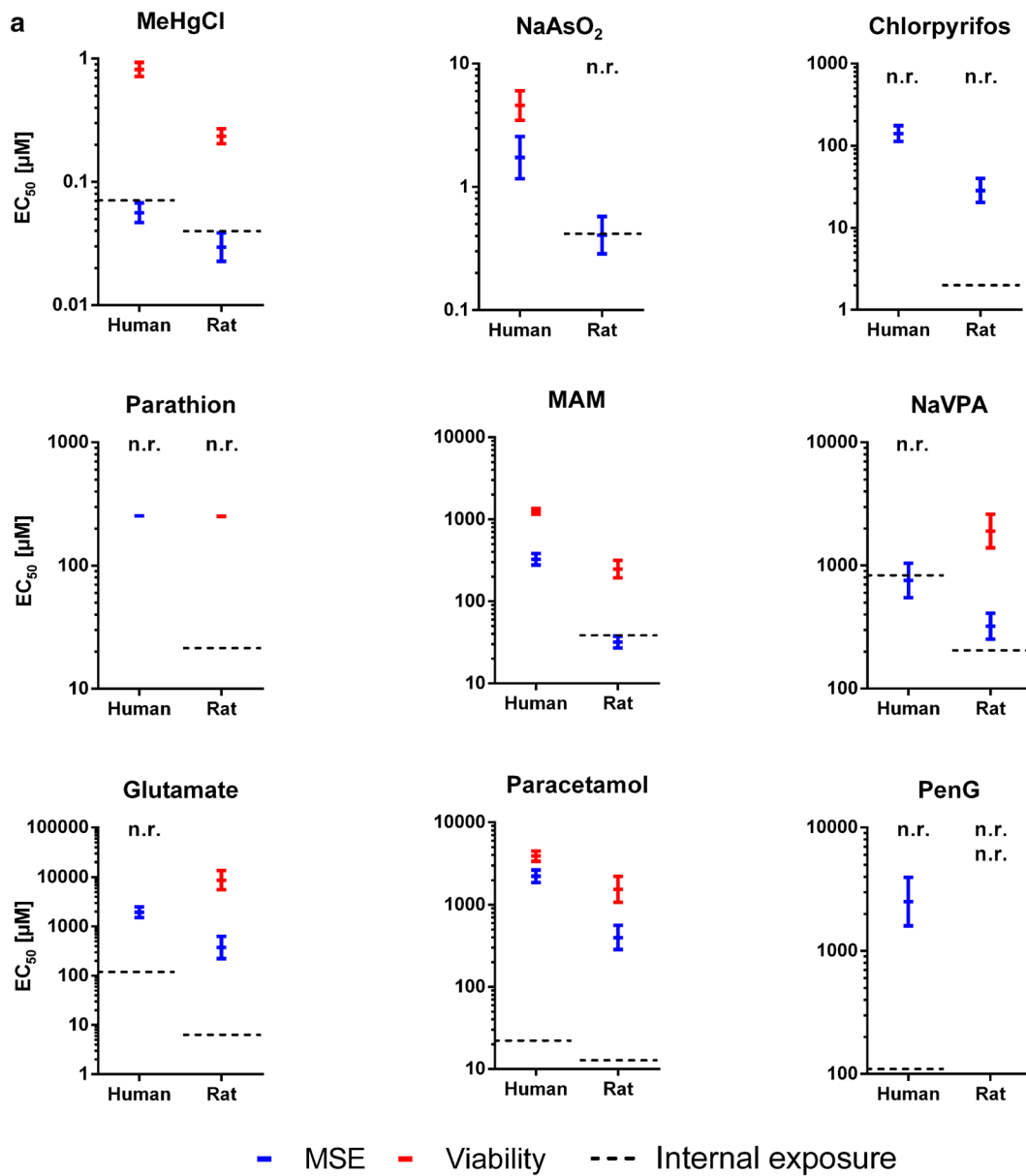
**Fig. 3** Pairwise comparison of the most sensitive endpoint and viability between human and rat neurospheres. **a**  $EC_{50}$  values of the most sensitive endpoint (MSE) and viability in human and rat neurospheres for each testing compound are shown with its 95 % confidence intervals and, if available, 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<sub>2</sub>—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)

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.

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<sub>2</sub> treatment, hNPC proliferation was the MSE ( $EC_{50} = 1.728$   $\mu$ M; Suppl. Fig. 2; Table 1), whereas neuronal differentiation was inhibited most potently in rNPC ( $EC_{50} = 0.4061$   $\mu$ M, Suppl. Fig. 4; Table 1).  $EC_{50}$  values for viability were either higher than the MSE (human: 4.574  $\mu$ M) or not reached at all (rat; Suppl. Fig. 2 and 4; Table 1), supporting specific DNT effects of NaAsO<sub>2</sub>. 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  $\mu$ M;



Suppl. Fig. 4; Table 1). In contrast, proliferation was the MSE in rNPCs (28.54  $\mu\text{M}$ ; Suppl. Fig. 2; Table 1) with the  $\text{EC}_{50}$  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  $\mu\text{M}$ ) and viability under differentiating conditions in rNPCs (251.2  $\mu\text{M}$ ; 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  $\text{EC}_{50}$  value for the endpoint migration was not reached, the highest concentration tested (257  $\mu\text{M}$ ) 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  $\text{EC}_{50}$  = 325.7  $\mu\text{M}$ , rat  $\text{EC}_{50}$  = 31.82  $\mu\text{M}$ ) as effects between proliferation and viability deviated most for both species (human  $\text{EC}_{50}$  = 1245  $\mu\text{M}$ , rat  $\text{EC}_{50}$  = 247  $\mu\text{M}$ ; 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 ( $\text{EC}_{50}$  = 756.3  $\mu\text{M}$ , Suppl. Fig. 2; Table 1) without affecting viability ( $\text{EC}_{50}$  not reached within tested concentration range). In contrast, NaVPA reduced proliferation and neuronal differentiation in rNPCs at similar concentrations ( $\text{EC}_{50}$  = 379.5  $\mu\text{M}$  and  $\text{EC}_{50}$  = 321.1  $\mu\text{M}$ , respectively) distinguishable from effects on viability ( $\text{EC}_{50}$  = 4019  $\mu\text{M}$  and  $\text{EC}_{50}$  = 1903  $\mu\text{M}$ , 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  $\text{EC}_{50}$  value of 1938  $\mu\text{M}$  (Suppl. Fig. 2; Table 1). In rNPCs, neuronal differentiation was specifically inhibited at lower concentrations ( $\text{EC}_{50}$  = 374.6  $\mu\text{M}$ , viability:  $\text{EC}_{50}$  = 8655  $\mu\text{M}$ ; Suppl. Fig. 4; Table 1), making the rat again the more sensitive species.

Paracetamol specifically inhibited proliferation in hNPCs ( $\text{EC}_{50}$  = 2219  $\mu\text{M}$ , viability:  $\text{EC}_{50}$  = 3884  $\mu\text{M}$ ; 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 ( $\text{EC}_{50}$  = 399.1  $\mu\text{M}$ , viability:  $\text{EC}_{50}$  = 1538  $\mu\text{M}$ ; Suppl. Fig. 4; Table 1).

Last, penicillin G only had a specific effect on proliferation in hNPCs ( $\text{EC}_{50}$  = 2512  $\mu\text{M}$ ), whereas in rNPCs the  $\text{EC}_{50}$  value was not reached for any of the endpoints (Fig. 2; Table 1) although the highest concentration (10,000  $\mu\text{M}$ ) 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).

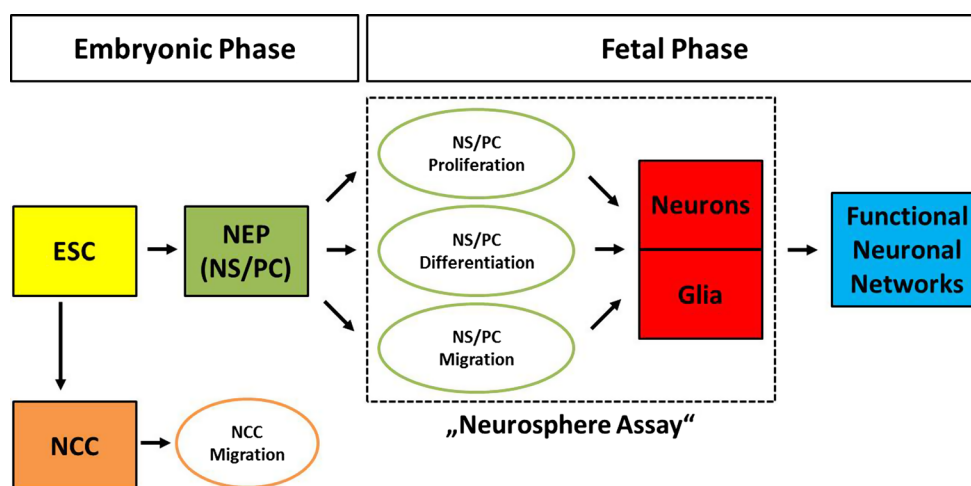
One way of determining whether these hazards are at all relevant to human health is implementation of exposure. Such an approach was already proposed for in vitro developmental toxicity testing (Daston et al. 2010) and successfully applied for in vitro testing for endocrine disruption (Rotroff et al. 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 LOELs 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 sub-cytotoxic 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  $\mu$ M (Ponce et al. 1994) and 3  $\mu$ M (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  $\mu$ M 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  $\mu$ M. 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  $\mu$ M (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 neuroepithelial 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  $\mu\text{M}$  (human) and 23–32  $\mu\text{M}$  (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  $\mu\text{M}$ , which is very similar to the effective concentrations in our rat in vitro results ( $EC_{50} = 31.82 \mu\text{M}$ ; 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  $\mu\text{M}$ ). Therapeutic plasma and CSF concentrations are several orders of magnitude lower than the effective concentration measured in the hNPC in vitro system (111  $\mu\text{M}$  and 2.4  $\mu\text{M}$ , 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  $\mu$ M), 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 *in vivo*, *in vitro*, *in silico* and *in chemico*—to support regulatory or safety decisions (Tollefsen et al. 2014).

**Acknowledgments** This work was supported by the German Ministry of Education and Research (BMBF Grants 0315522E and 16V0899). The authors thank Dr. Julia Tigges for critical review of the manuscript.

#### 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.

#### References

- Alépée N, Bahinski T, Daneshian M et al (2014) State-of-the-art of 3D cultures (organs-on-a-chip) in safety testing and pathophysiology. *ALTEX* 31(4):441–477
- Ankley GT, Bennett RS, Erickson RJ et al (2010) Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem* 29(3):730–741
- Ayuso-Sacido A, Moliterno JA, Kratovac S et al (2010) Activated EGFR signaling increases proliferation, survival, and migration and blocks neuronal differentiation in post-natal neural stem cells. *J Neurooncol* 97(3):323–337
- Bal-Price AK, Coecke S, Costa L et al (2012) Advancing the science of developmental neurotoxicity (DNT): testing for better safety evaluation. *ALTEX* 29(2):202–215
- Bal-Price A, Crofton KM, Leist M et al (2015a) International Stakeholder Network (ISTNET): creating a developmental neurotoxicity (DNT) testing road map for regulatory purposes. *Arch Toxicol* 89(2):269–287
- Bal-Price A, Crofton KM, Sachana M et al (2015b) Putative adverse outcome pathways relevant to neurotoxicity. *Crit Rev Toxicol* 45(1):83–91
- Bassanini S, Hallene K, Battaglia G et al (2007) Early cerebrovascular and parenchymal events following prenatal exposure to the putative neurotoxin methylazoxymethanol. *Neurobiol Dis* 26(2):481–495
- Baumann J, Barenys M, Gassmann K, Fritsche E (2014) Comparative human and rat “neurosphere assay” for developmental neurotoxicity testing. *Curr Protoc Toxicol* 59:12.21.1–12.21.24
- Bellanger M, Pichery C, Aerts D et al (2013) Economic benefits of methylmercury exposure control in Europe: monetary value of neurotoxicity prevention. *Environ Health* 12(1):3
- Bornhausen M, Müsch H, Greim H (1980) Operant behavior performance changes in rats after prenatal methylmercury exposure. *Toxicol Appl Pharmacol* 56(3):305–310
- Breier JM, Gassmann K, Kayser R et al (2010) Neural progenitor cells as models for high-throughput screens of developmental

- neurotoxicity: state of the science. *Neurotoxicol Teratol* 32(1):4–15
- Burbacher TM, Rodier PM, Weiss B (1990) Methylmercury developmental neurotoxicity: a comparison of effects in humans and animals. *Neurotoxicol Teratol* 12(3):191–202
- Burgess-Herbert SL, Euling SY (2013) Use of comparative genomics approaches to characterize interspecies differences in response to environmental chemicals: challenges, opportunities, and research needs. *Toxicol Appl Pharmacol* 271(3):372–385
- Castoldi AF, Coccini T, Ceccatelli S, Manzo L (2001) Neurotoxicity and molecular effects of methylmercury. *Brain Res Bull* 55(2):197–203
- Cattabeni F, Di Luca M (1997) Developmental models of brain dysfunctions induced by targeted cellular ablations with methylazoxymethanol. *Physiol Rev* 77(1):199–215
- Chen WJ, Bödy RL, Mottet NK (1979) Some effects of continuous low-dose congenital exposure to methylmercury on organ growth in the rat fetus. *Teratology* 20(1):31–36
- Clancy B, Kersh B, Hyde J, Darlington RB, Anand K, Finlay BL (2007) Web-based method for translating neurodevelopment from laboratory species to humans. *Neuroinformatics* 5(1):79–94
- Coecke S, Goldberg AM, Allen S et al (2007) Workgroup report: incorporating in vitro alternative methods for developmental neurotoxicity into international hazard and risk assessment strategies. *Environ Health Perspect* 115(6):924–931
- Crofton KM, Mundy WR, Lein PJ et al (2011) Developmental neurotoxicity testing: recommendations for developing alternative methods for the screening and prioritization of chemicals. *Altex* 28(1):9–15
- Croom EL, Shafer TJ, Evans MV et al (2015) Improving in vitro to in vivo extrapolation by incorporating toxicokinetic measurements: a case study of lindane-induced neurotoxicity. *Toxicol Appl Pharmacol* 283(1):9–19
- Dam K, Seidler F, Slotkin T (1998) Developmental neurotoxicity of chlorpyrifos: delayed targeting of DNA synthesis after repeated administration. *Dev Brain Res* 108(1):39–45
- Daston GP, Chapin RE, Scialli AR et al (2010) A different approach to validating screening assays for developmental toxicity. *Birth Defects Res B* 89(6):526–530
- De Groot DM, Hartgring S, Van de Horst L et al (2005) 2D and 3D assessment of neuropathology in rat brain after prenatal exposure to methylazoxymethanol, a model for developmental neurotoxicity. *Reprod Toxicol* 20(3):417–432
- Foti SB, Chou A, Moll AD, Roskams AJ (2013) HDAC inhibitors dysregulate neural stem cell activity in the postnatal mouse brain. *Int J Dev Neurosci* 31(6):434–447. doi:10.1016/j.ijdevneu.2013.03.008
- Gassmann K, Abel J, Bothe H et al (2010) Species-specific differential AhR expression protects human neural progenitor cells against developmental neurotoxicity of PAHs. *Environ Health Perspect* 118(1):1571–1577
- Gassmann K, Baumann J, Giersiefer S et al (2012) Automated neurosphere sorting and plating by the COPAS large particle sorter is a suitable method for high-throughput 3D in vitro applications. *Toxicol In Vitro* 26(6):993–1000
- Go HS, Kim KC, Choi CS et al (2012) Prenatal exposure to valproic acid increases the neural progenitor cell pool and induces macrocephaly in rat brain via a mechanism involving the GSK-3 $\beta$ / $\beta$ -catenin pathway. *Neuropharmacology* 63(6):1028–1041. doi:10.1016/j.neuropharm.2012.07.028
- Goldman LR, Koduru S (2000) Chemicals in the environment and developmental toxicity to children: a public health and policy perspective. *Environ Health Perspect* 108(Suppl 3):443
- Grandjean P, Landrigan PJ (2006) Developmental neurotoxicity of industrial chemicals. *The Lancet* 368(9553):2167–2178
- Grandjean P, Landrigan PJ (2014) Neurobehavioural effects of developmental toxicity. *Lancet Neurol* 13(3):330–338
- Harrill JA, Freudenrich TM, Robinette BL, Mundy WR (2011) Comparative sensitivity of human and rat neural cultures to chemical-induced inhibition of neurite outgrowth. *Toxicol Appl Pharmacol* 256(3):268–280
- Howard AS, Bucelli R, Jett DA, Bruun D, Yang D, Lein PJ (2005) Chlorpyrifos exerts opposing effects on axonal and dendritic growth in primary neuronal cultures. *Toxicol Appl Pharmacol* 207(2):112–124
- Jiang Y-Z, Wang K, Fang R, Zheng J (2010) Expression of aryl hydrocarbon receptor in human placentas and fetal tissues. *J Histochem Cytochem* 58(8):679–685
- Karlsson M, Hammers S, Nilsson-Ehle I, Malmberg A-S, Wretling B (1996) Concentrations of doxycycline and penicillin G in sera and cerebrospinal fluid of patients treated for neuroborreliosis. *Antimicrob Agents Chemother* 40(5):1104–1107
- Kisby G, Olivas A, Park T et al (2009) DNA repair modulates the vulnerability of the developing brain to alkylating agents. *DNA Repair* 8(3):400–412
- Kleinstreuer NC, Yang J, Berg EL et al (2014) Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms. *Nat Biotechnol* 32(6):583–591
- Krewski D, Acosta D Jr, Andersen M et al (2010) Toxicity testing in the 21st century: a vision and a strategy. *J Toxicol Environ Health B* 13(2–4):51–138
- Lein P, Silbergeld E, Locke P, Goldberg AM (2005) In vitro and other alternative approaches to developmental neurotoxicity testing (DNT). *Environ Toxicol Pharmacol* 19(3):735–744
- Lein P, Locke P, Goldberg A (2007) Meeting report: alternatives for developmental neurotoxicity testing. *Environ Health Perspect* 115(5):764–768
- Leist M, Hartung T (2013) Inflammatory findings on species extrapolations: humans are definitely no 70-kg mice. *Arch Toxicol* 87(4):563–567
- L'Episcopo F, Tirolo C, Testa N et al (2013) Aging-induced Nrf2-ARE pathway disruption in the subventricular zone drives neurogenic impairment in parkinsonian mice via PI3 K-Wnt/ $\beta$ -catenin dysregulation. *J Neurosci* 33(4):1462–1485
- Lewandowski T, Ponce R, Charleston J, Hong S, Faustman E (2003) Effect of methylmercury on midbrain cell proliferation during organogenesis: potential cross-species differences and implications for risk assessment. *Toxicol Sci* 75(1):124–133
- Moors M, Cline JE, Abel J, Fritsche E (2007) ERK-dependent and-independent pathways trigger human neural progenitor cell migration. *Toxicol Appl Pharmacol* 221(1):57–67
- Moors M, Rockel TD, Abel J et al (2009) Human neurospheres as three-dimensional cellular systems for developmental neurotoxicity testing. *Environ Health Perspect* 117(7):1131–1138
- NRC (2007) Toxicity testing in the 21st century: A vision and a strategy. National Academies Press, Washington
- OECD (2007) Test Guideline 426. OECD guideline for testing of chemicals. Developmental neurotoxicity study. In. <http://www.oecd-ilibrary.org/docserver/download/9742601e.pdf?expires=1424270931&id=id&acname=guest&checksum=B19A872A2C50D706CB32B2E5687B48>. Accessed 18 Feb 2015
- Patlewicz G, Simon T, Rowlands JC, Budinski RA, Becker RA (2015) Proposing a scientific confidence framework to help support the application of adverse outcome pathways for regulatory purposes. *Regul Toxicol Pharmacol* 71(3):463–477
- Ponce RA, Kavanagh TJ, Mottet NK, Whittaker SG, Faustman EM (1994) Effects of methyl mercury on the cell cycle of primary rat CNS cells in vitro. *Toxicol Appl Pharmacol* 127(1):83–90
- Rauh VA, Garfinkel R, Perera FP et al (2006) Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children. *Pediatrics* 118(6):e1845–e1859



- Rodier PM (1995) Developing brain as a target of toxicity. *Environ Health Perspect* 103(Suppl 6):73
- Rotroff DM, Wetmore BA, Dix DJ et al (2010) Incorporating human dosimetry and exposure into high-throughput in vitro toxicity screening. *Toxicol Sci* 117(2):348–358
- Rotroff DM, Martin MT, Dix DJ et al (2014) Predictive endocrine testing in the 21st century using in vitro assays of estrogen receptor signaling responses. *Environ Sci Technol* 48(15):8706–8716
- Schettler T (2001) Toxic threats to neurologic development of children. *Environ Health Perspect* 109(Suppl 6):813
- Seidle T, Stephens M (2009) Bringing toxicology into the 21st century: a global call to action. *Toxicol In Vitro* 23(8):1576–1579
- Somel M, Liu X, Tang L et al (2011) MicroRNA-driven developmental remodeling in the brain distinguishes humans from other primates. *PLoS Biol* 9(12):e1001214
- Spencer PS, Kisby GE, Ludolph AC (1991) Slow toxins, biologic markers, and long-latency neurodegenerative disease in the western Pacific region. *Neurology* 41(5 Suppl 2):62–66
- Timchalk C, Poet TS, Kousba AA (2006) Age-dependent pharmacokinetic and pharmacodynamic response in preweanling rats following oral exposure to the organophosphorus insecticide chlorpyrifos. *Toxicology* 220(1):13–25
- Tollefsen KE, Scholz S, Cronin MT et al (2014) Applying adverse outcome pathways (AOPs) to support integrated approaches to testing and assessment (IATA). *Regul Toxicol Pharmacol* 70(3):629–640
- USEPA (1998) Health Effects Test Guidelines: OPPTS 870.6300 Developmental neurotoxicity study. In: <http://www.regulations.gov#!documentDetail;D=EPA-HQ-OPPT-2009-0156-0042>. Accessed 18 Feb 2015
- Yamada KM, Cukierman E (2007) Modeling tissue morphogenesis and cancer in 3D. *Cell* 130(4):601–610
- Yang D, Howard A, Bruun D, Ajuja-Alemanj M, Pickart C, Lein PJ (2008) Chlorpyrifos and chlorpyrifos-oxon inhibit axonal growth by interfering with the morphogenic activity of acetylcholinesterase. *Toxicol Appl Pharmacol* 228(1):32–41
- Zhang YE, Landback P, Vibranovski MD, Long M (2011) Accelerated recruitment of new brain development genes into the human genome. *PLoS Biol* 9(10):e1001179