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CONTEMPORARY REVIEW

Current Availability of Stem Cell-Based *In Vitro* Methods for Developmental Neurotoxicity (DNT) Testing

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ABSTRACT

There is evidence that chemical exposure during development can cause irreversible impairments of the human developing nervous system. Therefore, testing compounds for their developmentally neurotoxic potential has high priority for different stakeholders: academia, industry, and regulatory bodies. Due to the resource-intensity of current developmental neurotoxicity (DNT) *in vivo* guidelines, alternative methods that are scientifically valid and have a high predictivity for humans are especially desired by regulators. Here, we review availability of stem−/progenitor cell-based *in vitro* methods for DNT evaluation that is based on the concept of neurodevelopmental process assessment. These test methods are assembled into a DNT *in vitro* testing battery. Gaps in this testing battery addressing research needs are also pointed out.

Key words: developmental neurotoxicity testing; DNT; neurological damage.

There is evidence that chemical exposure during development can cause irreversible impairments of the human developing nervous system (Andersen *et al.*, 2000; Bearer, 2001; Claudio, 2001; Grandjean and Landrigan, 2006, 2014; Mendola *et al.*, 2002; Rodier, 1995; Slikker, 1994). Neurological damage ranging from subtle to severe imposes significant burdens on affected individuals, their families, and society (Goldman and Koduru, 2000; Weiss and Lambert, 2000). Therefore, testing compounds for their developmentally neurotoxic potential has high priority for different stakeholders: academia, industry, and regulatory bodies (Bal-Price *et al.*, 2015; Crofton *et al.*, 2014; Fritsche *et al.*, 2017, 2018). Due to the resource intensity of current DNT *in vivo* guidelines, alternative methods that are scientifically valid and have a high predictivity for humans are especially desired by regulators (Bal-Price *et al.*, 2012, 2015, 2018a; Crofton *et al.*, 2011; Fritsche *et al.*, 2017, 2018; Lein *et al.*, 2005).

Development of these alternative methods are based on the strategy that the complex procedure of brain development is disassembled into spatiotemporal neurodevelopmental processes that are necessary for forming a brain. According to the adverse outcome pathway concept, such are key events for DNT that can be tested for adverse effects of compounds in *in vitro* assays (Bal-Price *et al.*, 2015, 2018a). To avoid species differences in responses to compound exposure (Baumann *et al.*, 2016; Dach *et al.*, 2017; Gassmann *et al.*, 2010; Harrill *et al.*, 2011a; Masjosthusmann *et al.*, 2018), key event-related DNT evaluation is preferably using human cells, ie,

neural stem/progenitor cells (NS/PC) including human-induced pluripotent stem cell (hiPSC)-derived NPC as a source (Bal-Price *et al.*, 2018b; Singh *et al.*, 2016). In this article, we will summarize the current state of the art on NS/PC-based methods for evaluation of neurodevelopmental toxicity. DNT methods published until April 2014 were assembled in a systematic review earlier (Fritsche *et al.*, 2015).

ESC DIFFERENTIATION TO NEUROEPITHELIAL PRECURSORS (NEP)/INDUCTION OF NEURONAL ROSETTES

Tests for studying compound effects on the early neurodevelopmental endpoint stem cell differentiation to NEP based on human embryonic stem cells (hESC) were developed (summarized in Fritsche *et al.*, 2015; Shinde *et al.*, 2015; Waldmann *et al.*, 2014). Here, rosette morphology and/or gene expression and viability are measured. In addition, a teratogenicity index was developed as a test method for distinguishing between DNT-specific and cytotoxic compound effects that promotes performing transcriptome-based DNT studies at noncytotoxic concentrations (Waldmann *et al.*, 2014). In a recent work, disturbance of neural rosette formation from hESC was studied in the context of toxicant-dependent altered DNA methylation (Du *et al.*, 2018). Similar to hESC, hiPSC also form neural rosettes that further mature to neurons when injected into mouse motorcortex (Malchenko *et al.*, 2014).

NPC PROLIFERATION

Proliferation of neural progenitor cells determines brain size (de Groot *et al.*, 2005). Different cell systems are available to study effects on proliferation *in vitro*: hESC-generated NPC, primary hNPC, hiPSC-derived NPC, the human umbilical cord blood (hUCB)-NSC line or ReNcell CX-based systems assessing proliferation in two-dimensional (2D) cultures with the bromodeoxyuridine (BrdU) or ethynyldeoxyuridine (EdU) assay that quantifies incorporation of the thymidine analogue BrdU or EdU into the DNA via fluorescence or luminescence-labeled antibodies, or by quantifying Ki67 expression. A variety of groups used hESC-based methods for studying the effects of single (summarized in Fritsche *et al.*, 2015; Sohn *et al.*, 2017; Wang *et al.*, 2016) or multiple (Behl *et al.*, 2015; Radio *et al.*, 2015) compounds on NPC proliferation. Lately, the frequency of cell cycles per day was calculated from the number of hESC-NSC, which was counted daily using a Neubauer hemocytometer (Vichier-Guerre *et al.*, 2017). While this is a cheap and easy method, it has a high variability and needs verification by an additional established method like the BrdU assay.

Also, 3D NPC aggregates generated from primary human material (Lonza, Belgium; NPC1 Assay; Bal-Price *et al.*, 2018a) or from hiPSC (Hofrichter *et al.*, 2017) called neurospheres are used for assessing compound effects on NPC proliferation. Here, the diameter increase of individual spheres over time or BrdU incorporation are two different ways of measuring cell replication (Baumann *et al.*, 2015, 2016; Fritsche *et al.*, 2015). The increase in sphere diameter over time of single spheres plated in wells of a 96-well plate is a fast and cheap possibly first tier screening method for analyzing cell proliferation.

NPC APOPTOSIS

Apoptosis is a well-balanced process during brain development with alterations in both directions, increase or decrease, having negative implications for organ development (Hakem *et al.*, 1998; Uzquiano *et al.*, 2018). It can be measured by different methods *in vitro* ranging from early events like mitochondria calcium or cytochrome c release or annexin V presentation, intermediate processes like caspase activation or late apoptotic activities like nuclear condensation, micronucleus formation, or chromatin disintegration. Several stem cell-based cell systems are suitable for detection of xenobiotic-induced apoptosis, ie, hESC-NPC and ReNcell CX culture, primary hNPC growing as monolayers in 2D, or as neurospheres in 3D (summarized in Fritsche *et al.*, 2015). Lately, the neurosphere system was used for studying the effects of gestational age and sex on methylmercury-induced apoptosis by quantification of condensed nuclei (Edoff *et al.*, 2017). With the ReNcell CX culture, a high content imaging analysis (HCA) assay based on multiplexed activated caspase-3/-7 (apoptosis) and protease (viability) activities. This method was applied to a comparative study of mouse cortical NPC (Millipore, Temecula, CA), immortalized NPC (ReNcell CX, Millipore), hESC-derived NSC (ArunA Biomedical, Athens, GA), and hiPSC-derived pure neuronal cultures (iCell, Cellular Dynamics International, Madison, WI) using 12 positive and negative compounds impressively demonstrating different susceptibilities toward compound-induced apoptosis between species and between brain cells of different developmental stages (Druwe *et al.*, 2015). Another comparative study assessed caspase-3/-7 activation by multiple compounds in a 384-well format using primary hNPC growing as monolayers (ThermoFisher, Waltham, Massachusetts), the neuroblastoma cell line SH-SY5Y, and the immortalized fetal mesencephalic cell line LUHMES. These different cell types show different sensitivities toward compound-induced caspase-3/-7 activation (Tong *et al.*, 2017). A different commercial hNPC source are ENStem-ATM hNPCs (ArunA Biomedical, Athens, Georgia). These cells growing in monolayers were used for multiplexed imaging analyses of live/dead/apoptotic cells by calcein AM/PI stainings in 96-well plates (Kim *et al.*, 2016).

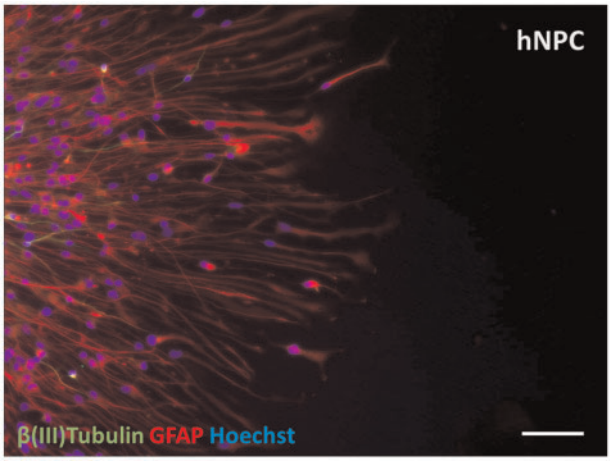

Figure 1. hNPC (Lonza, Verviers, Belgium) were plated onto poly D-lysin/laminin-coated glass slides. After 24 hours, cells were fixed with paraformaldehyde and stained with antibodies against GFAP and β(III)tubulin. Nuclei were stained with Hoechst. Scale bar = 50μm

RADIAL GLIA PROLIFERATION

The multitudes of radial glia cell types play diverse key roles during cerebral cortex development (Gotz and Huttner, 2005; Uzquiano *et al.*, 2018). Stem cell-based *in vitro* methods for studying compound effects on radial glia are sparse. Primary

hNPC (Lonza, Verviers, Belgium) growing as neurospheres is one cell system producing migrating, nestin+/GFAP+ cells that have radial glia-like morphology after 24 h in differentiation culture (Figure 1; Bal-Price *et al.*, 2018a). These differentiated hNPC express a variety of radial glia cell markers and respond to bone morphogenic protein (BMP)2 with increased astrocyte differentiation (Masjosthusmann *et al.*, 2018). Radial glia can also be differentiated from rosette-forming hESC or hiPSC (Malchenko *et al.*, 2014).

MIGRATION OF NEURAL CREST CELLS (NCC)/ RADIAL GLIA/NEURONS

Different neural cell types need proper migration during development. During embryogenesis, NCC migrate to distinct parts of the embryo developing into a variety of extracerebral cells and tissues causing diseases like cleft palate, hearing loss, Morbus Hirschsprung, or CHARGE syndrome when defective (Dupin and Sommer, 2012; Mayor and Theveneau, 2013). NCC migration can be studied with an *in vitro* assay, the "MINC Assay", based on neural crest cells (NCC) that are differentiated from hESC (Zimmer *et al.*, 2012). There are two different ways to perform the MINC Assay: the scratch method (Dreser *et al.*, 2015; Pallocca *et al.*, 2016; Zimmer *et al.*, 2012, 2014) or the recently developed stamp method (Nyffeler *et al.*, 2017) with the latter being more robust.

Cortex development involves radial glia migration leading to the development of a scaffold that is subsequently used by neurons to migrate along these glial fibers and reach their final cortical destination (Borrell and Gotz, 2014). One well-characterized migration assay is part of the "Neurosphere Assay" (NPC2; Bal-Price *et al.*, 2018a). Migration distance that cells cover by radially migrating out of the plated neurosphere is analyzed either manually using programs like ImageJ (Bal-Price *et al.*, 2018a; Barenys *et al.*, 2017; Baumann *et al.*, 2015, 2016; Edoff *et al.*, 2017; Fritsche *et al.*, 2015; Ivanov *et al.*, 2016) or by HCA using the software "Omnisphero" (Schmuck *et al.*, 2017). An important issue when evaluating effects of compounds on NPC migration with the 'Neurosphere Assay' is to distinguish between specific effects on migration and secondary migration effects due to cytotoxicity. Our recent data shows that migration distance or pattern, which determines the size of the total migration area, defines the magnitude of signal of viability assays like the Cell Titer Blue Assay (CTB Assay; Promega) because it is related to cell number. A different viability/cytotoxicity assay measuring a readout not directly dependent on cell number, like LDH leakage, indicates the specific effects of methylmercury (MeHgCl) on migration without producing cell death at two different time points (Figs. 2A and 2B). Similarly, epigallocatechin gallate (EGCG) inhibits adhesion and migration of hNPC thereby changing the migration pattern and area (Figure 2C; Bal-Price *et al.*, 2017; Barenys *et al.*, 2017). After 3 days of migration in the presence of EGCG, the CTB assay suggests that EGCG reduces cell viability (Figure 2D). However, FACS analyses identifying annexin V-/PI-positive cells clearly show that EGCG does not cause cell death, but diminishes the cell area with access to the CTB substrate (Figure 2E).

Migrated cells of the NPC2 assay form a 2-layered cell layer with neurons migrating on top of the glia cells (Alépée *et al.*, 2014; Baumann *et al.*, 2016). This enables measuring not only glia cell migration, but also the neuronal migration by assessing individual neuronal positions using the software Ominsphero

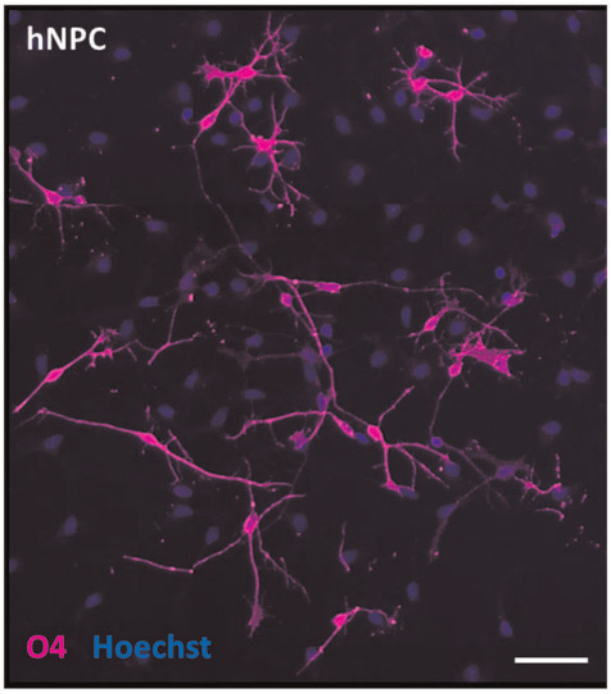

Figure 2. Oligodendrocyte differentiation of primary hNPC. Oligodendrocyte differentiation was assessed by immunocytochemical staining for the oligodendrocyte marker O4 of 5 days differentiated hNPC. Nuclei were counterstained with Hoechst. Scale bar = 50μm. The staining method was previously published in Baumann *et al.* (2014, 2015).

(Schmuck *et al.*, 2017). Hence, NPC2 can be utilized to assess radial glia and neuronal migration at the same time.

Migration analyses can also be performed with hiPSC-derived NPC (hiNPC2; Hofrichter *et al.*, 2017). Migration distance is similar between NPC2 and hiNPC. Yet, the first cells migrating from the hiNPC neurosphere are neurons and not radial glia as from the hNPC sphere.

ASTROCYTE DIFFERENTIATION/MATURATION

Astroglia differentiation is a crucial event during brain development because astrocytes obtain a variety of central functions in brain (Kettenmann and Verkhratsky, 2011). Astrocyte differentiation can be measured in developing mixed cell cultures by counting the percentage of, eg, GFAP+ or vimentin+ cells from the total number of differentiated hESC, primary hNPC or hUCB-NSC (summarized in Fritsche *et al.*, 2015; Edoff *et al.*, 2017). Lately, also hiPSC differentiation into the astrocyte lineage was employed in a toxicological context either in 2D (after 28 days) or in 3D (after 56 days) by creating "brain balls" in shaking cultures (Pamies *et al.*, 2017, 2018b; Pistollato *et al.*, 2014). Moreover, morphogen-induced astrocyte maturation can be studied in the context of the "Neurosphere assay" (Masjosthusmann *et al.*, 2018). Apart from toxicology, clearly more data on astrocyte differentiation is available on the basic science level, which is summarized, eg, in Chandrasekaran *et al.* (2016). Astrocyte function as the most relevant readout was recently compared between long-term self-renewing hiPSC-derived neuroepithelial-like stem cells (ltNES; Falk *et al.*, 2012)-astrocytes, human primary adult astrocytes (phaAstro), an astrocytoma cell line CCF-STTG1 (CCF), and hiPSC-derived astrocytes from Cellular Dynamics International (iCellAstro). Here, ltNES-astrocytes were the only ones expressing functional, glutamate

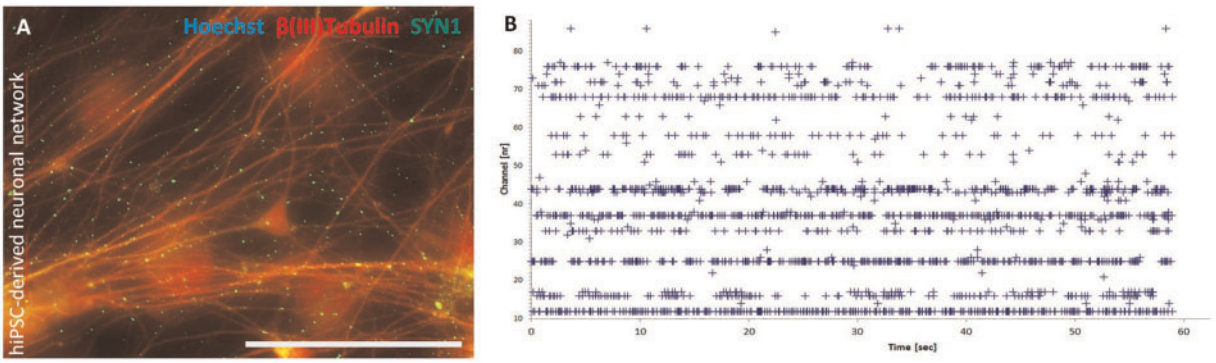

Figure 3. Synapse formation and neuronal network activity of a hiPSC-derived network. A, hiPSC (IMR-90, Wicell) were neurally induced to NPCs using the NIM-protocol (Hofrichter *et al.* 2017) and differentiated on D-lysin/laminin-coated glass slides for 28 days, fixed with paraformaldehyde and stained for β(III)-tubulin$^+$ neurons and synapsin1$^+$ presynaptic structures, respectively. Nuclei were stained with Hoechst. Scale bar=100 μm. B, Spikeraster plot of spontaneous electrical activity of a hiPSC-derived neuronal network cultivated for 20 days on D-lysin/laminin-coated microelectrode arrays.

transporting SLC1A3 protein, which is an assay suitable for higher throughput drug screening (Lundin *et al.*, 2018).

OLIGODENDROCYTE DIFFERENTIATION/ MATURATION

Oligodendrogenesis is necessary for proper brain functioning as oligodendrocytes form and keep myelin sheaths around axons (Baumann and Pham-Dinh, 2001). Data on chemical effects on hESC-, hNPC-, or hUCB-NSC-derived oligodendrocytes are summarized in Fritsche *et al.* (2015). Different groups have recently established further oligodendrocyte differentiation protocols using hESC/hiPSC growing feeder-free (Douvaras *et al.*, 2014; Gorris *et al.*, 2015; Madill *et al.*, 2016; Yamashita *et al.*, 2017) or in presence of feeder cells (Douvaras *et al.*, 2014; Ehrlich *et al.*, 2017; Gorris *et al.*, 2015; Madill *et al.*, 2016; Nicaise *et al.*, 2017) in a 2D format. Pamies *et al.* (2018b) used hiPSC-derived 3D "brain balls" to study differentiation of oligodendrocytes in a toxicological context. Oligodendrocytes emerge late during nervous system development and differentiation of hESC/hiPSC into the oligodendrocyte lineage following the above-mentioned protocols needs several weeks making medium-to-high throughput screening for oligodendrocyte toxicity using hESC/hiPSC a great challenge. In contrast, oligodendrocyte formation and maturation can be studied with hNPC growing as neurospheres with the NPC5/6 assay within a neurosphere differentiation time of 5 days (Figure 3; Bal-Price *et al.*, 2018a; Dach *et al.*, 2017). These assays are based on manual or automated oligodendrocyte quantification as a measure for oligodendrocyte formation (NPC5; Figure 3; Bal-Price *et al.*, 2018a; Barenys *et al.*, 2017), which is the normalization basis for subsequent thyroid hormone (TH)-dependent myelin basic protein (*MBP*) expression as a measure for oligodendrocyte maturation (Bal-Price *et al.*, 2018a; Dach *et al.*, 2017). Interference of a compound with the TH-induced oligodendrocyte maturation is thus an assay for identifying TH disruptors (NPC6). Oligodendrocyte precursor cells can also be enriched by isolation from gestational week 10–13 fetal human neurospheres (Lu *et al.*, 2015).

NEUROGENESIS

Neurogenesis is one of the most frequently studied endpoints for DNT evaluation. Compounds' effects on neuronal differentiation have been studied in hESC- (summarized in Ehashi *et al.*, 2014; Fritsche *et al.*, 2015; Schulpen *et al.*, 2015; Sohn *et al.*, 2017; Zeng *et al.*, 2016), hiPSC-derived (Pistollato *et al.*, 2017) mixed neuronal-glial or hUCB-NSC (summarized in Fritsche *et al.*, 2015; Kashyap *et al.*, 2015; Zychowicz *et al.*, 2014) cultures in 2D, as well as in hiPSC-derived mixed-culture "brain balls" in 3D (Pamies *et al.*, 2017) and hiPSC-generated (Hofrichter *et al.*, 2017) or primary neurospheres (summarized in Fritsche *et al.*, 2015 and Bal-Price *et al.*, 2018a; Masjosthusmann *et al.*, 2018) differentiating in "secondary 3D" structures. Within some of the above-mentioned studies, differences in sensitivity and specificity of DNT effects between neuronal cells toward methylmercury were observed. These are probably due to the large differences in stem cell cultivation and differentiation protocols concerning, eg, medium, feeder cell status, timing, differentiation through hESC-derived NPC or direct neuronal differentiation, amounts of glia present and level of quality control, just to mention some. Especially quality control and reporting standards are a large issue in current stem cell work that urgently need standardization as recently voiced by a workshop report on "Advanced good cell culture practice for human primary, stem cell-derived and organoid models' (Pamies *et al.*, 2018a).

Concerning cell-type composition, mixed neuron-glia cultures are advantageous for DNT testing as different cell types might have different susceptibilities toward compounds (Pei *et al.*, 2016), eg, astroglia might alter developmental toxicity to neurons (Wu *et al.*, 2017). In addition, the advantage of the monolayer differentiation protocols clearly lie in the more simple evaluation, eg, by high content image analyses (HCA), whereas the 3D differentiated methods are more complex to evaluate via immunostainings. Somewhat in-between are neurosphere-based methods that differentiate in so-called "secondary 3D" structures, ie, maintaining the multicellular organism aspect (Masjosthusmann *et al.*, 2018) despite plating of spheres on a 2D surface (Alépée *et al.*, 2014).

NEURONAL MATURATION

Dendritic and axonal (neurite) outgrowth followed by the formation of synapses are key cellular features associated with the functional maturation of the CNS. Neurite morphology can be measured with a variety of methods including neurite number, length, branching, or area using HCA, a fairly reliable and suitable image-based method for higher throughput applications (Harrill *et al.*, 2010, 2011a; He *et al.*, 2012; Wilson *et al.*, 2014). Cell

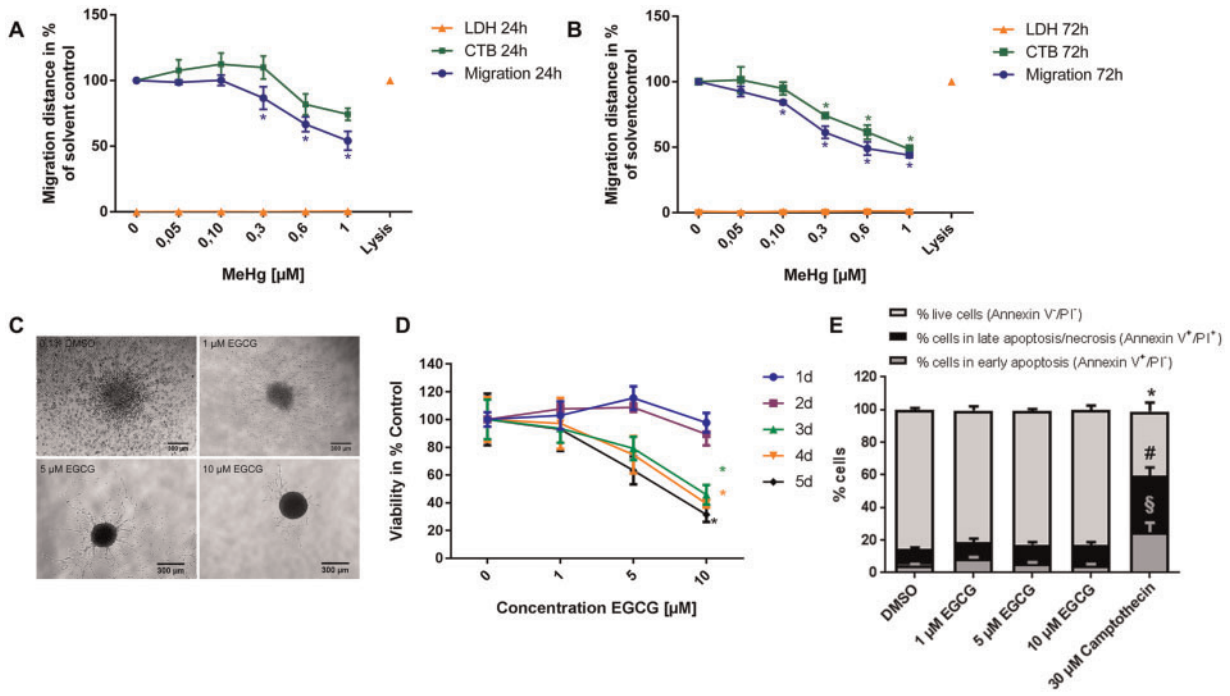

Figure 4. hNPC growing as neurospheres in proliferation culture, were plated for migration analyses onto poly D-lysin/laminin-coated glass slides in presence and absence of MeHgCl. After (A) 24 and (B) 72 h, migration distance was measured from the outer sphere rim to the furthest migrated cells at four opposite positions. Cell titer blue (CTB) and lactate dehydrogenase (LDH) assays were performed as described previously (Baumann *et al.* 2014). C, Neurospheres were plated as described in (A) in presence and absence of epigallocathechin gallate (EGCG). After 24 h the migration area was analyzed visually by phase contrast microscopy and for better visualization images were subjected to a black/white filter. D, Viability analyses using the CTB assay were performed on each day up to 5 DIV. E, On day 5, FACS analyses of dissociated hNPC were performed after annexin V/PI staining. As a positive control, spheres were treated with the topoisomerase I inhibitor camptothecin. (A, B, D) $^{*}p \leq .05$; (E) § $p \leq .05$ of annexin + /PI-cells; # $p \leq .05$ of annexin + /PI+ cells; $^{*}p \leq .05$ of live cells.

material for such analyses includes hESC-derived almost pure neuronal cultures (hN2TM, ArunA Biomedical Inc., Athens, Georgia; Behl *et al.*, 2015; Harrill *et al.*, 2011a; Wilson *et al.*, 2014) that were treated after neuronal specification had already taken place; hESC-derived hNP cells (hNP1^{TM} 00001, ArunA Biomedical Inc.) treated when cells were making the transition from proliferating NPC to postmitotic neurons (Wang *et al.*, 2016); hESC-derived hNPC (Chemicon-Millipore Norcross, Georgia; Zeng *et al.*, 2016); 3D aggregated hESC-derived embryoid bodies (He *et al.*, 2012); hiPSC-generated iCell neurons (Cellular Dynamics International; Ryan *et al.*, 2016); hiPSC-derived hNPC growing as neurospheres (Hofrichter *et al.*, 2017); primary NPC differentiating into mixed cultures (NPC4; Bal-Price *et al.*, 2018a; Edoff *et al.*, 2017; Schmuck *et al.*, 2017), ie, multiple parameters of the neurosphere assay including neurite morphology can be assessed with the algorithm Omnisphero (www.omnisphero.com; last accessed July 2018; Schmuck *et al.*, 2017); or LUHMES cells (Krug *et al.*, 2013; Scholz *et al.*, 2011).

NEURONAL SUBTYPE DIFFERENTIATION

During brain development, neural stem and progenitor cells produce a variety of neuronal subtypes, which differentiate at different stages and in different regions of the brain. Compounds' effects on neuronal subtype differentiation has mostly been assessed for dopaminergic (DA) neurons using hESC (Huang *et al.*, 2017; Stummann *et al.*, 2009; Zeng *et al.*, 2006), for DA as well as cholinergic neurons using hUCB-NSC (Kashyap *et al.*, 2015) and for DA, glutamate- and GABAergic neurons employing hiPSC (Pistollato *et al.*, 2017).

SYNAPTOGENESIS/NEURONAL NETWORK FORMATION

During early neurogenesis neurons start to mature, become electrically active and connect via synapses (Okado *et al.*, 1979; Zecevic and Antic, 1998). For the function of the CNS this neuronal maturation and the formation of synapses is crucial. So far, DNT testing for synaptogenesis and neuronal activity in the developing brain has mainly been performed using rat primary cells (Harrill *et al.*, 2011b; Hogberg *et al.*, 2011; Robinette *et al.*, 2011) and no *in vitro* DNT study has been published using hESC or hiPSC for assessing compound effects on neuronal network activity. However, there are a number of promising systems under development that have been used for acute neurotoxicity evaluations studying either synaptogenesis and/or neuronal electrical activity including hESC (Kapucu *et al.*, 2012; Oh *et al.*, 2016; Sandström *et al.*, 2017; Yla-Outinen *et al.*, 2010) or hiPSC neuronal network differentiation methods (Figure 3; Hofrichter *et al.*, 2017; Pellett *et al.*, 2015; Pistollato *et al.*, 2017; Toivonen *et al.*, 2013). Here, the use of hiPSC-derived neuronal networks growing directly on microelectrode arrays (MEAs) seems to be a promising method for screening neurodevelopmental toxins for their adverse effects on neuronal network formation.

What are the difficulties one faces with stem cell-based active neuronal networks for DNT evaluation? Every single neuronal network differentiates into a variable amount of neurons, ie, variable neuron/glia ratio, as well as neuronal subtypes that form neuronal connections by chance. Therefore, each network exhibits its own baseline activity level with high MEA-to-MEA variability making comparison of developmentally exposed neuronal networks to control networks very difficult. In

addition, network activity is generally not very high compared with rodent networks. Developing rat networks differentiate faster and show much higher activity levels and synchronization than stem cell-based human networks. This makes analyses of the adverse effects of toxicants on human networks much more difficult. To overcome these restrictions and make neuronal human stem cell-based networks more standardized and reproducible, one can envision making use of the relatively new method of 3D bioprinting (Zhuang *et al.*, 2018). Using this method, neural cells might directly be printed in a three-dimensional hydrogel precisely on MEAs (Tedesco *et al.*, 2018).

SUMMARY AND CONCLUSION

During the last 15 years, much effort has been put into establishment, scientific validation, and test method set up for DNT *in vitro* evaluation. In addition to primary rodent cultures, which are valuable cell methods for comparing compounds' effects *in vivo* to *in vitro*, stem/progenitor cell-based methods have become available that can now be assembled into a DNT *in vitro* testing battery (summarized in Bal-Price *et al.*, 2018a). Such a testing battery is necessary for covering the immense complexity of neurodevelopmental processes as well as timing aspects of brain development. However, the current state of the science concerning the testing battery is probably still at an early, immature state. While a variety of important key events are very well covered in the strategy, ie, neural proliferation, apoptosis, NPC migration, neuronal differentiation and neurite morphology, there are also some crucial aspects less well covered. These include glia differentiation and maturation, glia cell function, neuronal maturation, and neuronal network formation with assessment of electrical network activity. Moreover, the complexity of brain region-specific neural differentiation and function has not been addressed in DNT assays yet. However, basic science is moving down this path by creating brain region-specific organoids (Lancaster *et al.*, 2013; Qian *et al.*, 2016) that might be suited for studying region-specific effects of compounds on neurodevelopmental **key events**. In addition, hormone-related DNT has only been touched marginally with stem cell-based human DNT *in vitro* assays by studying interference with cellular thyroid hormone (Dach *et al.*, 2017) or glucocorticoid signaling (Moors *et al.*, 2012). Hormonal contributions to brain development are much more manifold and complex and chemicals with endocrine activities are thus suspected to interfere with neurodevelopment (WHO-UNEP, 2012). Here, interference with estrogen, androgen, retinoid, progesterone, peroxysome proliferator-activated receptor, or endocannabinoid signaling pathways might have implications for the developing brain at specific developmental stages. Especially sex hormone-related cellular and organ function is crucial for the development of gender-specific behavior, which follows species-specific traits (Wallen and Baum, 2002) . Molecular aspects of the development of such human sex-specific behavior is an understudied field of research posing a challenge for *in vitro* DNT evaluation. Yet, primary human cells show some sex-specific neurodevelopmental **key event** response differences toward methylmercury (Edoff *et al.*, 2017) without understanding the mechanistic implications behind these observations yet. Attempts are made to tackle this issue with rodent *in vitro* methods (Keil *et al.*, 2017). In this line, the largest challenge will be the understanding of disturbance of emotional and intellectual consciousness by chemical exposure in humans. Understanding physiology behind these human traits is a prerequisite that might enable establishment of adverse outcome pathways for these fundamental human aspects in the future.

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