Development and molecular characterization of electrically active neuronal networks on microelectrode arrays for neurotoxicity testing *in vitro*

Dissertation to obtain the degree
Doctor Rerum Naturalium (Dr. rer. nat.)
at the Heinrich-Heine-University Düsseldorf

submitted by

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from

Remscheid

Düsseldorf, June 2019

Entwicklung und molekulare Charakterisierung von elektrisch aktiven neuronalen Netzwerken auf Mikroelektroden Arrays für *in vitro* Neurotoxizitätstestung

Inaugural-Dissertation

Zur Erlangung des Doktorgrades der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf, Juni 2019

aus dem Leibniz Institut für umweltmedizinische Forschung (IUF)

an der Heinrich-Heine-Universität Düsseldorf gGmbH

Gedruckt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der

Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Ellen Fritsche

Korreferent: Prof. Dr. Vlada Urlacher

Tag der mündlichen Prüfung: Freitag, der 06. Dezember 2019

The difficulty lies not in the new ideas

but in escaping from the old ones.

(John Maynard Keynes)

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1. Introduction

1.1. Alternative methods to animal testing

Currently, neurotoxicity (NT) guideline studies are performed by resource-intensive *in vivo* animal experiments, regarding the numbers of animals and the time and costs required (OECD, 1997, EPA, 1998, OECD, 2007, Bal-Price et al., 2008). Moreover, only a restricted amount of new chemicals and a low compound throughput for neurotoxic potency emerged a lack of data that lead to false predictions of risk and the elimination of valuable products (Judson et al., 2009, Hartung, 2011). The European Centre for the Validation of Alternative Methods (ECVAM) recommends assigning alternative *in vitro* test methods for regulatory toxicological assessment. In addition, the current state of research strongly advises the limitations of the extrapolation of animal derived findings to the actual human hazard due to species differences (Perel et al., 2007, Matthews, 2008, Leist and Hartung, 2013). Thus a paradigm switch in toxicity assessment from apical endpoint evaluation in rodents towards a mode-of-action (MoA)-based testing in human-relevant *in vitro* methods was proposed (NRC, 2007). Our lab has taken up this challenge and has been developing a variety of *in vitro* mechanistic assays for neurotoxicity evaluation based on primary human cells that might increase predictivity of compound testing for humans due to the elimination of species differences and creating affordable, sensitive, and mechanism-based methods (Gassmann et al., 2010, Harrill et al., 2011, Baumann et al., 2016, Dach et al., 2017, Masjosthusmann et al., 2018c).
However, the use of human primary cells might bear ethical concerns and cell sources might be limited (Dunnett and Rosser, 2014). To overcome these issues human stem cell biology is commonly used and holds great potential to create *in vitro* models that predict neurotoxicity (Scott et al., 2013, Pistollato et al., 2014, Pei et al., 2016, Pamies et al., 2017).

1.2. Human induced pluripotent stem cells (hiPSC)

In 2012 the Nobel Prize for Physiology and Medicine was awarded to the research group of Shinya Yamanaka and John Gurdon who discovered that already differentiated mouse cells can be reprogrammed into induced pluripotent stem cells (iPSCs). They retrovirally transduced the four genes *octamer-binding transcription factor-4 (Oct-4), sex determining region Y-box 2 (Sox2), kruppel-like factor 4 (Klf4)* and *myelocytomatosis oncogene (c-Myc)* into mouse fibroblasts and gained cells with a pluripotent character (Takahashi and Yamanaka, 2006). Only one year later the reprogramming of human fibroblasts opened a wide opportunity for toxicological research as well as in regenerative medicine. Beside the introduction of the four mentioned Yamanaka factors (*Oct-4, Sox2, Klf4* and *c-Myc*) into human material (Takahashi et al., 2007), Yu and colleagues transduced cells with *Oct4* and *Sox2* together with *Nanog homeobox (Nanog)* and *lineage protein 28 (Lin-28;* Fig.1.4*)* which also resulted in a successful reprogrammed, pluripotent cell culture *(Yu et al., 2007)*. Human iPSCs exhibit most characteristics of embryonic stem cells (ESCs) like unlimited self-renewal capacity and differentiation into cell types of all three germ layers – ectoderm, mesoderm and endoderm (Gerecht-Nir and Itskovitz-Eldor, 2004, Zhang et al., 2012, Toivonen et al., 2013). Moreover, iPSCs express the ESC-specific proteins tumor rejection antigen (Tra)-1-60, Tra-1-81, stage-specific embryonic antigen (SSEA) -3 and SSEA4, validating that reprogramed iPSCs resemble stem cell characteristics (Takahashi et al., 2007, Chan et al., 2009, Zhao et al., 2013). The ability of reprogrammed somatic donor cells to differentiate into almost all cells of the body allows various applications. For example, neurological diseases or disorders are problematic to study due to limited sources of human brain tissue. The differentiation of hiPSC into the neural lineage could make them a valuable tool to allow human-based studies and circumvent ethical concerns (Figure 1-1) (Kastenberg and Odorico, 2008).

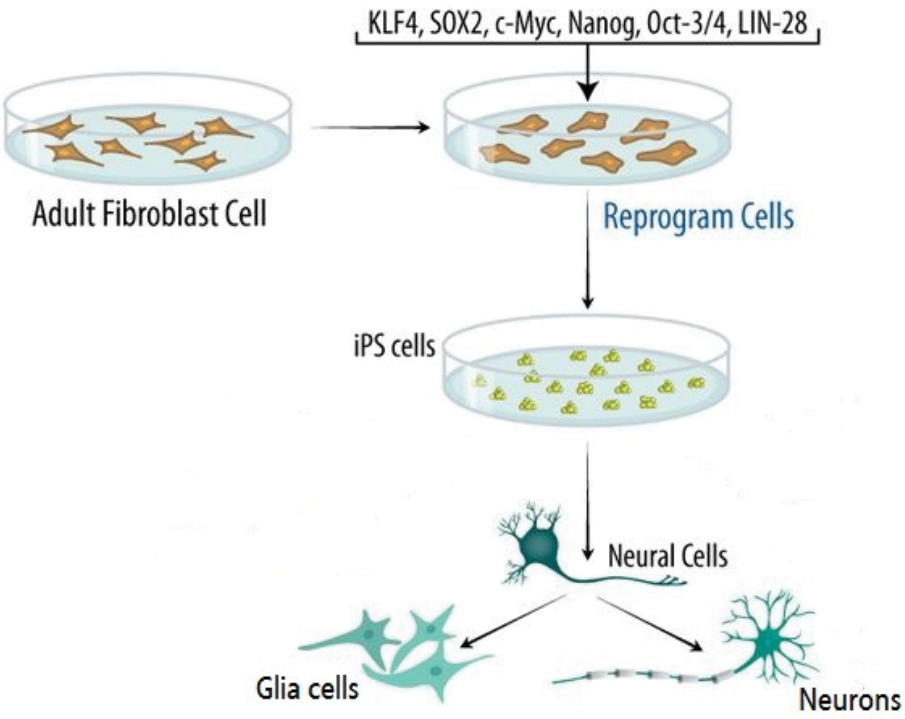

Figure 1-1: Schematic representation of the differentiation potential of induced pluripotent stem cells (iPSC). Adult somatic cells can be reprogrammed by transduction of specific expression vectors. A combination of four factors is used. The most common combined factors are KLF-4, SOX2, c-Myc and Oct-3/4. A successful alternative are the factors (Oct-3/4, SOX2, Nanog and LIN-28. Reprogrammed iPSCs gain a pluripotent character and are able to differentiate into all cell types of the body including neural cells like neurons and glia cells (Modified from Amabile and Meissner, 2009).

1.2.1. Neural induction of hiPSC

The central nervous system (CNS) develops during gastrulation from the dorsal ectoderm. To use hiPSC as an alternative human-based cell model for NT testing the cells need to differentiate into the ectodermal germ layer. To urge stem cells into the ectodermal lineage several strategies have been published. One possibility is to further differentiate EBs into neuroepithelial aggregates, called neural rosettes which can be replated and differentiated into a co-culture of neurons and glia cells (Stummann et al., 2009, Pistollato et al., 2014). Another option is to avoid the differentiation into mesodermal and endodermal direction by dual-SMAD inhibition. The abbreviation is derived from the homologies of genes concerning to Caenorhabditis elegans '*SMA*' ("small" worm phenotype) and Drosophila *'MAD*' ("Mothers Against Decapentaplegic"). It inhibits the transforming growth factor (TGF)-β1 and the bone morphogenetic protein

(BMP) pathway. TGF-β cooperates with the FGF signaling pathway and maintains pluripotency of the cells. When BMP is activated, it induces mesoderm differentiation. The inhibition of TGF-β and BMP signaling is known to result in a highly efficient differentiation into neural progenitor cells (NPC) (Chambers et al., 2009, Zhou et al., 2010, Neely et al., 2012, Telias et al., 2014). Therefore the cells can be cultured in a specific neural induction suspension, containing SMAD inhibition compounds. To ensure a successful neural induction, NPCs ought to be characterized by the expression of specific neural markers like nestin, Pax-6 and Sox2 (Chambers et al., 2009, Breier et al., 2010, Chandrasekaran et al., 2017). Those hiPSC-derived NPCs can be further differentiated into neurons and glia cells to mimic main processes of neurodevelopment (Hofrichter, Nimtz et al., 2017).

1.2.2. Neuronal differentiation and signal transmission

NPCs serve as stem cells for all neurons and glia cells -except for microglia- and differentiate through complex cascades of molecular signaling, migration and positioning in the CNS (Stiles and Jernigan, 2010). Neurodevelopment is characterized by proliferation of NPC, their directed migration to target locations, and the differentiation of NPC to specific cellular subtypes followed by a complex network formation with cell-cell interactions (Figure 1-2). The first neurons arise at the middle embryonic phase (post-conceptional week 4) and neurogenesis continues throughout the fetal phase and extends postnatally (Silbereis et al., 2016, Bystron et al., 2008). Once neurons are at their final position, they start to form dendrites and axons and connect with other neurons by forming points of communications – the synapses (Figure 1-2). Astrocytes play a major role in the formation and elimination of synapses (Clarke and Barres, 2013).

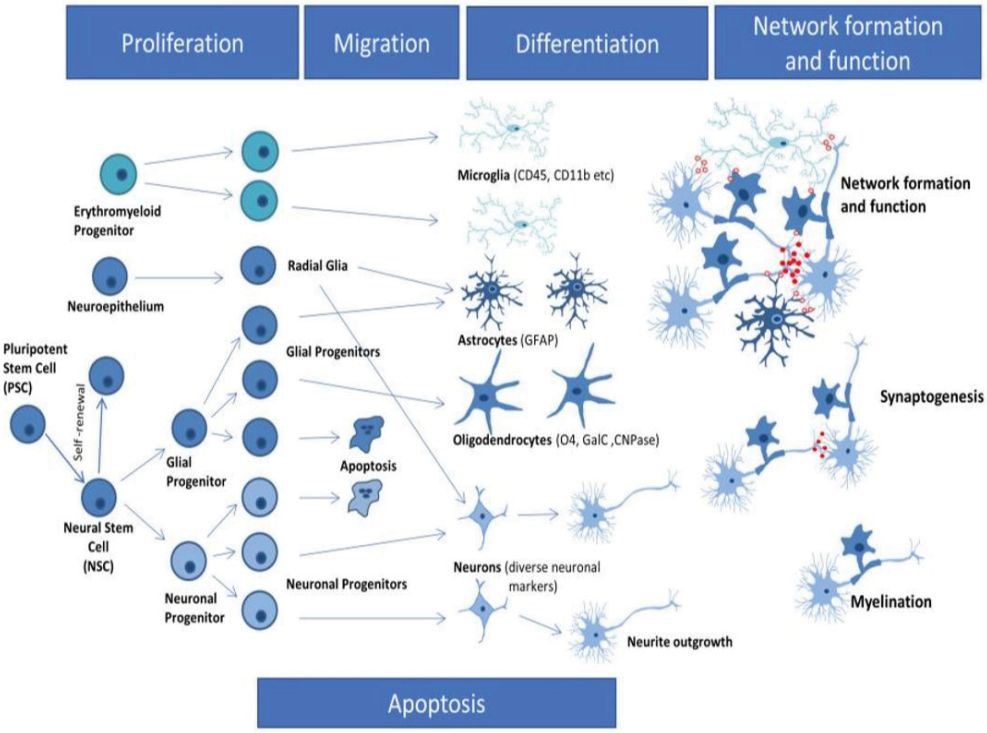

Figure 1-2: Overview of fundamental processes and timing of human brain development. Several processes are fundamental for neurodevelopment and can be modeled *in vitro*. From simple precursor cells (left) to a complex functional network (right). To resemble the *in vivo* biological nervous system these processes should be covered *in vitro* (Bal-Price et al., 2018a).

The controlling properties of the CNS concerning the entire organism lead to stimulating and inhibiting individual signal transmission processes. The postsynaptic membrane of thin branches (dendrites) receive an electrical stimulus, a so called action potential (AP), and transmit the signal via ion channels that allow charged atoms to pass through the membrane (Bean, 2007, Lovinger, 2008, Bender and Trussell, 2012). The changes in the membrane potential transmit the signal along the neuronal cell body to the axon where the presynaptic button of a synapse transfers the AP to the dendrites or the cell body of a connected second neuron (Figure 1-3). Two different types of synapses, chemical and electrical, carry the task of signal transmission between the cells. Here we will focus on the more common transmission form in the CNS via chemical synapses.

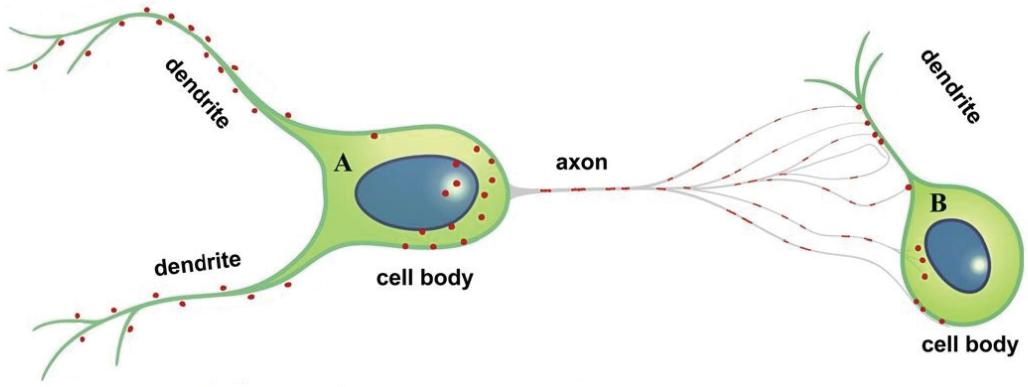

Figure 1-3: Schematic representation of morphological structures associated with synaptogenesis. The postsynaptic membrane of a dendrite receives an action potential and transmits the signal via changes in the membrane potential along the cell body (A) to an axon where the presynaptic bouton of a synapse transfers the action potential to the dendrites or the cell body of a connected second neuron (B). The cell nuclei are represented in blue, in green are the cell bodies and the respective dendrites connected with synaptic structures in red. The axons are represented in grey (Modified from Harrill et al., 2015).

Within a chemical transmission a neuronal stimulus can be transmitted across a synaptic gap of 20-50 nm by the release of neurotransmitters serving as messengers that activate specific answering receptors (Hyman, 2005, Lovinger, 2008). To be classified as a neurotransmitter a substance must be synthesized in the presynaptic neuron, stored in presynaptic vesicles and is released by nerve stimulation to act on pre- or postsynaptic receptors and alters the activity of a receiving neuron (Katz, 1971, Eccles, 1990, Hyman, 2005). Beside large molecules, the neuropeptides, the most common neurotransmitters are amino acids and amines like Glutamate, γ-aminobutyric acid (GABA), acetylcholine (ACH), serotonin or dopamine (DA) (Hyman, 2005, Lovinger, 2008, Samano et al., 2012). Neurons consist of different amounts of specific receptors which can be divided in ligand-gated ion channel receptors and G-protein-coupled receptors (Figure 1-4) (Hyman, 2005, Lovinger, 2008). Ligand-gated receptors can be activated through the binding of a specific agonist - the neurotransmitter molecule. When activated the receptor protein forms an ion pore in the membrane and charged ions can pass along their gradients. This either depolarizes or hyperpolarizes the membrane and leads to a receptor-specific excitatory or inhibitory effect within 1 to 2 milliseconds (Hyman, 2005, Lovinger, 2008). Most receptors have excitatory tasks like the glutamate-dependent α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and N-

methyl-D-aspartate (NMDA)–receptors. The activation leads to stimulating effects through depolarization of the postsynaptic neuron (Nedergaard et al., 2002, Lovinger, 2008, Silbereis et al., 2016). If the prevalent receptors are inhibitory GABA receptors the activated neuron acts inhibitory to the target process (Breakefield and Giller, 1976, Lovinger, 2008, Mendu et al., 2012, Silbereis et al., 2016). It prevents the release of an AP by hyperpolarization of the postsynaptic neuron. Receptors can lead to either an inhibitory or an excitatory effect (Hyman, 2005, Edwards, 2007). For example, DA, serotonin, or ACH receptors can result in both – stimulation and inhibition of signal transmission, depending on what kind of receptor type is involved. The activation of the serotonin-dependent 5-hydrotryptamine 1 (5-HT1) receptor of hippocampal pyramid cells opens neuronal potassium (K^+) channels which results in a hyperpolarization of the post-synapse (Barnes and Sharp, 1999). In contrast the 5-HT3 receptor is a ligand-gated ion channel that results in passing of sodium (Na^{2+}) and K^+ ions along their electrochemical gradients if activated – Na^{2+} streams into the cell and K^+ leaves the cell which depolarizes the postsynaptic membrane (Barnes and Sharp, 1999, Lovinger, 2008).

ACH, glutamate and GABA also have G-protein-coupled receptors which bind the specific neurotransmitter and subsequently activate an intracellular biochemical cascade (Figure 1-4) (Hyman, 2005, Lovinger, 2008). The G-protein, a small intracellular protein, exchanges GTP for GDP which separates the G-protein into the α- and the β-subunit and dissociates from the receptor. The α-subunit activates an enzyme which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP plays a major role in enzyme-mediated phosphorylation that alter functions of cellular proteins. The β-subunit directly activates ion channels that allow K^+ ions flow across the neuronal membrane and produce neuronal inhibition. This kind of receptor activation is slower and longer lasting compared to the ligand-gated receptor activation.

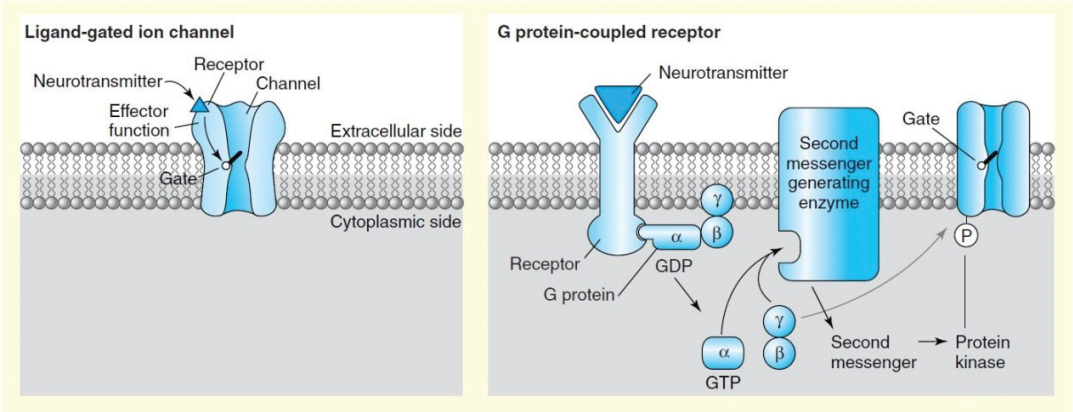

Figure 1-4: Neurotransmitter receptors. The most neurotransmitter receptors are ligand-gated channels (left) or G-protein-coupled receptors (right). They have an extracellular ligand binding domain and a mechanism to convert the ligand binding to a cellular signal. The ligand-gated channel has a central pore that opens and permits ions to pass. The G-protein coupled receptor transmits a signal through the membrane to activate a G-protein and their related signaling cascades. This activates or inhibits second messenger generating enzymes (Modified from Hyman, 2005).

Altogether, these pre- and postnatal immensely complex and strictly spatially and temporally regulated molecular and cellular processes as well as cellular interactions are essential for the precise formation of a functional neuronal network that is the prerequisite for a healthy brain. Any effect on such signaling pathways or processes involved in brain development might lead to an adverse outcome on the organ level (Andersen et al., 2000, Bearer, 2001, Claudio, 2001, Mendola et al., 2002, Grandjean and Landrigan, 2006, Grandjean and Landrigan, 2014).

1.3. Neurotoxicity testing *in vitro*

1.3.1. Developmental neurotoxicity (DNT)

Disturbances and interferences of the developing CNS are of important scientific and socio-political concerns, due to often irreversible outcomes. Several common substances are known to be developmentally neurotoxic, including lead, mercury and many pesticides (Costa et al., 2008, Caito and Aschner, 2015, Aschner et al., 2017). Even a small influence on cognitive abilities might have an enormous impact on the distribution of societal intelligence (Schettler, 2001). The development of a functional

human CNS is constituted of a variety of key neurodevelopmental processes and different developmental timings (Figure 1-5). Thus a strategy for *in vitro* DNT testing needs to be set up that closely resembles human physiology and covers the major neurodevelopmental key events (KE).

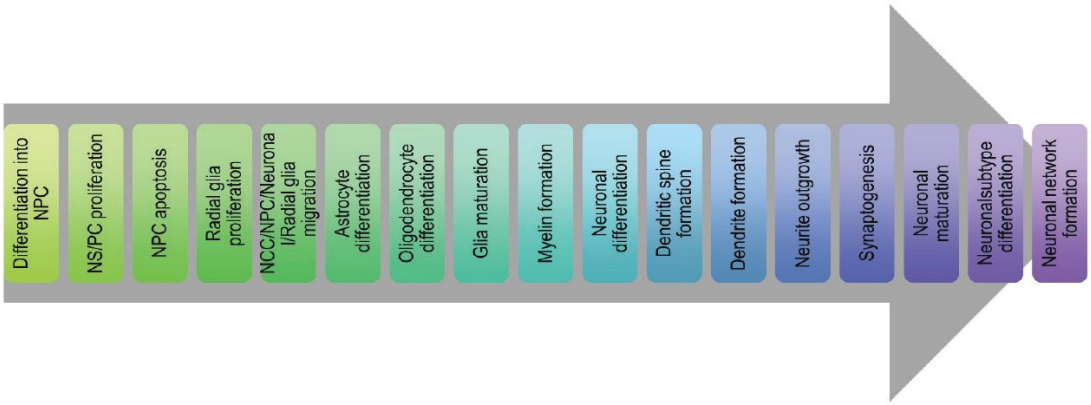

Figure 1-5: Neurodevelopmental key events (Fritsche, 2016).

In the last decades a huge selection of alternative methods for *in vitro* DNT testing were produced that represent developmental processes in different species, brain regions and cell types, and developmental stages (Breier et al., 2010, Fritsche et al., 2015, Fritsche et al., 2018). The most abundant and best studied cell system addressing developmental timing and processes are primary rat cells which are useful for a variety of applications yet still hold the issue of extrapolation to human physiology (Coecke et al., 2007, Fritsche et al., 2015, Fritsche et al., 2017). Recently 6 human-based cell models (hESC, hiPSC, hNPC, ReNcell CX, hUCBSC and LUHMES cells) were identified that mimic 16 neurodevelopmental KE *in vitro.* Such methods are extremely useful but are not comprehensive as they fail to cover neurodevelopmental processes like radial glia proliferation, glia maturation, dendritic spine formation, dendrite formation, axonal growth, and neuronal maturation and neuronal network formation (Fritsche, 2016, Bal-Price et al., 2018a). For *in vitro* DNT testing single assays need to be assembled into a test battery covering various neurodevelopmental processes. One of the test systems that are useful for studying neurodevelopmental KE are primary human neural progenitor cells (NPC). These cells, cultured as 3D neurospheres, mimic multiple

processes of brain development (Moors et al., 2009a, Fritsche et al., 2011, Baumann, 2014). They are positive for the neural stem/progenitor marker nestin and spontaneously differentiate into a co-culture of β-III-tubulin$^+$ neurons, GFAP$^+$ astrocytes and O4$^+$ oligodendrocytes (Reynolds et al., 1992, Piper et al., 2001, Reubinoff et al., 2001, Hofrichter, Nimtz et al., 2017; Moors et al., 2009). NPC can be isolated from a variety of species like rodents and humans (Lonza, Verviers, Belgium; Reynolds and Rietze, 2005, Moors et al., 2009b, Workman et al., 2013, Baumann, 2014, Baumann et al., 2016) and grow in 3D as neurospheres. Due to the 3D nature of the culture they are thought to resemble the *in vivo* situation better than 2D monolayer cultures with regard to advantages for the complexity of directional growth, cell-to-cell- and cell-to-matrix-connectivity (Yamada and Cukierman, 2007, Alepee et al., 2014). Neurospheres suit as an appropriate *in vitro* model to analyze neurodevelopmental processes like apoptosis, proliferation, migration, and differentiation into neurons, astrocytes, and oligodendrocytes (Moors et al., 2009b). To reveal adverse effects of potential neurodevelopmentally toxic compounds the so-called 'Neurosphere Assay'(Figure 1-6) provides analysis of some of the main cellular processes of neurodevelopment and is a useful tool for DNT testing (Moors et al., 2009b, Baumann, 2014, Baumann et al., 2016).

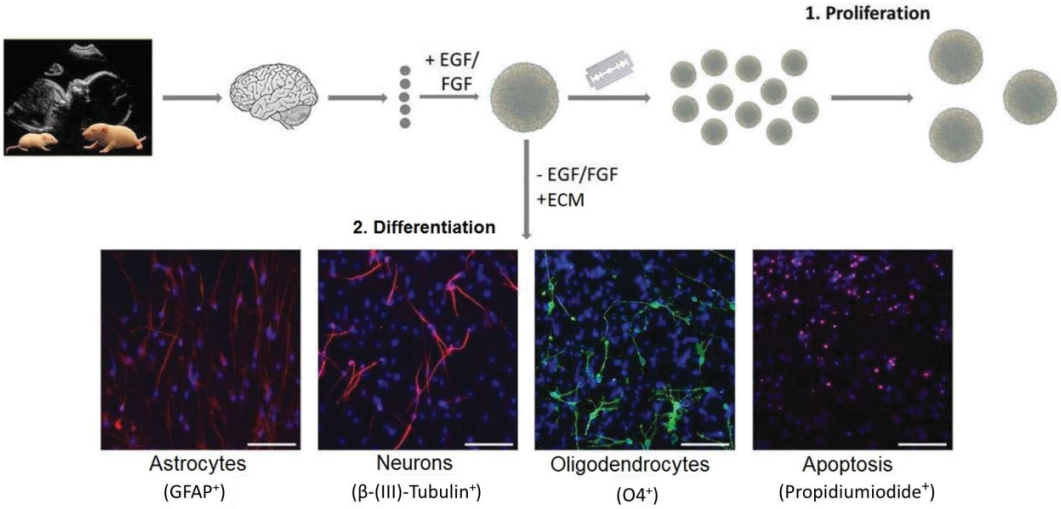

Figure 1-6: The Neurosphere assay. In the presence of growth factors (EGF, FGF) freshly prepared NPC from full brain homogenates form free-floating neurospheres. 1. Proliferation: Under these conditions neurospheres proliferate and can be expanded by mechanically chopping with a razorblade. 2. Differentiation: By withdrawing growth factors and providing an extracellular matrix (ECM) neurospheres adhere, migrate and differentiate into the three main cell types of the CNS and can transit into apoptosis. Shown are proliferating human neurospheres and immunocytochemistry stainings of differentiating astrocytes (red), neurons (red) and oligodendrocytes (green), as well

as stainings of propidiumiodide-positive apoptotic cells (red). Cell nuclei are stained with Hoechst (blue). Scale bar = 100µm.

1.3.2. Neurotoxicity (NT)

Beside neurodevelopmental impairments of potentially toxic chemicals globally used agents like pesticides, possess adult neurotoxic properties and contribute to neurodegenerative disorders and diseases like Alzheimer's and Parkinson's disease. NT testing of compounds for regulatory concern is performed by *in vivo* animal experiments (OECD, 1997). Due to controversially discussed animal consumption tied to inter-species variations, it is recognized that the chemical safety assessment must shift from *in vivo* experiments to functional mechanistic *in vitro* endpoint evaluation. In 2018, 27 endpoint categories were defined that are able to reflect key events of neurotoxicity (Masjosthusmann et al., 2018b). These endpoints in general are associated with inhibition/stimulation of neurotransmitter-dependent neuronal transmission, as well as directed neuronal ion channel modifications (e.g. sodium, potassium, calcium and chloride channel) or cellular endpoints like mitochondrial dysfunction, neuroinflammation, enzyme inhibition etc. that can be used to study MoA-dependent adverse effects. But still most endpoints are covered by either rat (21 endpoint categories) or mouse (18 endpoint categories) primary cell models. The highest rates for human-based cell models were achieved using human stem/progenitor cells that covered 8 endpoint categories. Comparisons of *in vivo* cellular and molecular functions with *in vitro* models are crucial and need special attention. But with regards to species differences working with human cell-derived systems *in vitro* is highly warranted. Using hiPSC-derived neural cells offer the possibility to provide human data, circumventing ethical concerns of human primary cells, and prospectively substitute rodent-based cell systems for NT evaluation (Singh and Kashyap, 2016, Bal-Price et al., 2018b). Nevertheless rodent cell models are well characterized and currently the gold-standard for NT testing (OECD, 1995a, OECD, 1995b, OECD, 1997, OECD, 2007). By using human stem cell-derived testing strategies species comparisons with rodent models as a reference are probably very helpful. Especially functional endpoints like electrical activity of a neuronal network or neuronal subtype differentiation is well characterized in

rodents, yet sparse with hiPSC cultures. Moreover, the standardization of assays and establishment of reliable protocols will be necessary to enhance regulatory assurance and application of non-animal alternatives for neurotoxicity.

1.4. Neuronal networks on microelectrode arrays (MEA)

The formation of a complex neuronal network, consisting of several subtypes of neurons offers the possibility to analyze molecular mechanisms, disturbances of neurotoxicants and neurological diseases. If a neuronal network developed appropriate functional synapses with both excitatory and inhibitory signal transmission, it can be validated by measuring and analyzing electrophysiological activities (Tukker et al., 2016). hiPSC-derived neurons exhibit electrically active properties (Carpenter et al., 2001), and spontaneously form an active functional neuronal network (Heikkila et al., 2009). A widely used and common laboratory technique to analyze electrophysiological brain waves is the so-called patch clamp method where APs are evoked by injecting depolarizing current pulses into a single neuron and spontaneous synaptic currents are recorded (Weick et al., 2011, Pina-Crespo et al., 2012, Telias et al., 2014, Tang-Schomer et al., 2014, Petersen, 2017). The main limitation of this method is that the stimulus transmission of just a single neuron can be measured. A functional neuronal network consists of around 86 billion neurons with over a quadrillion neuronal connections that are able to communicate rapidly across long distances (Azevedo et al., 2009, Herculano-Houzel et al., 2015, Silbereis et al., 2016). The use of substrate-integrated MEA bypasses this limitation. It allows simultaneous extracellular recordings of electrical activity from an entire neuronal network. A MEA consists of a defined number of conductive material-coated microelectrodes, which are integrated into a glass slide. Conductive materials could be indium tin oxide, titanium, palladium or gold. The actual recording field contains the electrodes arranged in a grid that covers up to 2 mm^2. By coating the glass surface with an extracellular matrix cells can be differentiated on top of these electrodes. In real-time an external amplifier transmits the electrical signals to a computer for digital conversion where it is filtered, analyzed and stored. Recording-analysis of these extracellular APs provides important information of network structures

and maturation stages which is impossible to obtain using other electrophysiological techniques. The typically accessed extracellular potentials recorded by a MEA are the rate of APs, named “Spikes”, and a train of APs, named “Bursts” (Figure 1-7). Those parameters can be recorded from each electrode and analyzed regarding single spike and burst rates or overall network rates.

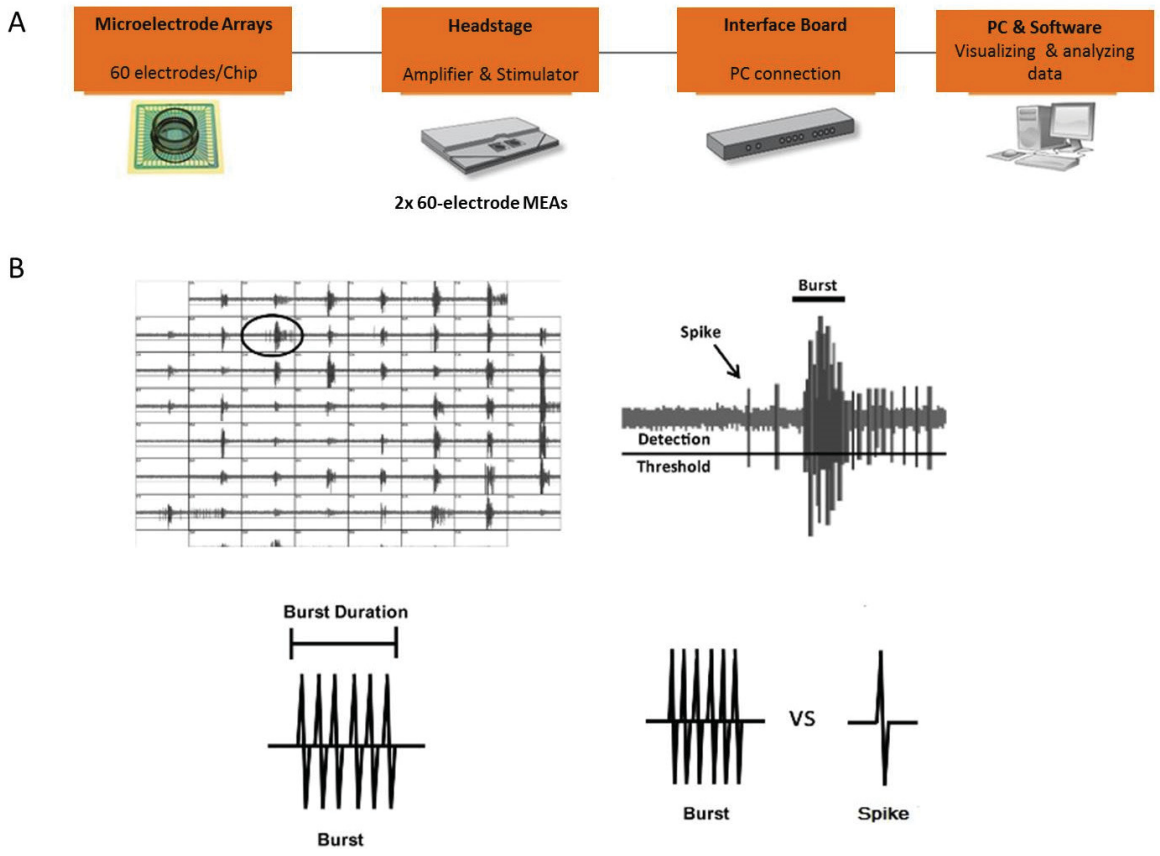

Figure 1-7: Experimental setup and electrical activity visualization of microelectrode arrays (MEAs). A) The recording field of a MEA-Chip consists of 60 electrodes. Two chips can be placed into a head stage which relates to a personal computer (PC) via an interface board. Recording and analyzing software processes the incoming data. **B)** Shown is a representative measurement of electrical activities from a MEA. Each rectangular box represents one individual electrode which must detect spike or burst activities passing a defined threshold. The burst duration and the comparison of burst with single spikes is schematically represented. (Modified from Mack et al., 2014).

Many additional parameters from spiking and bursting may be analyzed using specified software – e.g. how many electrodes detected activity, mean firing rate (spikes/minute), inter-spike-intervals, mean bursting rate (bursts/minute), inter-burst-intervals, burst duration, number of spikes per burst (Kapucu et al., 2012, Cotterill et al., 2016a).

MEA approaches are also suitable for network characterization by determination of neuronal subtypes and receptor constitutions. Pharmacological agonists and antagonists like GABA, bicuculline, Glutamate, NBQX, AP-5 and DA can lead to changes in activity patterns and give indications of the presence of network states (Heikkila et al., 2009, Yla-Outinen et al., 2010, Kapucu et al., 2012, Odawara et al., 2014, Fukushima et al., 2016, Odawara et al., 2016b, Kasteel and Westerink, 2017).
Due to species differences it will be unavoidable to focus on a research based on human material. Especially analyses of neurological diseases or disturbances on the developing neural system are in a need of concentrating on human stem cell derived neural tissue, owing to primary cell source limitations. As specified in chapter 1.3. the MEA technology can serve as a useful tool to analyze and validate key neurological processes like neuronal maturation and neuronal network formation.

1.5. Aim of this thesis

For studying molecular and cellular aspects of neurotoxicity currently used animal models are labor-intensive, time-consuming, costly and ethically debated. Thus alternative human-based *in vitro* methods that avoid species-differences and are able to substitute *in vivo* animal studies are needed. However, the use of primary human cells often bears cell source limitations and ethical concerns. Therefore the goal of this thesis was to generate hiPSC-derived NPC, to overcome these issues, and to differentiate hiPSC-derived electrophysiologically active neuronal networks. These functional neuronal networks offer the possibility to understand and analyze the complex procedures of brain development and the orchestrations of neuronal interworking with the final goal to test for adverse outcomes of potentially neurotoxic compounds. The aims of this study were:

1. Generation of hiPSC-derived NPC via a neural induction protocol cultured as three-dimensional neurospheres and comparative analysis to primary human fetal neurospheres.

2. Molecular and functional characterization of human iPSC-derived and rat electrophysiological active neuronal networks on microelectrode arrays.

3. Identification of activity performance across hiPSC-derived and rat neuronal networks with a set of pharmacological compounds identifying neuronal subtype constitutions.

2. Manuscripts

The present thesis consists of four manuscripts.

The first manuscript 2.1, ‘Comparative performance analysis of human iPSC-derived and primary neural progenitor cells (NPC) grown as neurospheres *in vitro*’ provides protocols to neurally induce hiPSC into neurospheres and compares them with human primary neurospheres. We tested two different neural induction protocols for the generation of hiPSC-derived neurospheres. We further molecularly characterized the hiPSC-derived neurospheres and compared them to well established human primary neurospheres to validate their performance in the ‘Neurosphere Assay’. The aim of this work was the establishment of a neural induction protocol for hiPSC and their application in the ‘Neurosphere Assay’ to mimic neurodevelopmental processes. Based on this development of a human stem cell-based *in vitro* method for DNT testing, we reviewed in the second manuscript 2.2, ‘Current Availability of Stem Cell-Based *In Vitro* Methods for Developmental Neurotoxicity (DNT) Testing’, the availability to date of stem cell-based *in vitro* methods for DNT evaluation that focusses on the concept of neurodevelopmental process assessment. Here we summarized the current state of the art on neural stem/progenitor cell-based methods including a variety of specific endpoints for the evaluation of DNT. With the third publication 2.3, ‘Development of the Concept for Stem Cell-Based Developmental Neurotoxicity Evaluation’, we further pointed out the scientific rationales for endpoints that are currently selected for assay establishment with human stem/progenitor cells. The findings of these publications provide the fundamentals for the manuscript 2.4, ‘Characterization and application of electrically active neuronal networks established from human induced pluripotent stem cell-derived neural progenitor cells for neurotoxicity evaluation’. Here we changed the medium composition used in manuscript 2.1 to a so-called CINDA medium that aims at acceleration of neuron and glia maturation. Both, conventional and CINDA medium were compared by investigating gene and protein expression. Moreover, hiPSC-derived electrically active neuronal networks were pharmacologically characterized.

Manuscripts

2.1 Comparative performance analysis of human iPSC-derived and primary neural progenitor cells (NPC) grown as neurospheres *in vitro*.

Maxi Hofrichter, **Laura Nimtz**, Julia Tigges, Yaschar Kabiri, Friederike Schröter, Brigitte Royer-Pokora, Barbara Hildebrandt, Alexey Epanchintsev, Stephan Theiss[e], James Adjaye, Jean-Marc Egly, Jean Krutmann, Ellen Fritsche

Stem Cell Research

Entwicklungsneurotoxizität (ENT)-Tests, die häufig an Ratten durchgeführten werden, sind ressourcenintensiv (Kosten, Zeit, Tiere) und Ergebnisse sind hinsichtlich der Spezies-Unterschiede nicht auf den Menschen übertragbar. Daher sind zuverlässige alternative Ansätze auf der Basis menschlicher Zellen erforderlich, um beispielsweise die Toxizität von Chemikalien für die Entwicklung menschlicher Neurone vorherzusagen. Human induzierte pluripotente Stammzellen (hiPSCs) bilden die Grundlage für solch eine alternative Methode, die möglicherweise als Teil einer alternativen ENT-Teststrategie verwendet werden kann.

Hier vergleichen wir zwei Protokolle der neuralen Induktion von hiPSC zu neuralen Progenitorzellen, den sogenannten 3D-Neurosphären: ein Protokoll verwendet das Protein Noggin und eine Kultivierung der Zellen in einem speziellen neuralen Induktionsmedium (NIM-Protokoll). Die Ausbildung von Nestin + / SOX2 + -HiPSC-abgeleiteten neuralen Vorläuferzellen (NPCs) wurde mit primären humanen NPCs verglichen. Im Allgemeinen differenzieren primäre hNPCs zuerst in Nestin + - und / oder GFAP + -radiale glia-ähnliche Zellen, während die von hiPSC abgeleiteten NPCs (hiPSC-NPC) sich zuerst in βIII-Tubulin + -Neurone differenzieren. Dies deutet auf ein früheres Entwicklungsstadium von hiPSC-NPC hin. Im "Neurosphere Assay" schnitten die mit dem NIM-Protokoll erzeugte hiPSC-NPC besser ab als solche mit dem Noggin-Protokoll. Nach einer Langzeitdifferenzierung bildete hiPSC-NPC neuronale Netzwerke, die nach 85 Tagen auf Mikroelektrodenarrays elektrische Aktivitäten aufwiesen. Ein erster toxikologischer Test mit der Substanz Methylquecksilberchlorid inhibierte die Migration von hiPSC-NPC und hNPC mit ähnlichen Potenzen.

Dies zeigt, dass sich hiPSC-NPCs, die als Neurosphären wachsen, für ENT-Tests eignen, da sie in der Lage sind die frühe neurale Entwicklung *in vitro* nachzuahmen. Weitere Charakterisierungen dieses Zellsystems durch Substanztestung sind allerdings erforderlich, um Vertrauen in diese Methode zu gewinnen.

Stem Cell Research 25 (2017) 72–82

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Comparative performance analysis of human iPSC-derived and primary neural progenitor cells (NPC) grown as neurospheres *in vitro*☆

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ARTICLE INFO

Article history:
Received 28 October 2016
Received in revised form 17 October 2017
Accepted 23 October 2017
Available online 26 October 2017

Keywords:
In vitro
Testing
Brain development
Stem cell
MEA

ABSTRACT

Developmental neurotoxicity (DNT) testing performed in rats is resource-intensive (costs, time, animals) and bears the issue of species extrapolation. Thus, reliable alternative human-based approaches are needed for predicting neurodevelopmental toxicity. Human induced pluripotent stem cells (hiPSCs) represent a basis for an alternative method possibly being part of an alternative DNT testing strategy.

Here, we compared two hiPSC neural induction protocols resulting in 3D neurospheres: one using noggin and one cultivating cells in neural induction medium (NIM protocol). Performance of Nestin$^+$/SOX2$^+$ hiPSC-derived neural progenitor cells (NPCs) was compared to primary human NPCs. Generally, primary hNPCs first differentiate into Nestin$^+$ and/or GFAP$^+$ radial glia-like cells, while the hiPSC-derived NPCs (hiPSC-NPC) first differentiate into βIII-Tubulin$^+$ neurons suggesting an earlier developmental stage of hiPSC-NPC. In the 'Neurosphere Assay', NIM generated hiPSC-NPC produced neurons with higher performance than with the noggin protocol. After long-term differentiation, hiPSC-NPC form neuronal networks, which become electrically active on microelectrode arrays after 85 days. Finally, methylmercury chloride inhibits hiPSC-NPC and hNPC migration with similar potencies. hiPSC-NPCs-derived neurospheres seem to be useful for DNT evaluation representing early neural development *in vitro*. More system characterization by compound testing is needed to gain higher confidence in this method.

1. Introduction

Research performed in animals helped understanding physiology and disease and thus lead to the development of treatment strategies over the past decades. However, there seem to be limitations in translation of animal data to humans (Leist and Hartung, 2013), which is one of the discussed causes for failures in drug development (Kenter and Cohen, 2006; Leist and Hartung, 2013; Seok et al., 2013). For this reason, models that resemble human physiology on the molecular level and thus might help predicting drug efficacy and adversity in humans are needed (Leist and Hartung, 2013). Thus, the discovery that differentiated somatic cells can be reprogrammed into human induced pluripotent stem cells (hiPSCs) with the potential to self-renew and differentiate into most cell types of the body (Takahashi et al., 2007) has been raising excitement within the scientific community. Such hiPSCs are human-based and circumvent the ethical issues associated with primary human material or human embryonic stem cells (hESCs) (Kao et al., 2008; Kastenberg and

Abbreviations: BDNF, brain-derived neurotrophic factor; BMPs, bone morphogenic proteins; DNT, Developmental neurotoxicity; EBs, embryoid bodies; GDNF, glial cell line-derived neurotrophic factor; GW, gestational week; hESCs, human embryonic stem cells; hiPSCs, Human induced pluripotent stem cells; hiPSC-NPC, hiPSC-derived NPCs; LOAEC, lowest observed adverse effect concentration; MEA, microelectrode arrays; MeHgCl, methylmercury chloride; nd, not detectable; NDM, neural differentiation medium; NIM, neural induction medium; NPCs, neural progenitor cells; NPM, neural proliferation medium; TGF-β3, transforming growth factor β-3; TTX, tetrodotoxin.

☆ Acknowledgment of grants, equipment, or drugs for research support: This work was funded by iBrain – the interdisciplinary Graduate School for brain research and translational neuroscience at the Heinrich-Heine-University Duesseldorf and CERST-NRW (State Government of Northrhine Westphalia, Germany).

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https://doi.org/10.1016/j.scr.2017.10.013

Odorico, 2008; Singh et al., 2015). Moreover, hiPSCs provide unlimited cell sources, might render the possibility to overcome the species-dependent shortcomings of animal cells and are thus possible alternative models for basic research, disease modeling, drug development and toxicity screening (Robinton and Daley, 2012).

The development of the human brain is a highly complex procedure relying on a large variety of neurodevelopmental processes including proliferation, migration, differentiation, synaptogenesis and apoptosis of neural progenitor cells (NPCs). The need for a spatiotemporally concerted action of such complex processes makes it especially vulnerable to adverse effects of exogenous compounds (Rodier, 1995; Rice and Barone, 2000). Identification of substances with adverse effects on these processes is important, as resulting neurodevelopmental defects may lead to cognitive and intellectual disability as well as to neurological disorders (Grandjean and Landrigan, 2006). These do not only pose a burden on individuals but also provide a socio-economic deficit for society (Bellanger et al., 2013; Trasande et al., 2015).

At present, the gold standards for developmental neurotoxicity (DNT) testing are the rat EPA 870.6300 DNT Guideline (EPA, 1998) and the draft OECD TG426 (OECD, 2007). Performing either one involves high costs (approx. € 1,000,000/compound), takes up to one year and engages a large number of animals. Still there are uncertainties in the guidelines' methodology, evaluation, and regulation (Tsuji and Crofton, 2012). Thus, there is a need for alternative methods evaluating DNT potential of pharmaceuticals and industrial compounds that has been voiced by different stakeholders (Lein et al., 2007; Crofton et al., 2011; Bal-Price et al., 2012, 2015a; Fritsche et al., 2017).

Different *in vitro* methods have been published over the last decade, which evaluate a variety of DNT-related endpoints (Bal-Price et al., 2012, Fritsche et al., 2015). Amongst models from different species (human, mouse, rat) and methods (tumor, primary, stem/progenitor cells), human stem/progenitor cell methods seem to be the most promising as they involve the correct species, resemble physiologically relevant developmental processes and cover a large variety of relevant endpoints (Fritsche et al., 2015). Such methods when based on human embryonic stem cells have the drawback that they are prone to raising ethical concerns when it comes to their usage in compound testing (Kastenberg and Odorico, 2008; Dunnett and Rosser, 2014).

With the goal to overcome the species-dependent and ethical hurdles of current *in vitro* test methods, this study aimed to develop a hiPSC-based neural progenitor cell (hiPSC-NPC) *in vitro* assay based on hiPSC-derived neurospheres that allows assessment of multiple neurodevelopmental endpoints. In addition to assessment of the hiPSC-neurosphere assay performance by comparing two different neural induction protocols, hiPSC-NPCs' performance is compared to primary human NPCs, which are considered as the gold standard for this study (Moors et al., 2009).

2. Material and methods

2.1. hiPSC culture

The hiPSC lines A4, (Wang and Adjaye, 2011), and CRL2097 (characterization in Figs. S1–S6) were cultured in mTeSR1 medium (Stemcell Technologies, Germany) under feeder-free conditions on Matrigel (BD Biosciences, Germany). Medium was changed every day and cells were passaged mechanically using a 0.8 mm × 40 mm syringe needle (BD Biosciences, Germany). Both hiPSC lines were regularly tested for the expression of the pluripotency markers (Fig. S7) and their chromosomal integrity (Fig. S2).

2.2. Neural induction

2.2.1. Noggin protocol

Neural induction was performed as previously described (Denham and Dottori, 2011). Briefly, hiPSC colonies with hESC-like morphology (Fig. S8) were treated with induction medium (KoDMEM (Invtirogen, USA); 20% Knockout Serum Replacement (KSR, Invitrogen, USA); 2 mM L-Glutamine (PAA Laboratories GmbH, Germany); 1×10^{-4} M non-essential amino acids (NEAA, Biochrom, Germany); 1×10^{-4} M β-Mercaptoethanol (Invitrogen, USA); 1% Penicillin/Streptomycin (P/S, Pan Biotech, Germany)) without growth factors but with 500 ng/mL noggin (Peprotech, Germany) for 14 days. Afterwards, colonies were cut and cultured in ultra-low-attachment (ULA) plates (Oehmen, Germany) in neural proliferation medium (NPM) consisting of DMEM (Life Technologies, USA) and Hams F12 (Life Technologies, USA; 3:1) supplemented with 1× B27 (Invitrogen GmbH, Germany), 20 ng/mL epidermal growth factor (EGF; Biosource, Germany) and 20 ng/mL basic fibroblast growth factor (bFGF, R&D Systems, Germany) until they formed free-floating three-dimensional neurospheres and were referred to as hiPSC-NPCs which were kept in culture for an additional 28 days before starting experiments (Fig. 1B).

2.2.2. NIM protocol

This neural induction protocol was modified from a previously described one (Hibaoui et al., 2014). Briefly, hiPSC colonies with hESC-like morphology (Fig. S8) were cut and hiPSC clumps were cultured in ULA plates (Oehmen, Germany) in neural induction medium (NIM) consisting of DMEM (Life Technologies, USA) and Hams F12 (Life Technologies, USA; 3:1) supplemented with 1× B27 (Invitrogen GmBH, Germany), 20 ng/mL EGF (Biosource, Germany) and 1× N2 supplement (Invitrogen, Germany) for 7 days as embryoid bodies (EBs). Subsequently, EBs were transferred into new ULA plates with NIM containing 10 ng/mL bFGF (R&D Systems, Germany) for another 14 days. Afterwards, EBs were referred to as hiPSC-NPCs, transferred into new ULA plates (Oehmen, Germany) and cultured as free-floating three-dimensional neurospheres in NPM.

hiPSC-NPCs from both protocols were cultured under these conditions for at least another 28 days before starting experiments (Fig. 1C).

Three neural inductions of each of the Noggin and the NIM protocol were performed as independent experiments.

2.3. hiPSC-NPC and hNPC culture

Primary fetal hNPCs (GW 16–18) were purchased from Lonza Verviers SPRL (Verviers, Belgium) and were kept in culture as passage 0 for 4 weeks after thawing. Primary hNPCs and hiPSC-NPCs were cultured as 3D free-floating spheres in un-coated and ULA 100 mm petri dishes, respectively, in NPM. Medium was changed every 2–3 days. Proliferating neurospheres were passaged using a McIlwaine tissue chopper to cut them into smaller pieces with a diameter of 200 μm (Mickle Laboratory, UK (Fritsche et al., 2011; Baumann et al., 2014)). After chopping, spheres were allowed to recover for 2 days. This procedure was also performed two days prior to experiments to obtain sphere populations of equal sizes.

2.4. Flow cytometry analysis

For FACS analysis 30 hiPSC-NPCs and primary hNPCs spheres each with a diameter of 300 μm were collected. Cells were singularized by incubation with Accutase (Invitrogen, USA) for 20 min at 37 °C and 5% CO_2. Afterwards, cells were fixed with 4% paraformaldehyde (PFA, Sigma Aldrich, Germany) for 30 min at 37 °C and washed with PBS (Life Technologies, USA). Subsequently, cells were permeabilized with 0.1% PBS-T (Triton-X (Sigma Aldrich, Germany) in PBS) at room temperature for 15 min. Finally, cells were stained with anti-Nestin-Alexa647 (BD Bioscience, Germany) and anti-Sox2-PE (BD Bioscience, Germany) in 0.1% PBS-T in the dark at 4 °C for 30 min. Samples were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, Germany).

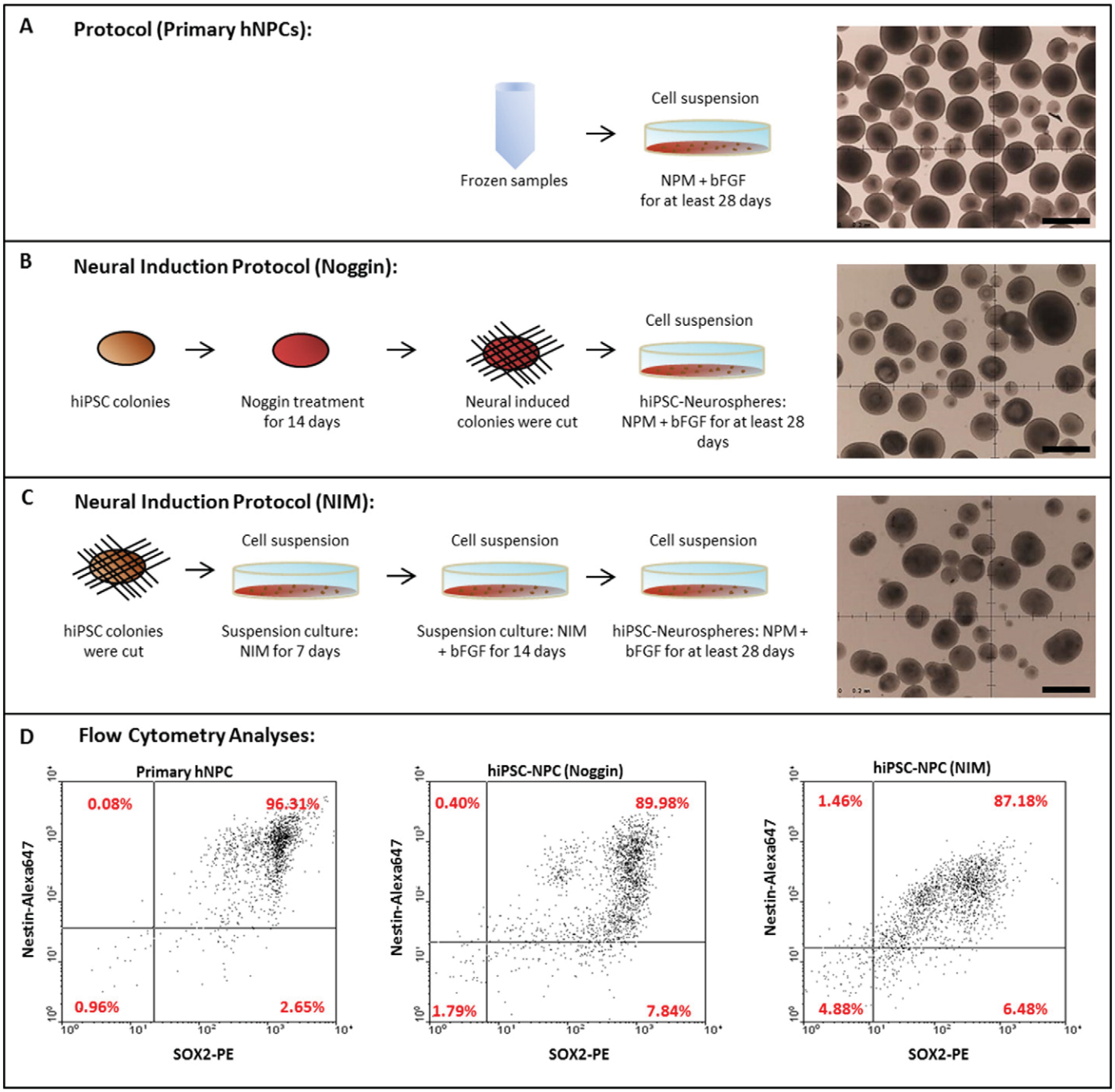

Fig. 1. Neural Induction Protocols to differentiate hiPSCs into neurospheres. hiPSCs were differentiated into neurospheres resembling primary human fetal neurospheres (A) using two different protocols. (B): For the noggin protocol hiPSCs colonies were treated with 500 ng/μL noggin for 14 days. Afterwards they were cut into pieces and cultured as suspension culture in neural proliferation medium (NPM) containing basic fibroblast growth factor (bFGF; Denham and Dottori, 2011). (C): For the NIM protocol hiPSC colonies were cut into pieces and directly cultured as suspension culture in NIM. After 7 days, bFGF was added to the culture for additional 14 days. Finally, hiPSC-derived neurospheres were cultured in NPM containing bFGF for at least 28 days (modified from Hibaoui et al., 2014). Scale bars = 500 μm. (D): hiPS-derived neurospheres generated with the Noggin protocol (B) and the NIM protocol (C) were analyzed for their expression of the neural stem/progenitor markers Nestin and SOX2 *via* flow cytometry analyses and compared to primary human neurospheres. Number of analyzed cells = 2000.

2.5. Quantitative reverse-transcription PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's protocol. For reverse transcription, 300 ng RNA was transcribed into cDNA using the QuantiTect Rev. Transcription Kit (Qiagen, Germany) according to manufacturer's protocol. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using the Rotor Gene Q Cycler (Qiagen, Germany) with QuantiTect SYBR green PCR Master Mix (Qiagen, Germany) following manufacturer's instructions. Primer sequences are presented in supplementary Table 1. Analysis was performed with the ddCT method and the detection limit was set to 0.0001/β-actin. Lower expressions are indicated as 'not detectable' (nd).

2.6. Neurosphere assay

2.6.1. Proliferation assay

Six 300 μm neurospheres per condition were placed into 96-well plates (Greiner, Austria) and cultured either in NPM containing 20 ng/mL EGF (Biosource, Germany) and 20 ng/mL bFGF (R&D Systems,

Germany) or in NPM without growth factors for 14 days. Neurospheres were visualized under a light-optical microscope (Olympus, Germany) and photographed (camera: Visitron Systems, Germany) every 3–4 days. Diameter was measured using ImageJ (National Institutes of Health, USA). Medium was changed every 2–3 days during this assay (Baumann et al., 2014).

2.6.2. Migration assay

NPC migration was assessed as described previously (Moors et al., 2007; Baumann et al., 2014, 2016). For detailed description refer to Supplementary material and methods.

2.6.3. Differentiation assay and immunocytochemistry

Five neurospheres per condition with a diameter of 300 μm were plated in neural differentiation medium (NDM) containing DMEM (Life Technologies, USA) and Hams F12 (Life Technologies, USA; 3:1) supplemented with 1× B27 (Invitrogen GmBH, Germany) and 1× N2 supplement (Invitrogen, Germany) and cultured for 7 or 28 days. Medium was changed once a week. Afterwards, cells were fixed with 4% PFA (Sigma Aldrich, Germany) for 30 min at 37 °C and washed with PBS. Cells were stained with the primary antibodies (mouse-anti-βIII-Tubulin and rabbit-anti-GFAP (Sigma Aldrich, Germany)) for 1 h at 37 °C. For the co-staining of neurons and synapses, cells were stained with mouse-anti-βIII-Tubulin and rabbit-anti-PSD-95 (Abcam, UK) or mouse-anti-Synapsin1 (Synaptic Systems, Germany) for 1 h at 37 °C. After washing with PBS, cells were incubated with the secondary antibodies anti-mouse-Alexa-546 (Invitrogen, USA) and anti-rabbit-Alexa488 (Invitrogen, USA) for 30 min at 37 °C. Nuclei were stained with Hoechst33258 (Sigma Aldrich, Germany). Samples were analyzed using a fluorescent microscope (Carl Zeiss, Germany) and the AxioVision Rel.4.8 software (Carl Zeiss, Germany).

2.6.4. Quantification of immunocytocemistry

βIII-Tubulin staining of the obtained fluorescent images (Fig. 3D) was analyzed using a Fiji Macro (Schindelin et al., 2012). Briefly, images were converted to 8-bit and a local background subtraction was performed using the rolling ball method with a radius of 800 pixels. Consequently, thresholds of the images were defined with fixed background values obtained through averaging Otsu threshold values of three images per experiment. Resulting binary images were filtered for particles larger than 100 pixels, to only obtain neuronal structures. Binary images were analyzed for their area, and neurite mass was calculated by dividing this area by the number of cell nuclei present in a given image. Neurite mass is given as μm^2/nucleus.

The total number of synapses, stained for SYNAPSIN-1 or PSD95, which are co-localized with βIII-Tubulin$^+$ neuronal cells (Fig. 4E, F), were analyzed utilizing a self-written script in the Omnisphero software (Schmuck et al., 2017). Two different thresholding methods were used to pre-process βIII-Tubulin staining in the images. In a first step foreground pixels were removed, using the imopen command (https://www.mathworks.com/help/images/ref/imopen.html) with a non-flat structuring element (ball) with different sizes to get an estimation of the uneven background. This background was subsequently subtracted from the image. On this image an adaptive thresholding method was applied (https://www.mathworks.com/matlabcentral/fileexchange/8647-local-adaptive-thresholding). The resulting binary image was used as a mask to only extract areas from the initial thresholded image, located within the mask. Consequently, the image was thresholded with a fixed threshold and calculated with the isodata algorithm (Ball and Hall, 1965). In a second approach, the same initial background corrected images were processed with various edge detecting algorithms (canny, prewitt, sobel, Roberts and log; Canny, 1986; Lim, 1990; Parker, 1997). Resulting binary images of edges were summed. This image was then added to those obtained from the adaptive thresholding and small holes were closed, utilizing the imfill function. PSD95 and SYNAPSIN-1 were also processed by removing the foreground pixel to estimate the background. Consequently, an isodata threshold was applied and large particles, not considered synapses, were filtered out. In a final step all synapses not located on a binary component of the neuron image were removed. As output the area of the binary neuronal components was calculated, as well as the number of synapse particles and the relative density of these particles as a measure of particle per area. Quantified synapses are shown as synapses/neurite area [μm^2].

We calculated the number of synapses co-localizing with βIII-Tubulin positive neurites in relation to the respective neurite area in three representative images of three independent neural inductions.

2.7. Methylmercury chloride (MeHgCl) treatment and viability assay

Neurospheres were plated as described for the migration assay in the presence of the indicated concentrations of MeHgCl dissolved in DMSO or 0.1% DMSO as solvent control in NDM for 24 h. Migration distance was determined as described above and cell viability was assessed using the Cell Titer-Blue® (CTB) Viability Assay (Promega, Germany) according to manufacturer's instructions. Briefly, primary and CRL2097 and A4 hiPSC-based neurospheres were incubated with the CTB solution for 2, 3.5 and 4.5 h before fluorescence (540Ex/590Em) was measured using a multimode microplate reader (Tecan, Switzerland).

2.8. Neuronal network differentiation on microelectrode arrays (MEA)

Recordings of electrical activity were performed using MEAs with a square array of 59 substrate-embedded titanium nitride microelectrodes (30 μm diameter, 200 μm inter-electrode distance), and an internal reference electrode (200/30iR-Ti-gr, Multichannel Systems MCS GmbH, Reutlingen, Germany). MEA chips were coated with PDL (0.1 mg/mL, 500 μL for 48 h at 4 °C; Sigma Aldrich) and after washing and air-drying with a 50 μL-drop of Laminin (0.01 mg/mL, 48 h at 4 °C; Sigma Aldrich). Afterwards, ~200 hiPS-derived neurospheres (100 μm diameter) were seeded onto MEAs and incubated at 37 °C and 5% CO_2 in NDM medium. For tetrodotoxin (TTX) treatment, 1 μM of TTX (Tocris Bioscience, UK) was added directly to the culture during recordings of the MEA. Afterwards, cultures were washed three times with PBS and fresh NDM medium was added. The same MEA was again measured after 48 h.

2.9. MEA recordings

Recordings of each MEA were performed under sterile conditions for 10 min at one-minute intervals. Therefore, MEAs were placed in the MEA-amplifier (MEA 2100-2x60-System, Multichannel Systems, Germany) and equilibrated to 37 °C for 2 min using the TC02 temperature controller (Multichannel Systems, Germany). Recordings were performed with the MC_Rack-Software (MC_Rack, Multichannel Systems, Germany), electrical activity was sampled at 25 kHz and a background noise below 20 μV. To remove baseline variations, high- and lowpass filters (MCS Filter V 1.0.7, Multichannel Systems, Germany) with cut-off frequencies of 100 Hz and 3500 Hz, respectively, were used. Recordings were analyzed by SpAnNer Software (SpAnNer XBD 3.6, Result GmbH), which detected spikes with a threshold of 8.0 times the standard deviation of the baseline noise level.

2.10. Statistics

All statistical analyses were performed using GraphPad Prism 6.00 for Windows (GraphPad Software, La Jolla Carlifornia USA, www.graphpad.com). To compare different groups of PCR analyses an unpaired *t*-test was performed (Fig. 2A–C). All other data were analyzed using a two-way ANOVA followed by Bonferroni test to correct for multiple testing.

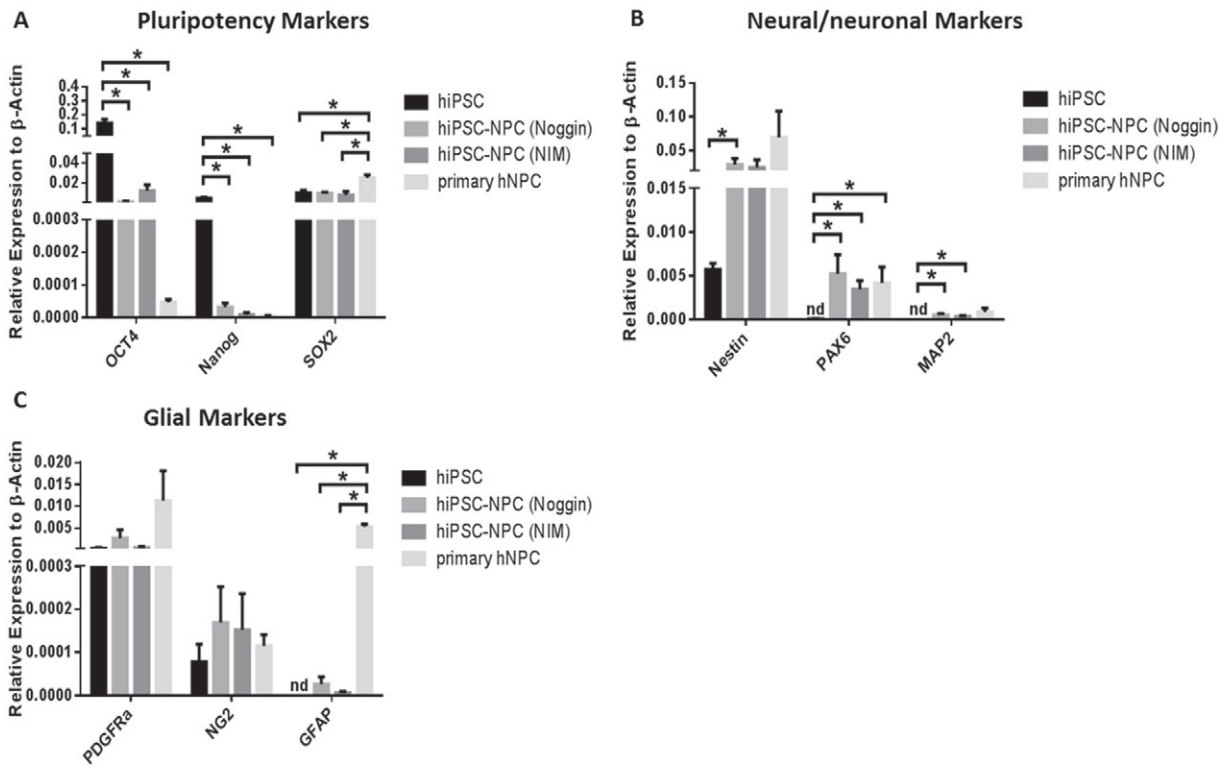

Fig. 2. qRT-PCR analyses of proliferating hiPSC-derived NPCs. hiPSC-derived proliferating neurospheres generated with the noggin and the NIM protocol were analyzed for their mRNA expression profile regarding pluripotency (A; Oct4, Nanog, Sox2), neural/neuronal (B; Nestin, Pax6, Map2) and glial (C; PDGFRα, NG2, GFAP) markers using qRT-PCR analysis. Gene expressions of hiPSC-derived NPC are compared to expressions of these markers in undifferentiated hiPSCs and primary hNPCs. Values are depicted with ddCT method as mean + SEM of relative marker expression to β-actin, n = 3; each n represents data from an independent neural induction. Statistical significance was calculated using the unpaired *t*-test; * significant difference compared to undifferentiated hiPSCs ($p < 0.05$). # significant difference compared to primary hNPCs ($p < 0.05$). nd = not detected (>0.0001/β-actin).

3. Results

3.1. Generation of NPCs from hiPSCs

Two different neural induction protocols were used to create 3D neurospheres consisting of NPCs from hiPSCs. For the first approach hiPSCs were treated with 500 ng/mL noggin (referred to as noggin protocol Denham and Dottori, 2011; Fig. 1B), a protein playing a crucial role in neurogenesis by inhibition of bone morphogenic proteins (BMPs; Lamb et al., 1993; Moreau and Leclerc, 2004). The second protocol was based on a serum-free neural induction medium (referred to as NIM protocol; modified from Hibaoui et al., 2014; Fig. 1C) containing B27 and N2 supplements without additional SMAD inhibitors. Both protocols were tested in two different hiPSC lines, A4 (Wang and Adjaye, 2011) and CRL2097 (Fig. S1–S6) and resulted in free-floating aggregates with spheroid morphology comparable to primary fetal human neurospheres (Moors et al., 2009; Fig. 1A–C).

3.2. Characterization and comparison of proliferating hiPSC-NPCs to primary hNPCs

To ensure that resulting hiPSC-spheres consisted of NPCs, proliferating spheres were singularized and analyzed for the expression of the neural stem cell/progenitor markers Nestin and SOX2 using flow cytometry analyses. Primary hNPCs were analyzed in parallel (Fig. 1D). Almost 100% of primary hNPCs were double-positive for both markers (Fig. 1D). In comparison, hiPSC-NPCs obtained with the different protocols also consisted of 89.98% and 87.18% (Fig. 1D) of Nestin$^+$/SOX2$^+$ cells, respectively, and another approx. 8% of the cells were either Nestin or SOX2 positive, indicating that both differentiation protocols successfully produced hiPSC-NPCs.

For molecular characterization of proliferating hiPSC-NPCs in comparison with primary hNPCs and undifferentiated hiPSC cultures, we studied the mRNA expression profiles of the pluripotency genes *OCT4*, *NANOG* and *SOX2*, which are highly expressed in hiPSCs (Takahashi et al., 2007; Warren et al., 2010; Vuoristo et al., 2013). Expectedly, undifferentiated hiPSC expressed the highest amount of *OCT4* mRNA, which was one and several orders of magnitude higher than in hiPSC-NPCs and primary NPCs, respectively (Fig. 2A). This pattern was similar for *NANOG* mRNA expression (Fig. 2A). In case of *SOX2*, which is a pluripotency and an NPC marker (Breier et al., 2010; Zhang and Cui, 2014), expression did not change during neural induction of hiPSC in accordance with our expectations. The statistically significant difference of *SOX2* mRNA expression between hiPSC-NPCs and hNPC, however, most likely has no biological relevance as expressions range in the same order of magnitude (Fig. 2A).

Expression of the NPC marker *Nestin* as well as of the early neuroectodermal marker *PAX6* and the advanced neuronal maturation marker *MAP2* (Fig. 2B) were analyzed as neural/neuronal markers. *Nestin* expression was very low in undifferentiated hiPSCs (~0.006/β-actin), but increased after neural induction in both hiPSC-NPCs reaching an expression level similar to primary hNPCs (Fig. 2B). A similar gene expression pattern was detected for *PAX6* and *MAP2*, which were not detectable (nd; <0.0001/β-actin) in undifferentiated hiPSCs, but up-regulated in both hiPSC-NPCs to levels comparable to primary hNPCs (Fig. 2B). Expression of the neuronal marker *MAP2* was not detectable in hiPSCs whereas it was measurable in both hiPSC-NPCs and primary hNPCs just above the detection limit (>0.005/β-actin; Fig. 2B).

The expression profile of glial markers (*PDGFRα*, *NG2*, *GFAP*; Fig. 2C) revealed very low abundance compared to pluripotency or neuronal markers. Markers for oligodendrocyte progenitor cells, *PDGFRα* and *NG2*, were similarly expressed in both hiPSC-NPCs compared to undifferentiated hiPSCs and in primary hNPCs (Fig. 2C). The astrocyte marker

GFAP was not detectable in undifferentiated hiPSCs and just above the detection limit in hiPSC-NPCs, whereas it was well detectable in primary hNPCs (Fig. 2C). Taken these results together, hiPSC-NPCs and primary hNPCs exert a similar gene expression regarding neural and neuronal markers. However, expression of the glial marker *GFAP* revealed a difference between hiPSC-NPCs and primary hNPCs.

3.3. hiPSC-NPCs mimic the main processes of neurodevelopment

The previously established 'Neurosphere Assay' (Fig. 3A) studies compounds' effects on hNPC proliferation, migration and differentiation (Moors et al., 2007, 2009; Gassmann et al., 2010; Baumann et al., 2015). Hence, here we evaluated these endpoints in a comparative study by using primary hNPC and hiPSC-derived NPC generated by two different protocols, NIM and noggin.

Monitoring neurosphere size over time (14 days) revealed that hiPSC-neurospheres from the NIM protocol exhibited similar proliferative capacity compared to primary human neurospheres resulting in a diameter increase of 550 μm and 450 μm, respectively, whereas hiPSC-neurospheres from the noggin protocol proliferated less, resulting in a diameter total increase of approximately 300 μm (Fig. 3B).

The migration assay revealed that after 3 days hNPCs as well as hiPSC-NPCs covered a migration distance of approximately 700 μm (Fig. 3C).

To assess the neuronal differentiation potential of hiPSC-NPCs compared to primary hNPCs, neurospheres were stained cells for the neuronal marker βIII-Tubulin after 7 days of differentiation. All neurospheres were able to differentiate into βIII-Tubulin$^+$ neurons (Fig. 3D), but in contrast to primary human neurospheres and hiPSC-neurospheres from the NIM protocol, the noggin protocol resulted in neurons with weaker βIII-Tubulin staining (Fig. 3D). Moreover, quantification of the

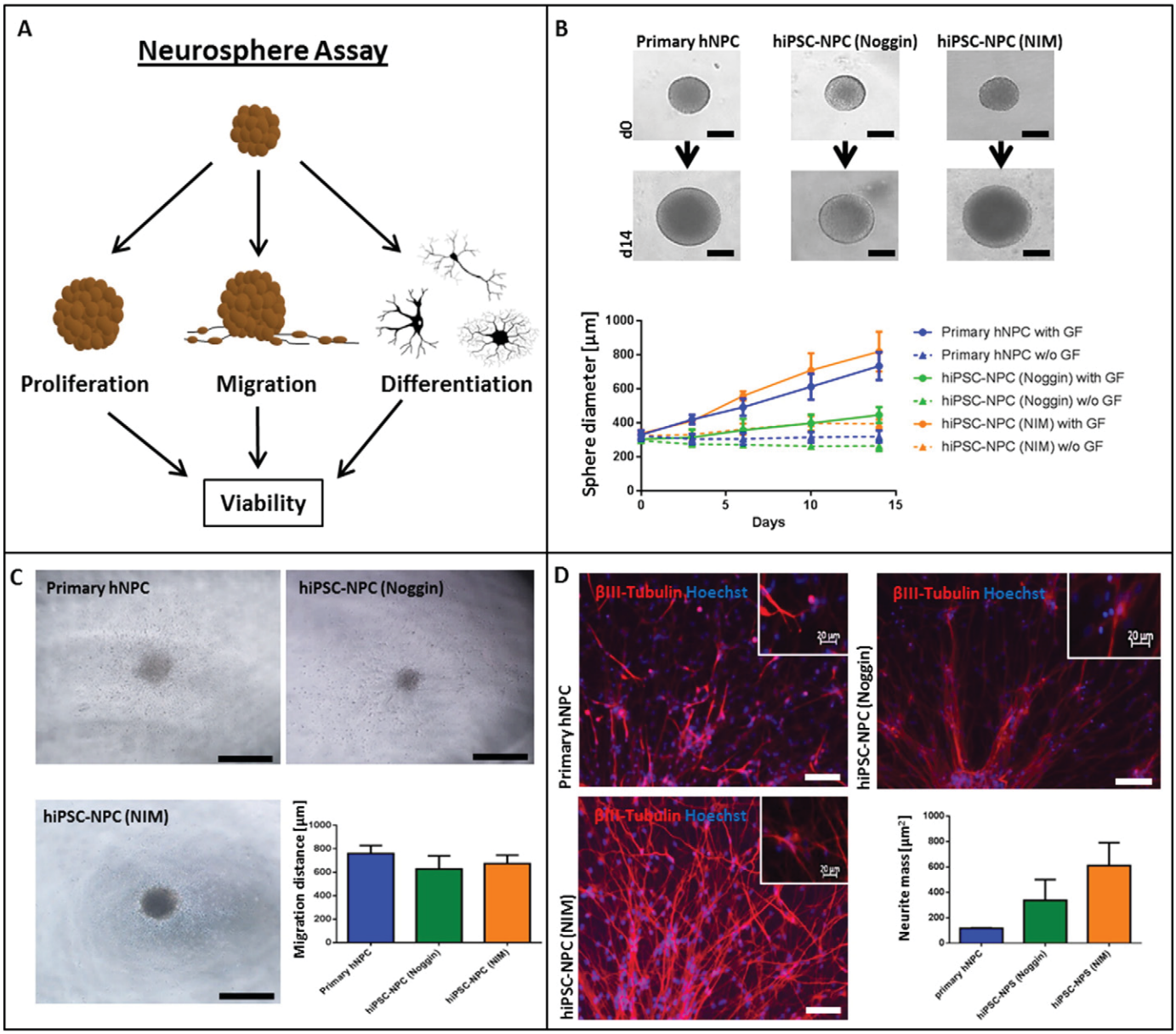

Fig. 3. Comparative functional analyses of hiPSC-neurospheres and primary human neurospheres using the 'Neurosphere Assay'. (A): The 'Neurosphere Assay' is a method to determine basic processes of neurodevelopment (proliferation, migration, differentiation and viability) *in vitro*. (B): Proliferation was measured for 14 days in neural proliferation medium (NPM) with (solid line) or without growth factors (GF; dotted line). Values represent mean ± SD, n = 3. Scale bars = 200 μm. (C): Migration distance of migrating cells 3 days after plating of neurospheres on Poly-D-Lysin (PDL)/Laminin coated plates in NDM. Values represent mean + SEM, n = 3. Scale bars = 500 μm. (D): Immunocytochemical stainings and quantification of at least five representative immunocytochemical images per neural induction for the neuronal marker βIII-Tubulin in 7 days differentiated NPCs of different origins plated on PDL/Laminin coated plates in NDM. Nuclei were stained with Hoechst 33258 (blue). Each n represents an independent neural induction; Scale bars in large images = 100 μm; scale bar in insertions = 20 μm.

immunocytochemical stainings might suggests that hiPSC-neurospheres generated with the NIM protocol contain a larger area of βIII-Tubulin$^+$ neurite staining than primary hNPC or hiPSC-NPC generated with the noggin protocol (Fig. 3D). In addition, the images from the cultures derived from the noggin protocol reveal a higher number of condensed nuclei indicating spontaneous apoptosis (insertions of Fig. 3D). The displayed image is a representative example for this finding.

Taken together these results show that the NIM protocol results in hiPSC-neurospheres, which better resemble primary human neurospheres and display more promising neuronal differentiation than spheres generated with the noggin protocol. Therefore, all following experiments were performed using the NIM protocol.

3.4. hiPSC-NPCs are able to differentiate into functional neuronal networks with spontaneous electrophysiological activity

After 28 days of differentiation, immunocytochemical staining revealed βIII-Tubulin$^+$ neurons and GFAP$^+$ astrocytes in primary as well as hiPSC-neurospheres (NIM; Fig. 4A + B). In contrast to primary hNPC, neurons of hiPSC-neurospheres built a dense neuronal network possibly indicating a higher neuronal maturation state (Fig. 4C, D). This observation is supported by immunocytochemical stainings for the pre-synaptic marker SYNAPSIN-1 and the post-synaptic marker PSD-95, of which the quantification of the staining (Fig. 4G) reveals an approximately similar number of SYNAPSIN-1 and PSD95-positive dots/neurite area: whereas hiPSC-derived neurons co-stain for βIII-Tubulin and SYNAPSIN-1 or PSD-95 (Fig. 4E, F), neurons of primary NPCs are negative for these markers (Fig. S9).

In order to study the functionality of these neuronal networks, hiPSC-neurospheres were cultivated on MEAs in NDM for up to 6 months. The first spontaneous electrophysiological signals were measured after 3 weeks of differentiation (data not shown). After 85 days multiple single spikes were detected (Fig. 5A), indicative of an immature neuronal network. A detailed analysis revealed the typical morphology of monophasic spikes characteristic for physiological action potentials (Fig. 5B, C). Additionally, hiPSC-derived neuronal networks were

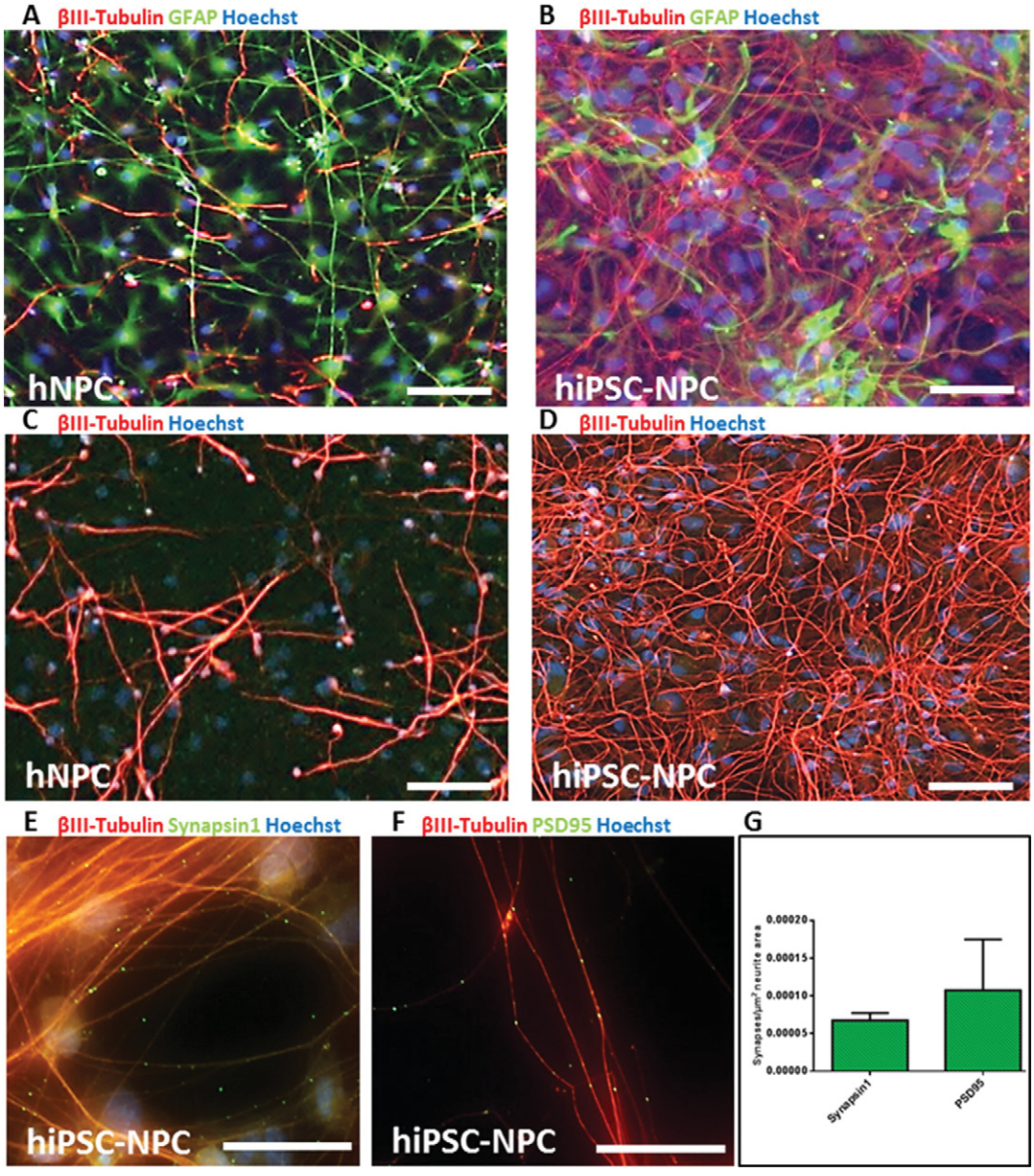

Fig. 4. Comparative immunofluorecent stainings of 28-days differentiated neurospheres. Primary hNPCs (A/C) and hiPSC-NPCs (B/D) were stained for βIII-Tubulin (neurons, red, A–D) and GFAP (astrocytes/NPCs, green, A/B) after 28 days of differentiation. hiPSC-NPCs show co-localization of βIII-Tubulin (red) with the synapse markers Synapsin1 (green, (E)) and PSD-95 (green, (F)) and three representative images of results from three independent neural inductions were quantified (G). Nuclei are counter-stained with Hoechst 33,258. Scale bars A–F = 100 μm.

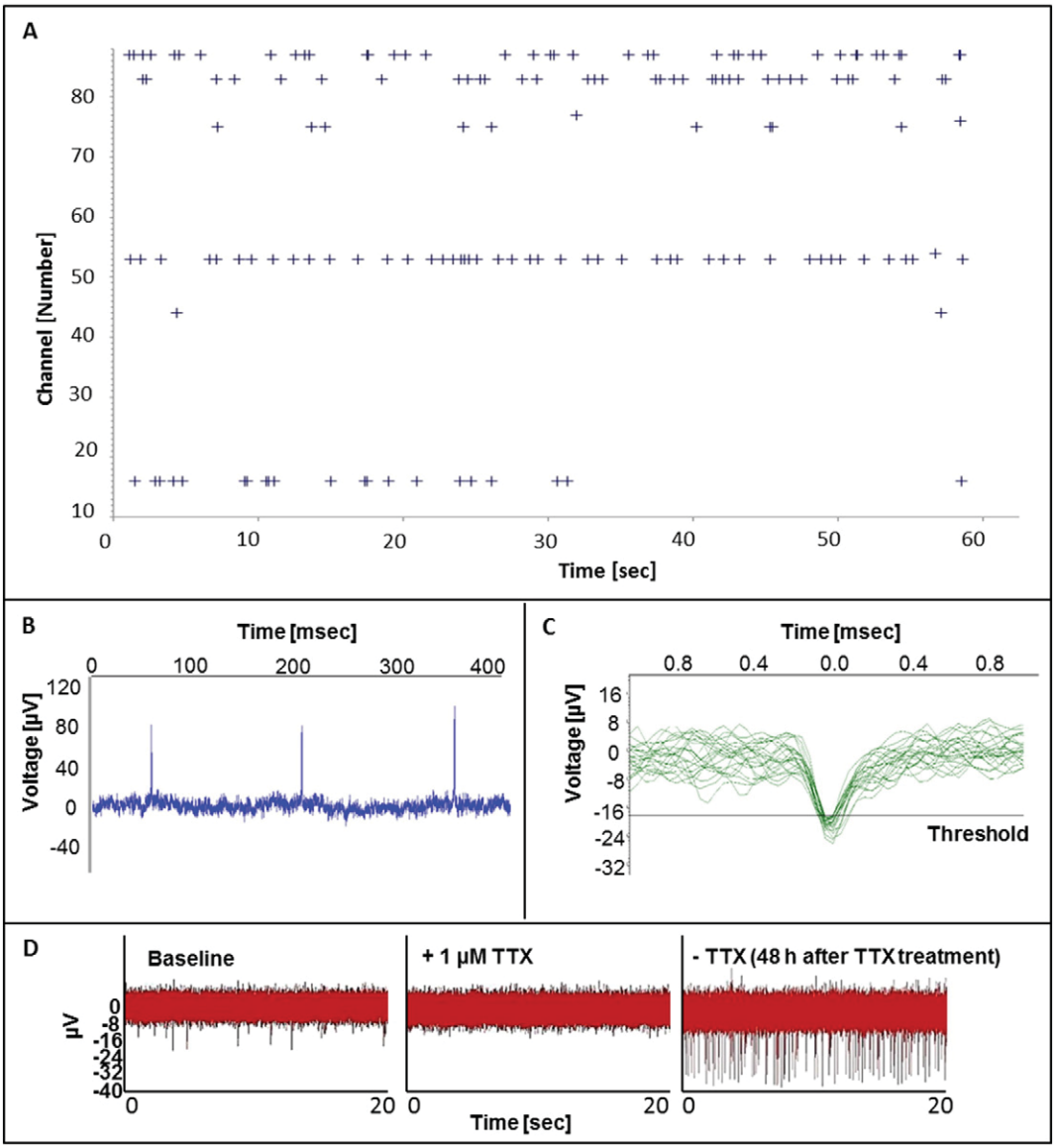

Fig. 5. Electrophysiological activity of hiPSC-derived neuronal networks grown on MEAs. (A): Representative spike raster plot of hiPSC-NPCs differentiated for 85 days on a MEA. Each cross denotes a spike representing an action potential. (B): Spike train of hiPSC-NPCs after 82 days of differentiation. (C): Detected spikes after 85 days of differentiation in a spike overlay. Depicted is one representative out of 7 electrically active chips. (D): After treatment with 1 µM TTX no action potentials (APs) were detected in a hiPSC-NPCs culture differentiated on MEAs for 84 days. After removal of TTX the networks recovered after 48 h resulting in APs with higher frequency and higher amplitude.

sensitive to TTX, demonstrating that the electrophysiological activity in our culture was sodium ion channel-dependent (Lee and Ruben, 2008). This blockage was reversible, as action potentials increased in number and magnitude after a 48 h recovery period in NDM (Fig. 5D), pointing to an adaptive mechanism like homeostatic plasticity.

3.5. hiPSC-NPCs identify disturbances of migration by MeHgCl

To determine if the hiPSC-neurospheres generated with the NIM protocol are a useful model for DNT *in vitro* testing, we performed a proof-of-principle study by treating primary human and hiPSC-derived neurospheres with the DNT model compound MeHgCl (Kadereit et al., 2012). This compound causes mental retardation and developmental delay in children exposed *in utero* (Grandjean and Landrigan, 2006, 2014). DNT of MeHgCl is amongst others due to its inhibition of cell migration resulting in global brain disorganization (Schettler, 2001). Using the 'Neurosphere Assay' we showed that MeHgCl exposure inhibited hNPC migration *in vitro* (Moors et al., 2007, 2009; Baumann et al., 2015). In this study, exposure to increasing concentrations of MeHgCl for 24 h decreased migration distance and viability of hiPSC-derived and primary human neurospheres in a concentration-dependent manner (Fig. 6) with a lowest observed adverse effect concentration (LOAEC) for migration of 0.3 µM MeHgCl for both cell types, whereas the LOAEC for viability was 3 and 1 µM, respectively (Fig. 6A, B). Despite similar LOAECs for migration inhibition, EC_{50} values differed between hiPSC-derived NPC (0.39 µM) and primary hNPC (0.77 µM) for this endpoint, while EC_{50} values for viability did not differ significantly from each other (2.74 and 2.35 µM, respectively, Table 1).

4. Discussion

Fetal hNPCs cultured as neurospheres represent a valuable model for studying neurodevelopmental processes (Svendsen et al., 1998) and thus we have been establishing the hNPC-based 'Neurosphere Assay' (Fig. 3A) as an alternative method for analyzing neurodevelopmental toxicity *in vitro* (Moors et al., 2007, 2009; Gassmann et al., 2010; Baumann et al., 2015). Timing is an important trait during development, thus a possible *in vitro* testing strategy should cover different stages of

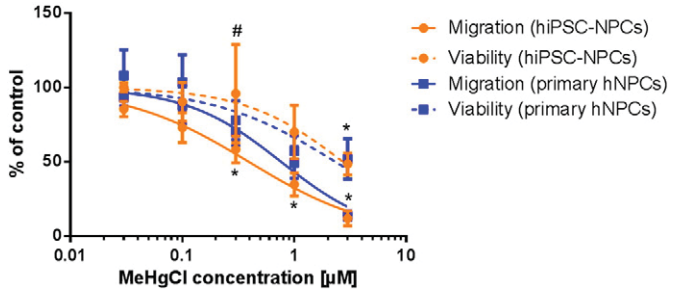

Fig. 6. Effect of the DNT model compound MeHgCl on neurosphere migration. Primary hNPCs and hiPSC-NPCs were treated with different concentrations of MeHgCl (30 nM – 3 μM) for 24 h. Migration distance (solid lines) and viability (dotted lines) were measured. Comparison of MeHgCl effects on migration and viability of primary hNPCs (blue) and hiPSC-NPCs (NIM; orange). Data is presented as concentration-response curve fits and is plotted for comparison of endpoints between cell systems. * significant difference compared to the respective control. # significant difference between the two measured endpoints. All values represent mean ± SD, n = 3; each n represents data from an independent neural induction, $p < 0.05$.

development. Therefore, we set up a hiPSC-NPC 'Neurosphere Assay', which we compared to primary human neurospheres (Fig. 3) as we expected these cells to closer resemble the embryonic period.

hiPSCs bear great potential for future research, as they (i) provide unlimited human cell material, (ii) do not provide the ethical concerns of hESCs and (iii) hold the possibility for human compound hazard assessment and disease modeling (Hatakeyama and Goto, 2016; Mlody et al., 2016; Xie and Tang, 2016). Thus, they are thought to be a useful tool for neuropharmacology/−toxicology (Jennings, 2015). There are numerous publications dealing with the differentiation of hiPSCs into the neuroectodermal lineage either under 2D (Espuny-Camacho et al., 2013; Palm et al., 2015) or 3D (Karumbayaram et al., 2009; Mahairaki et al., 2014) culturing conditions. Neural differentiation is induced either through BMP and transforming growth factor β3 (TGFβ3) inhibition, also known as dual SMAD inhibition (Chambers et al., 2009; Denham and Dottori, 2011; Du et al., 2012; Qiang et al., 2013; Naujock et al., 2014), or by N2 and/or B27 medium supplements (Brennand et al., 2011; Lancaster et al., 2013; Hibaoui et al., 2014). In the present study, we reproduced, optimized and compared two differentiation protocols: (i) BMP inhibition *via* noggin (noggin protocol; Denham and Dottori, 2011) and (ii) neural induction *via* N2 and B27 medium supplements (NIM protocol; modified from Hibaoui et al., 2014). Neural induction was performed in a 3D spherical format as cells grown as organoids seem to be closer to the *in vivo* situation than 2D monolayer cultures (Yamada and Cukierman, 2007; Alepee et al., 2014). The cells derived from both differentiation protocols were compared to primary hNPCs derived from fetal brains. Both protocols resulted in free-floating spheres, which were comparable to primary cells with regard to morphology (Fig. 1A–C), expression of neural stem/progenitor markers (Fig. 1D) and the expression profile of pluripotency as well as neural/neuronal markers (Fig. 2A, B). With respect to glial markers hiPSC-NPCs from both protocols resulted in cells, which seem to represent an earlier maturation stage than primary hNPCs (Fig. 2C). These findings are in line with previous studies reporting that hiPSC-NPCs and so-called EZ spheres, which resemble an early hiPSC-derived neural stem cell (NSC) stage, expressed significantly less glial markers, including *GFAP* and *S100β*, compared to primary hNPCs (Shofuda et al., 2013; Sareen et al., 2014). However, these two studies compared hiPSC-derived NPC to primary embryonic hNPCs of GW 10 (Yamane et al., 2011) and 8, respectively, while the primary NPCs used in our study were of later gestation, *i.e.* fetal origin (GW 16–18). That fetal proliferating NPCs express GFAP is probably due to the fact that GFAP-expressing radial glia, which are in fact NPC (Merkle et al., 2004), play a major role in corticogenesis and are thus present in the progenitor cell preparation of GW 16–18 human brains. Because hiPSC-derived NPCs are generated from stem cells, these cells seem to reflect an earlier developmental stage hardly expressing GFAP, but Nestin and SOX2. This is supported by the observation that 72 h after start of migration neurons migrate on top of $GFAP^+/Nestin^+$ and $GFAP^-/Nestin^+$ glia cells in hNPC and hiPSC-derived NPC, respectively (Fig. S10). Glia cell differentiation from stem cells takes longer than neuronal differentiation *in vitro* (Hu et al., 2010) and neurons also arise before glial cells *in vivo* (Kolb and Gibb, 2011). Thus, gene expression analyses of primary fetal hNPC and hiPSC-derived NPC indicate that each model reflects its respective time of development, *i.e.* fetal and embryonic, respectively.

Performing the 'Neurosphere Assay' (Moors et al., 2007; Breier et al., 2010; Gassmann et al., 2010; Fritsche et al., 2011; Gassmann et al., 2012; Baumann et al., 2014, 2015) with hiPSC-derived neurospheres we analyzed basic processes of early brain development (Fig. 3): NPC proliferation and differentiation of hiPSC-NPC generated with the NIM protocol were more similar to primary human neurospheres and more effective than hiPSC-NPC generated with the noggin protocol, which produced neurons with weaker βIII-Tubulin staining (Fig. 3B, D). Therefore, this protocol was used for all further experiments.

To study if neurons differentiated from either neurospheres were functionally active, we differentiated hNPC and hiPSC-derived neurospheres for 28 days and then immunocytochemically stained them for different markers. hiPSC-neurospheres formed neuronal networks consisting of $βIII\text{-}Tubulin^+$ neurons and $GFAP^+$ astrocytes (Fig. 4B, D), which seemed to be more mature, dense and connected than those formed by primary human neurospheres (Fig. 4A, C). This was confirmed by immunocytochemical stainings for the synaptic markers SYNAPSIN1 and PSD-95, which were only present in hiPSC-derived neurospheres (Fig. 4E–G, Fig. S9). This is in line with previous studies, which reported the expression of the pre- and postsynaptic markers SYNAPTOPHYSIN and PSD95 in hiPSC after 4 weeks of differentiation under 2D culturing conditions (Palm et al., 2015).

In hiPSCs-derived neural cultures neuronal activity on MEAs was monitored in form of asynchronous single spikes starting from 3 weeks of differentiation (Fig. 5), whereas no activity could be measured from primary human neurosphere networks (data not shown). Over the whole incubation period of up to 6 months we observed in turn periods with more and periods with less activity (data not shown), but neither increased number of single spikes, nor synchronicity. The data from Heikkilä and co-workers, who worked with hESC-derived neurons (Heikkilä et al., 2009), indicate that our data represents an immature neuronal network. Nevertheless, our hiPSC-derived neuronal networks exhibited positive and negative monophasic spikes (Fig. 5B, C) as previously described (Heikkilä et al., 2009). The same holds true for the spike amplitude of +/− 20–80 μV (Fig.5B, C). Moreover, hiPSC-derived neuronal networks were sensitive to TTX (Fig. 5D), as previously shown by patch clamp analyses (Palm et al., 2015). As TTX is a potent neurotoxin which blocks voltage-gated sodium channels, these data show that the electrophysiological activity of our culture is sodium channel-dependent (Lee and Ruben, 2008). The effect of TTX was reversible as networks started firing again with even higher amplitudes and frequencies after a recovery period. This is in line with the literature, as TTX treatment influences miniature excitatory postsynaptic current amplitude and frequency depending on the culture time *in vitro* (Turrigiano et al., 1998; Wierenga et al., 2006). At this time we can only speculate on the absence of synchronous signals. One possible explanation is insufficient

Table 1
Calculated EC_{50} values for MeHgCl treatment on migration distance and viability.

EC_{50} values	Primary hNPC	hiPSC-NPCs
Migration	0.77 μM	0.39 μM
95% confidence interval (migration)	0.59–0.99 μM	0.30–0.53 μM
Viability	2.35 μM	2.74 μM
95% confidence interval (viability)	1.17–4.73 μM	1.29–5.81 μM

maturation of the neurons differentiated from hiPSC-derived NPCs. Recently, Palm and co-workers reported that hiPSC-derived neurons exhibited presynaptic, but no postsynaptic currents in voltage clamp recordings after 4 weeks of differentiation illustrating an incomplete neuronal network even though both pre- and postsynaptic markers were present (Palm et al., 2015). These observations reflect our data and might indicate that maturation of hiPSC-derived neurons either takes longer compared to hESC-derived neurons or requires special medium supplements. This is in line with recent findings indicating that complete network maturation of commercially available iPSC-derived cerebral cortical neurons takes 20 to 30 weeks (Odawara et al., 2016) and that co-culture with 1% rat astrocytes and 20% rat primary cortical astrocyte-conditioned medium increases the firing rate (Odawara et al., 2014). Additional experiments, *e.g.* supplementation of creatine, cholesterol or estrogen (Brewer et al., 2008) to the medium, are needed to study if hiPSC-neurosphere differentiation on MEAs can also be enhanced to form synchronized neuronal networks. Also, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), TGF-β3, dibutyryl-cAMP (dbcAMP) or ascorbic acid induce neuronal maturation and might lead to synchronous signals in our system (Heikkilä et al., 2009; Palm et al., 2015).

It has recently been suggested that iPSCs could provide 'the future of *in vitro* toxicology' (Jennings, 2015) and there is consensus that alternative *in vitro* methods might be suitable for DNT hazard and potency evaluation (Tsuji and Crofton, 2012; Bal-Price et al., 2015a). Amongst others, the 'Neurosphere Assay' using primary fetal hNPCs (Moors et al., 2009; Gassmann et al., 2010; Fritsche et al., 2011; Baumann et al., 2015) already complies with this approach. In the present study we provide first evidence that hiPSC-NPCs generated with the NIM protocol might be a useful addition to a possible DNT testing strategy as they seem to resemble an earlier stage of brain development. As a-proof-of-principle the DNT-compound MeHgCl, which causes mental retardation and disturbs neural migration in children exposed *in utero* (Choi et al., 1978; Choi, 1986), inhibits hiPSC-derived NPC migration (Fig. 6). IC_{50} values for inhibition of migration and effects on viability were comparable between primary human and hiPSC-neurospheres (Table 1). This is not surprising because MeHgCl's mode of action, interference with SH-groups of proteins and other molecules and production of oxidative stress, which disturbs cytoskeletal function necessary *e.g.* for migration (Bal-Price et al., 2015b), explains similar migration disturbance in both models despite different developmental stages. To better understand the application domain for neural migration analyses using hiPSC-derived NPC, further studies are needed evaluating the functionality of signaling molecules/pathways known to contribute to normal migration like *e.g.* PLCγ1, GDNF-RET, BDNF/TrkB, PDGFR, FGFR, mTORC1 and Reelin-Dab (Lee, 2015; Ohtaka-Maruyama and Okado, 2015; Kang et al., 2016). In addition, chemicals with known DNT activity need to be tested in this system.

5. Summary and conclusion

In this study we show that hiPSC-derived NPC proliferate, migrate and differentiate similarly to primary fetal hNPCs. However, hiPSC-NPC seem to reflect an earlier developmental stage with primarily neuronal and slower glia cell differentiation than fetal hNPC. In addition, neurons differentiated from hiPSC-derived NPC exert measurable electrical activity. These data suggest that the hiPSC-based neurosphere assay might be a tool to complement the primary neurosphere assay for DNT *in vitro* testing by (i) covering an earlier stage of development and (ii) adding the endpoint of neuronal network formation to a potential neurosphere-based DNT testing battery we suggested earlier (Baumann et al., 2015).

Acknowledgments

The authors would like to thank Drs. Marta Barenys, Jenny Baumann, Katharina Dach and Janette Goniwiecha for useful discussions and comments. Furthermore, we thank Gabriele Freyberger from the Institute of Human Genetics of the HHU for technical assistance.

Disclosure of potential conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2017.10.013.

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Supplementary Material and Methods:

Reprogramming of CRL2097

1 x 10^6 primary neonatal human fibroblast (CRL-2097, ATCC, Manassas, VA, USA) of early passage were nucleofected with 1 µg each of following plasmids: pCXLE-hSK (SOX2, KLF4), pCXLE-hUL (L-MYC, LIN28) and pCXLE-hOCT3/4-shp53-F (OCT3/4, shRNAp53)[1] according to standard Amaxa protocol (VPD-1001, program U-020). Afterwards cells were cultured in DMEM with 10% FCS at 37°C and 5% CO_2. After one week cells were replated onto a feeder layer of mouse embryonic fibroblasts (MEFs) and cultured with human embryonic stem (ES) cell medium (DMEM/F12 containing 20% KSR (Invitrogen, Carlsbad, CA, USA), 10 ng/ml bFGF (PeproTech, Rocky Hill, NJ, USA), 1 mM L-glutamine, 100 µM non-essential amino acids, 100 µM β-mercaptoethanol, 50 U/ml penicillin and 50 mg/ml streptomycin), in the presence of 3 µM MEK1 (Axon, PD184352, London, UK), 1 µM GSK3 (Axon, CHIR99021[2-4] and histone deacetylace (HDAC) inhibitor butyrate 0,5 mM (Sigma, 303410, L' lsle D'Abeau, France[5].

For first passaging, hiPSC clones were mechanically isolated and further singularized enzymatically using dispase (temporally in the presence of ROCK inhibitor, (Tocris, Bristol, UK). Afterwards cells were again cultured on MEFs with human ES media.

The total RNA of hiPSC was analyzed for endogenous and exogenous (episomal) expression of reprogramming factors using qRT-PCR. Respective primers were used as previously published[1]. Practically a complete disappearance of expression of exogenous reprogramming factors was confirmed at passage 10 (Fig. S5).

***in vitro* hiPSC differentiation and teratoma formation.**

A spontaneous *in vitro* differentiation of hiPSCs into embryoid bodies (EBs) was performed as described elsewhere[6]. Briefly, hiPSC colonies were harvested using dispase and left to form round-shaped EBs in low-attachment cell culture plates. Spontaneous differentiation was induced by withdrawal of bFGF from hES media for two weeks. Total RNA from EBs

were characterized by qRT-PCR for decline of pluripotency associated genes and appearance of markers bound to developmental progression of major tissues[6]; Fig. S6).

Following the positive outcome of spontaneous *in vitro* differentiation we performed a teratoma formation assay in NUDE mice[7,8]. Briefly, when hiPSC culture reached 75% confluency, clones were pre-treated with ROCK inhibitor, enzymatically harvested by dispase to dispose of MEFs and shortly trypsinized by TripLE (Invitrogen, Carlsbad, CA, USA) in order to get a single cell suspension. 1 x 10^7 hiPSC were resuspended in 200 µl of 1:1 matrigel/KO DMEM in the presence of 10 µM of ROCK inhibitor. Cell suspension was injected intraperitoneally and intramuscularly into NUDE mice (Charles River, Saint-Germain-sur-l'Arbresle, France, Crl:NU(Ico)-Foxn1Nu). Six to eight weeks post injection mice were sacrificed and tumors were surgically removed. Each tumor was approx. 0.5 - 1 cm in diameter. Tissues were then, either fixed in 4% PFA, embedded in paraffin and sliced for IHC (Fig. S4) or snap frozen in liquid nitrogen for further analysis. All work on teratoma and histological analysis has been performed at the IGBMC animal house, in association with the Institut de la Clinique de la Souris (ICS), Illkirch, France.

Immunofluorescence (IF) and immunohistochemistry (IHC) were done as previously described[6].

Briefly, for IF, hiPSC clones were grown on glass chamber slides, fixed with 4% PFA, washed, permeabilized (staining for nuclear factors) or left untreated (staining for transmembrane proteins), blocked with serum and stained with specific antibodies, as well for Alkaline Phosphatase (AP; Sigma, AB0300, L' Isle D'Abeau, France).

Briefly, for IHC, slides with teratoma tumors were deparaffinized, hydrated, pre-heated with citrate buffer, blocked and stained with specific antibodies or H&E, respectively (Fig. S4).

Antibodies: NANOG (Abcam, ab21603, Paris, France), OCT3/4 (Santa Cruz, sc-5279, Dallas, TX, USA), TRA-1-60 (Millipore, MAB4360, Molsheim, France), SSEA4 (Millipore, MAB4304, Molsheim, France), GFAP (DAKO, Z033429, Bollschweil, Germany), TuJ/β3-tubulin (Covance, MMS-435P, Geneva, Switzerland), Smooth muscle actin (Sigma, A-5228,

L' Isle D'Abeau, France), Chondroitin (Sigma, C8035, L' Isle D'Abeau, France), FoxA2 (R&D Systems, AF2400, Minneapolis, MN, USA) and AFP (DAKO, A000829, Bollschweil, Germany).

Microarray and SNP array profiling of CRL-2097 hiPSCs.

Total RNA from CRL-2097 hiPSC (passage 10) was subjected to a library preparation and hybridized on Affymetrix Human 1.0 ST Array (IGBMC gene expression and sequencing facility, Illkirch, France). Gene expression profile was aligned and compared to already published mRNA profiles of WT hiPSC (GSM676321), H9 hESC (GSM676323) and WT fibroblast (GSM676320) analyzed on the same Affymetrix platform[9]; Fig. S3).

To characterize genomic integrity of CRL-2097 hiPSC, the library was prepared from the genomic DNA and hybridized onto The High Density Single Nucleotide Polymorphism (SNP) Mapping Array (250K) (IGBMC gene expression and sequencing facility). Further detailed analysis at a low resolution revealed the absence of big chromosomal changes, such as deletions or duplications (Fig. S2A). Higher resolution, a window size of 100 kb, revealed insignificant chromosomal changes represented by heterozygous deletions or amplifications (Fig. S2A, red or blue arrow heads). These detected regions did not contain any cancer-associated genes.

Flow cytometry analysis of hiPSCs

For flow cytometry analysis hiPSCs were treated with 10 µM ROCK inhibitor (R&D Systems, Germany) for 1 h. Subsequently, cells were singularized by incubation with Accutase (Invitrogen, USA) for 20 min at 37°C and 5% CO_2. Afterwards, cells were stained with anti-SSEA4-APC (Miltenyi, Germany) and anti-TRA-1-60-PE (Miltenyi, Germany) antibodies in PBS (Life Technologies, USA) in the dark at 4°C for 30 min. Samples were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, Germany; Fig. S7).

Cytogenic analysis of hiPSC line A4

Cytogenetic analyses were performed by the Institute of Human Genetics at the Heinrich-Heine University Düsseldorf, Germany. Cytogenetic analyses were done using GTG-banding of chromosomes as described preciously[10]. 1 x 10^6 cells were grown to 80% confluency in mTeSR1 medium (Stemcell Technologies, Germany) and treated with colcemid 6.5 h before harvesting. Preparation of chromosomes was carried out according to standard procedures[11] accredited for clinical diagnostics. Two independent cultures were set up and a total of 30 and 32 metaphases were analysed, respectively. Chromosomes were identified and sorted manually using a bright-field microscope.

Migration assay

Five neurospheres per condition with a diameter of 300µm were plated on 0.1mg/mL poly-D-lysin-hydrobromide (PDL, Sigma Aldrich, Germany) and 0.01mg/mL Laminin (Sigma Aldrich, Germany) coated eight-chamber slides (BD Biosciences, Germany) in neural differentiation medium (NDM) containing DMEM (Life Technologies, USA) and Hams F12 (Life Technologies, USA; 3:1) supplemented with 1x B27 (Invitrogen GmBH, Germany) and 1x N2 supplement (Invitrogen, Germany). After 24h or 72h individual neurospheres were photographed and radial migration distance was assessed by measuring the length at four distinct locations between the edge of the sphere and the furthest migrated cells using ImageJ (National Institutes of Health, USA). For endpoint specific controls, neurospheres were treated either with 20ng/mL EGF (Biosource, Germany) or 10µM PP2 (Sigma Aldrich, Germany) which led to an increase or decrease of migration area, respectively.

Figure S1

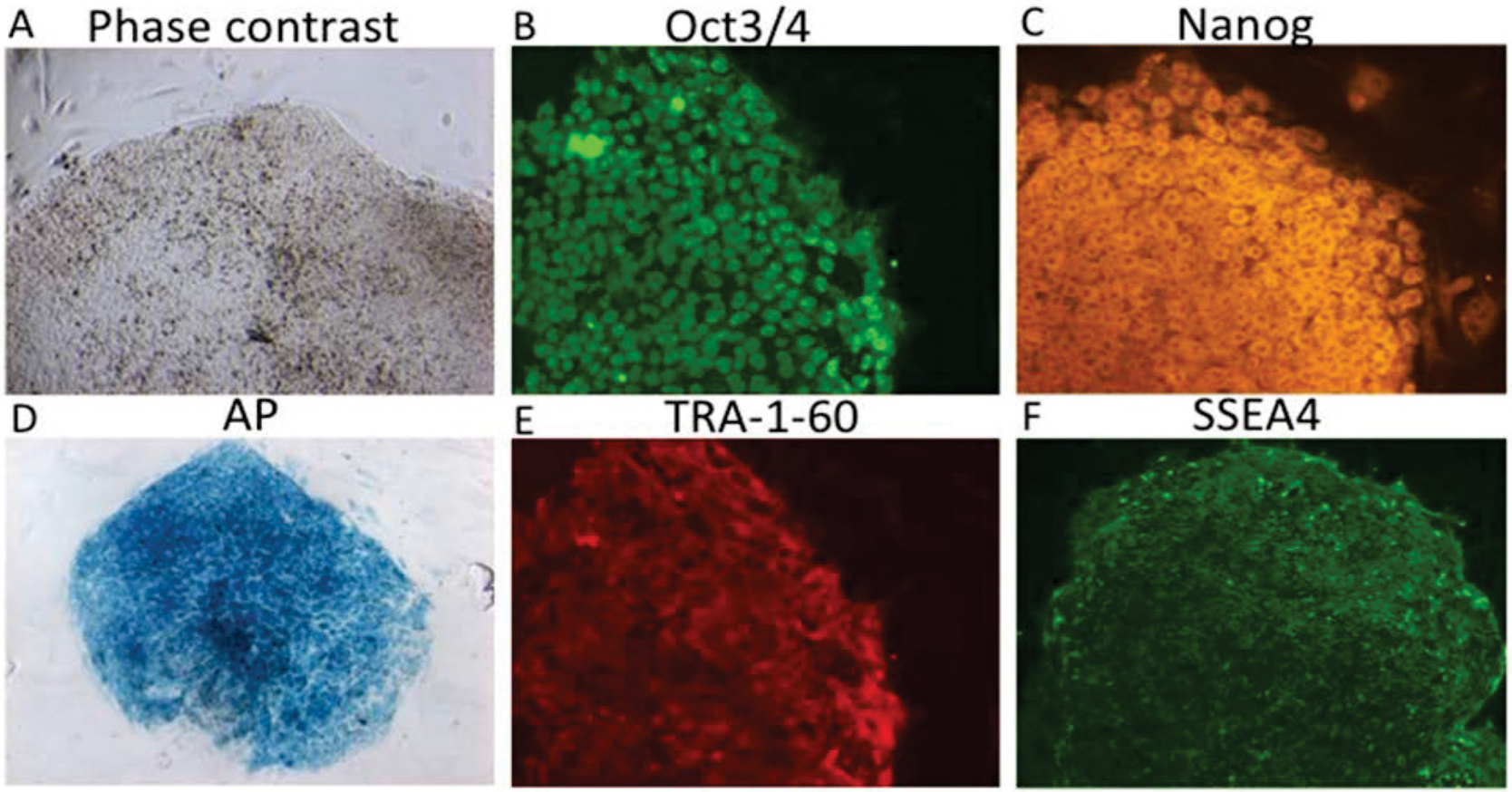

Fig. S1: Characterization of hiPSC CRL2097 for pluripotency markers. Representative immunofluorescent stainings of hiPSC colonies on feeder cells either in phase contrast **(A)** or stained for standard pluripotency markers: nuclear factors **(B+C)** and transmembrane proteins **(E+F)**. **D:** Alkaline phosphatase (AP) staining.

Figure S2

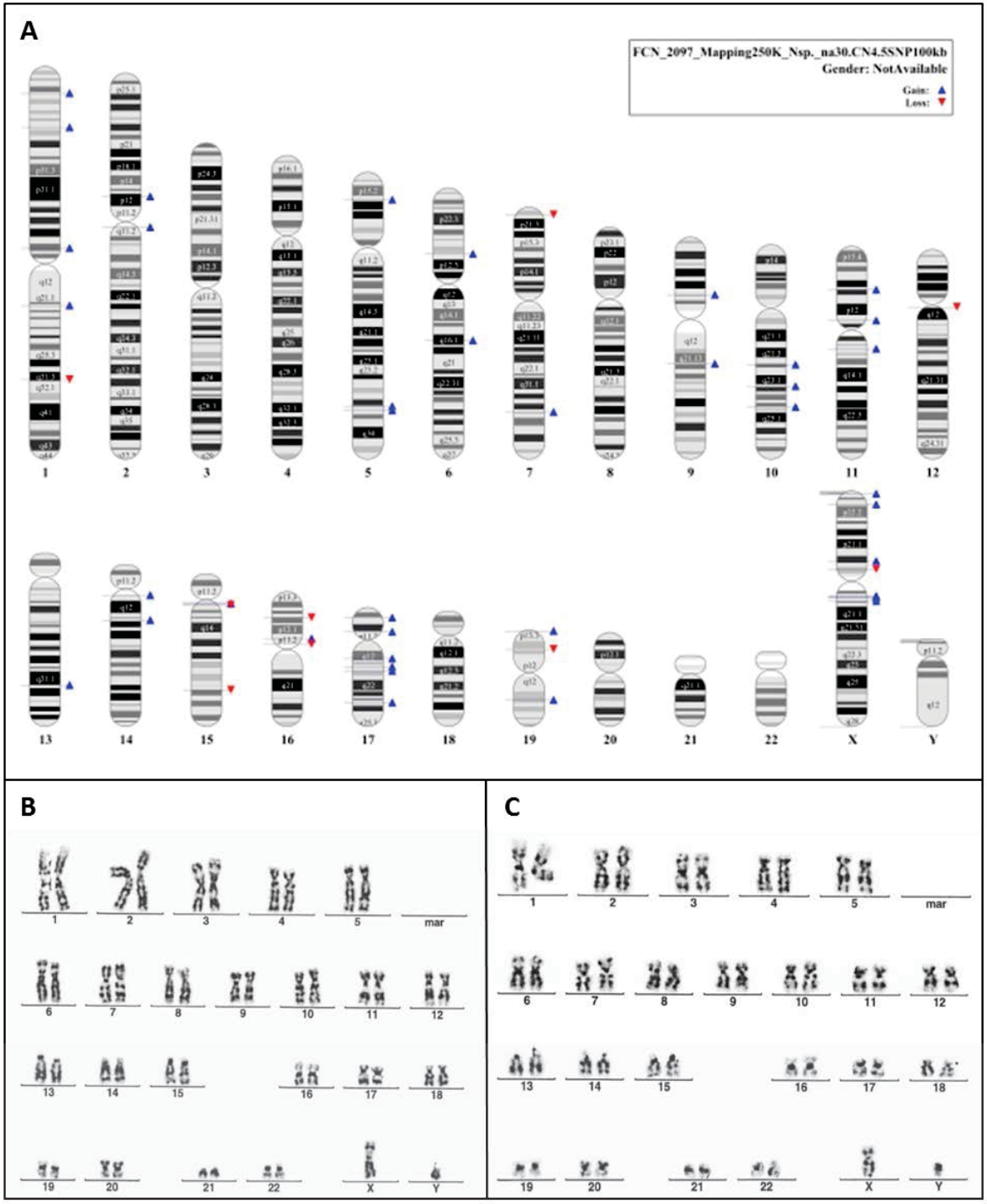

Fig. S2: Karyotype analysis of used hiPSC lines. (A): Representative analysis of a digital chromosomal content of CRL2097 (passage 10) hybridized to SNP/250K Mapping Array. Overall analysis shows absence of significant chromosomal alterations and confirms that CRL2097 hiPSCs resemble diploid genome after reprogramming and culturing conditions. **(B-C):** Karyotype analysis of hiPSC line A4 (passage 47 **(B)** and passage 74 **(C)**), indicating a normal diploid karyotype of the cells.

Figure S3

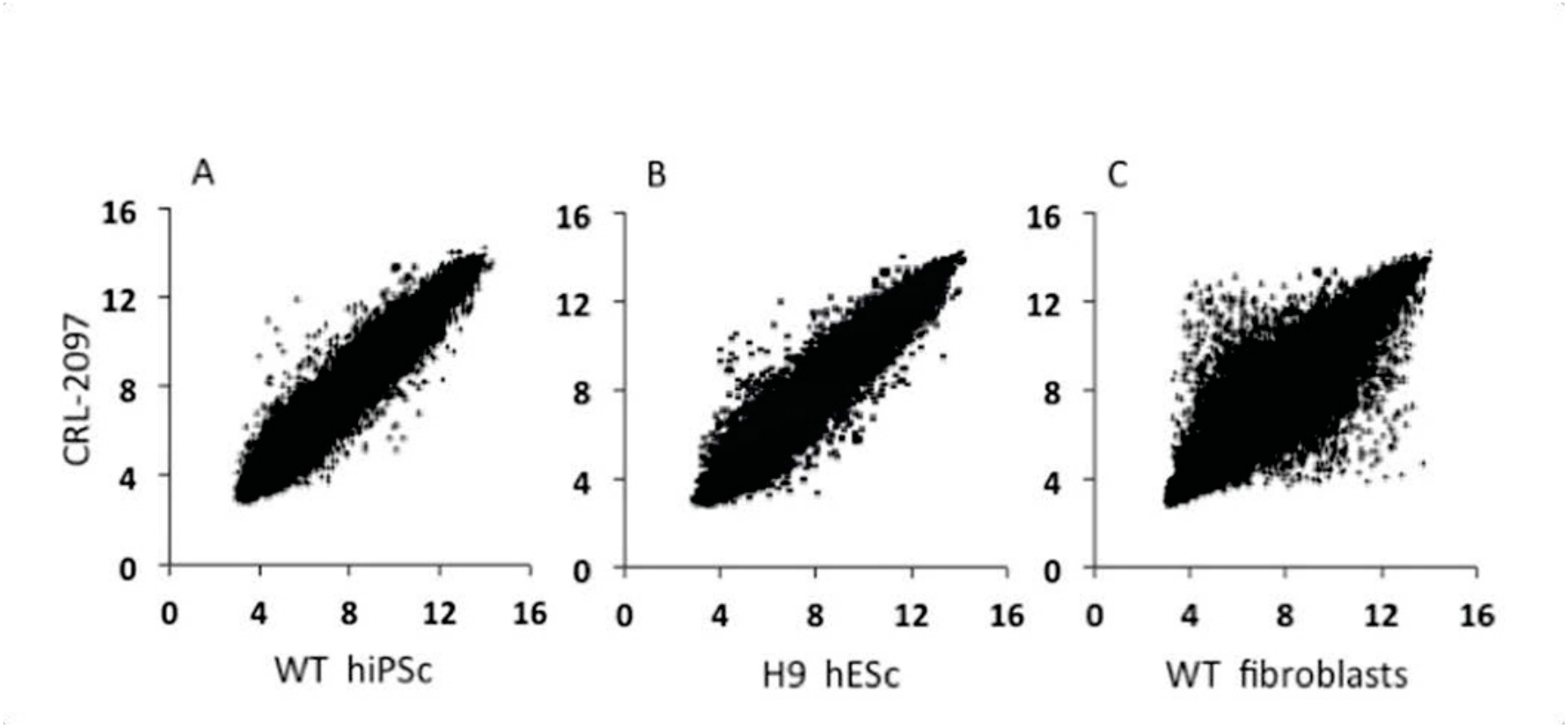

Fig. S3: Microarray characterization of hiPSC line CRL2097. Dot-plots show alignment of gene expression profile of CRL2097 (y-axis) against previously characterized (Stelzer et al., 2011) expression profile of WT hiPSCs **(A)**, H9 hESCs **(B)** and WT fibroblasts **(C)** (respective x-axis).

Figure S4

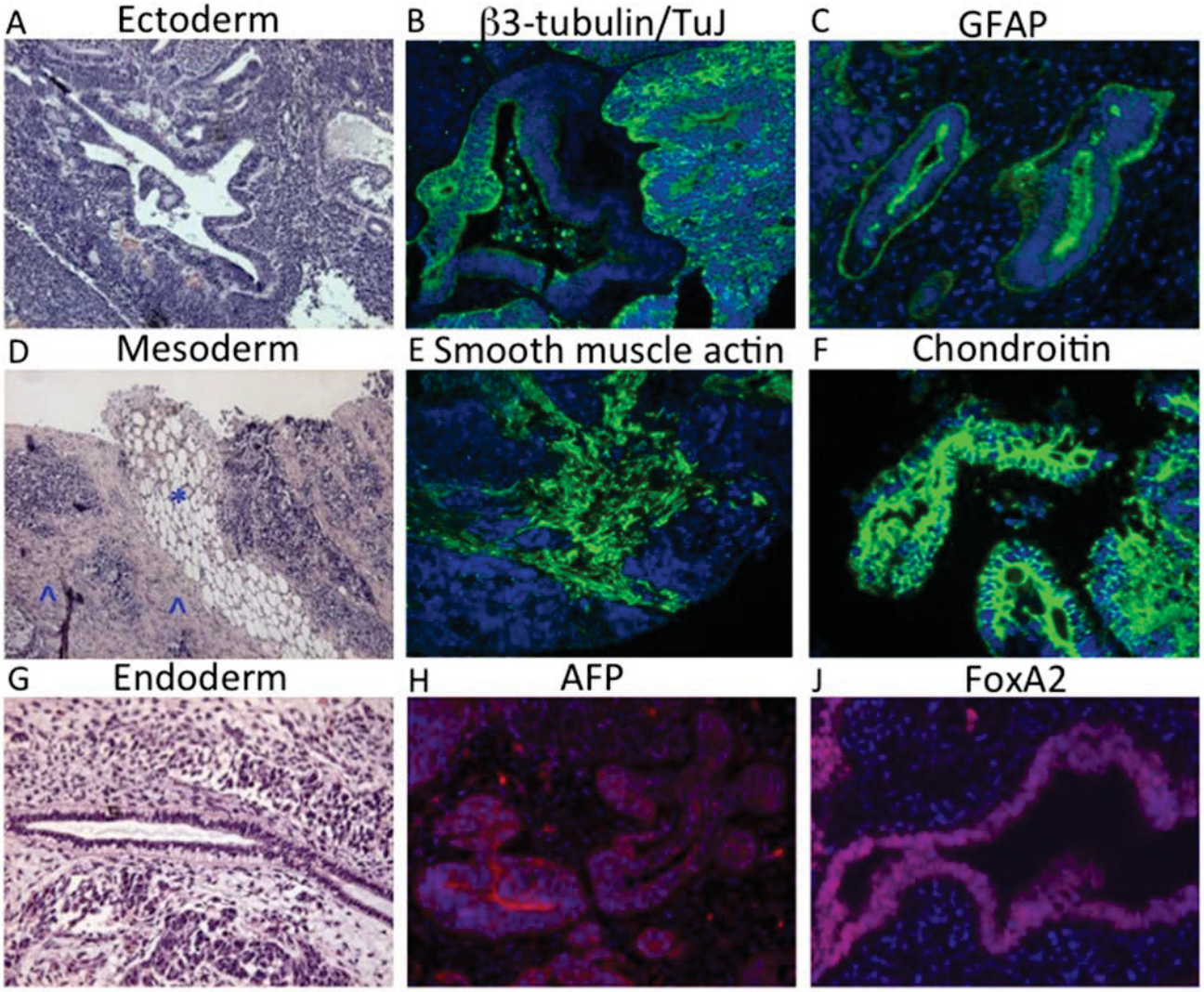

Fig. S4: Characterization of teratoma derived from hiPSC CRL2097 injected into NUDE mice. Representative H/E and immunofluorescent images of the different germ layers: ectoderm/neural tube (**A:** H/E; **B:** neuronal marker βIII-tubulin/TuJ; **C:** glial marker GFAP), mesoderm/adipose tissue (*) and smooth muscles (^) (**D:** H/E; E: smooth muscle actin; **F:** cartilage marker chondroitin) and embryonic endoderm/ yolk sac (piano key-like structure) (**G:** H/E; H: α-fetoprotein (AFP); **J:** FoxA2).

Figure S5

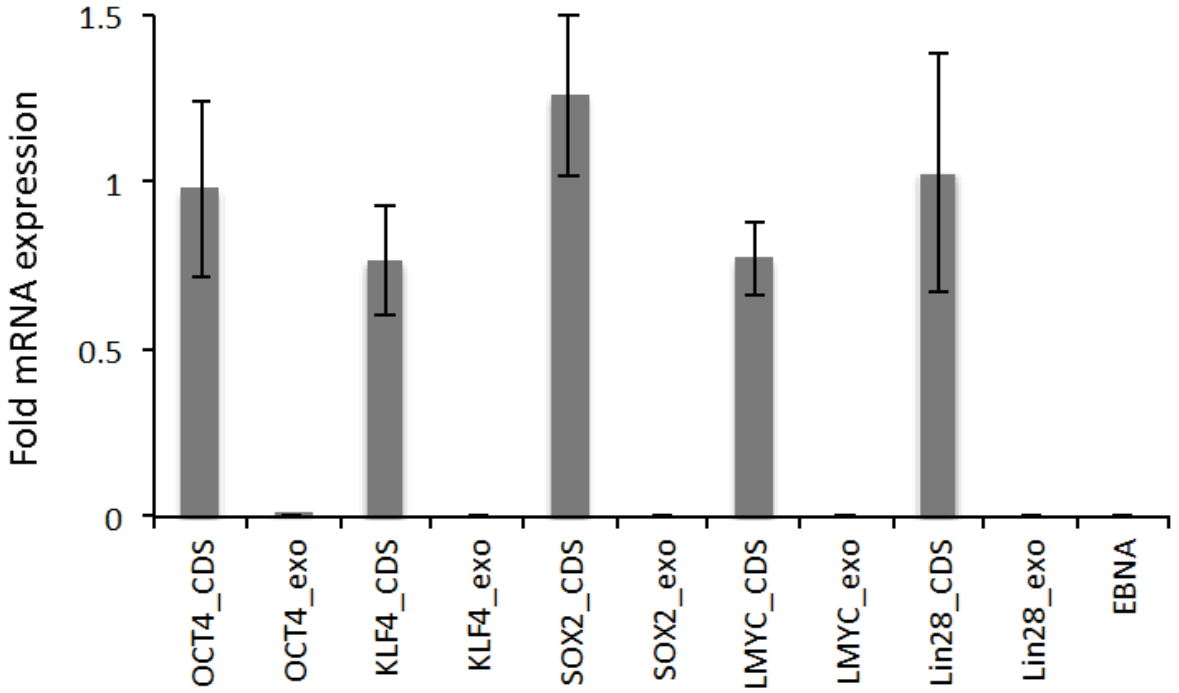

Fig. S5: Quantitative real-time (qRT-PCR) analysis of hiPSC line CRL2097. Detection of endogenous expression of reprogramming factors (CDS) and remaining presence of cDNA expressed from episomal vectors (exo) of respective reprogramming factors. Analysis was performed in passage 10 and 0, respectively. Results are represented as mean +/- SD. n = 3.

Figure S6

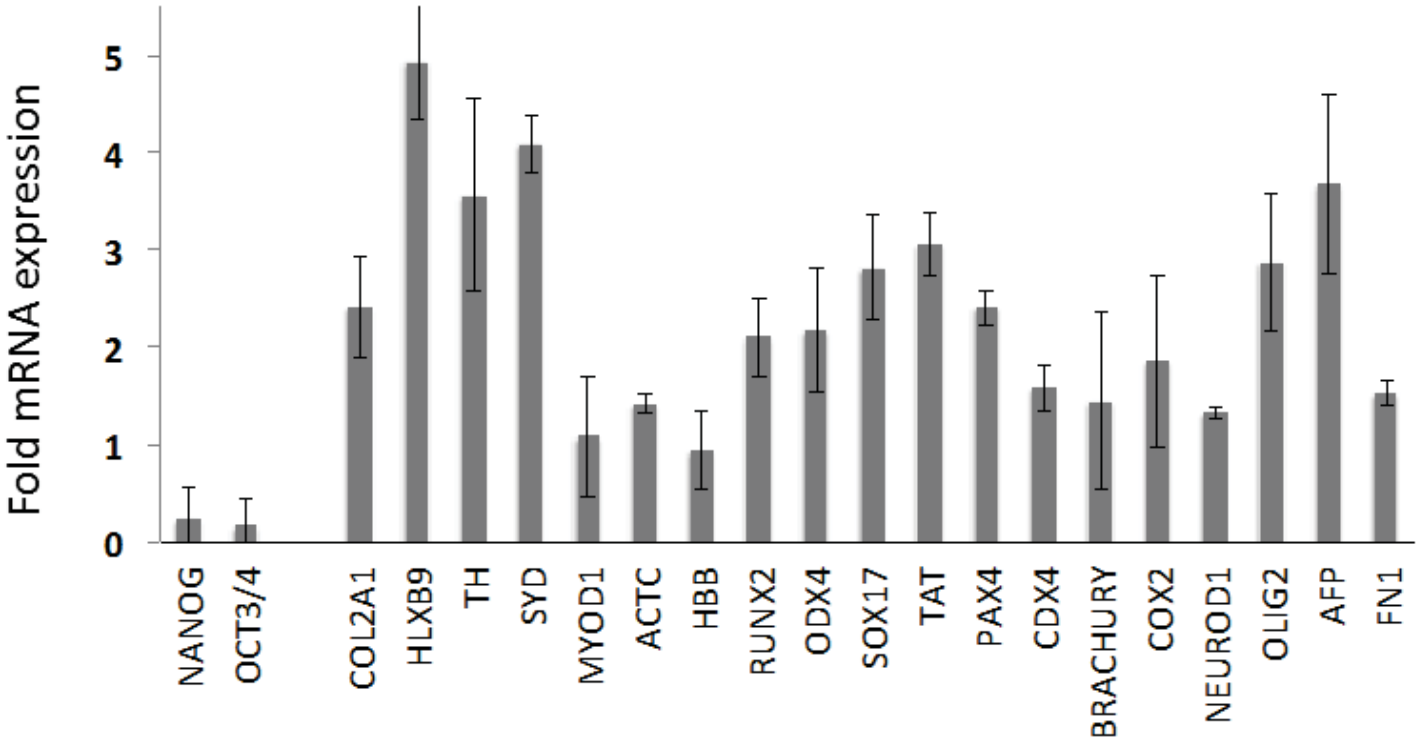

Fig. S6: Quantitative real-time (qRT-PCR) analysis of spontaneously differentiated embryoid bodies (EBs). Analysis was performed with EBs vs. undifferentiated CRL2097 hiPSCs. Results are represented as mean +/- SD. n = 3.

Figure S7

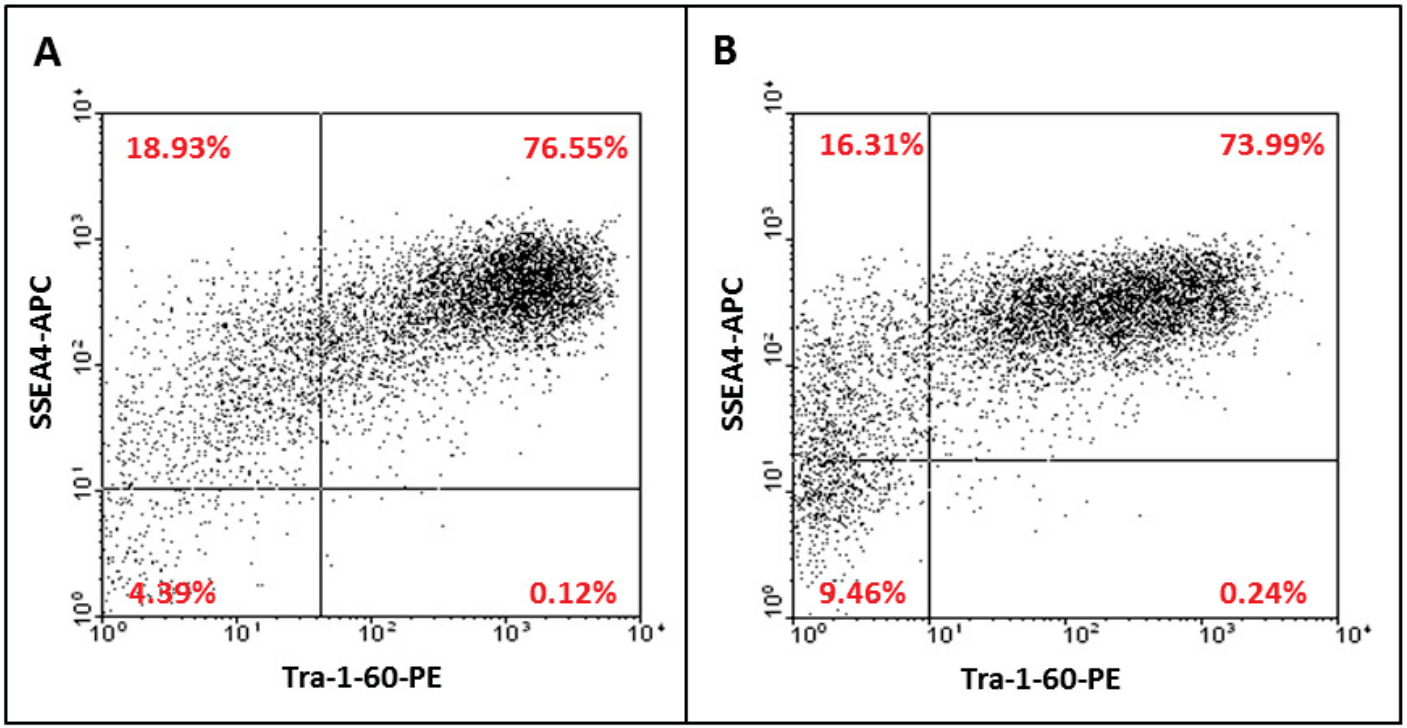

Fig. S7: Flow cytometry analysis of hiPSC lines A4 (A) and CRL2097 (B). hiPSCs were analyzed for the expression of the pluriopotency markers TRA-1-60 (x-axis) and SSEA-4 (y-achsis). Number of analyzed cells = 7000.

Figure S8

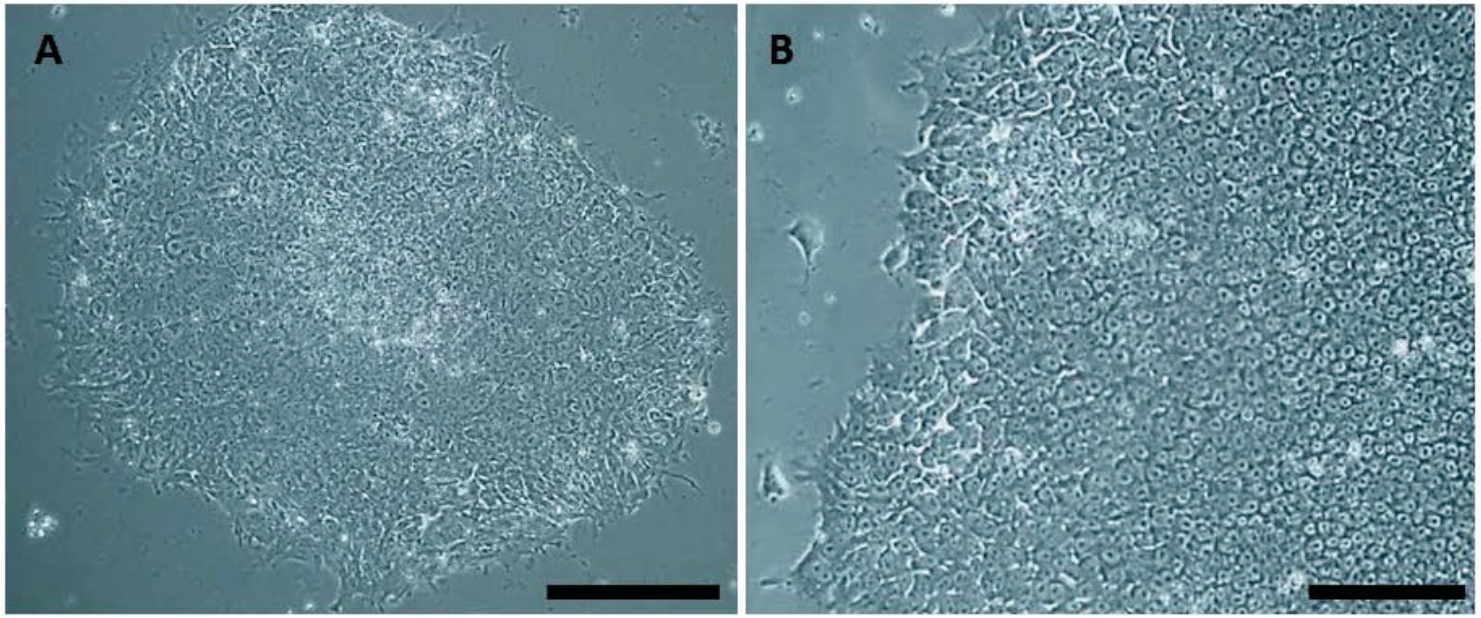

Fig. S8: Cell morphology of hiPSC line A4 colonies under feeder-free conditions. Representative phase contrast images of an A4 hiPSC colony with typical hESC-like morphology. Scale bars = 500 µm **(A)** and 100 µm **(B)**.

Figure S9

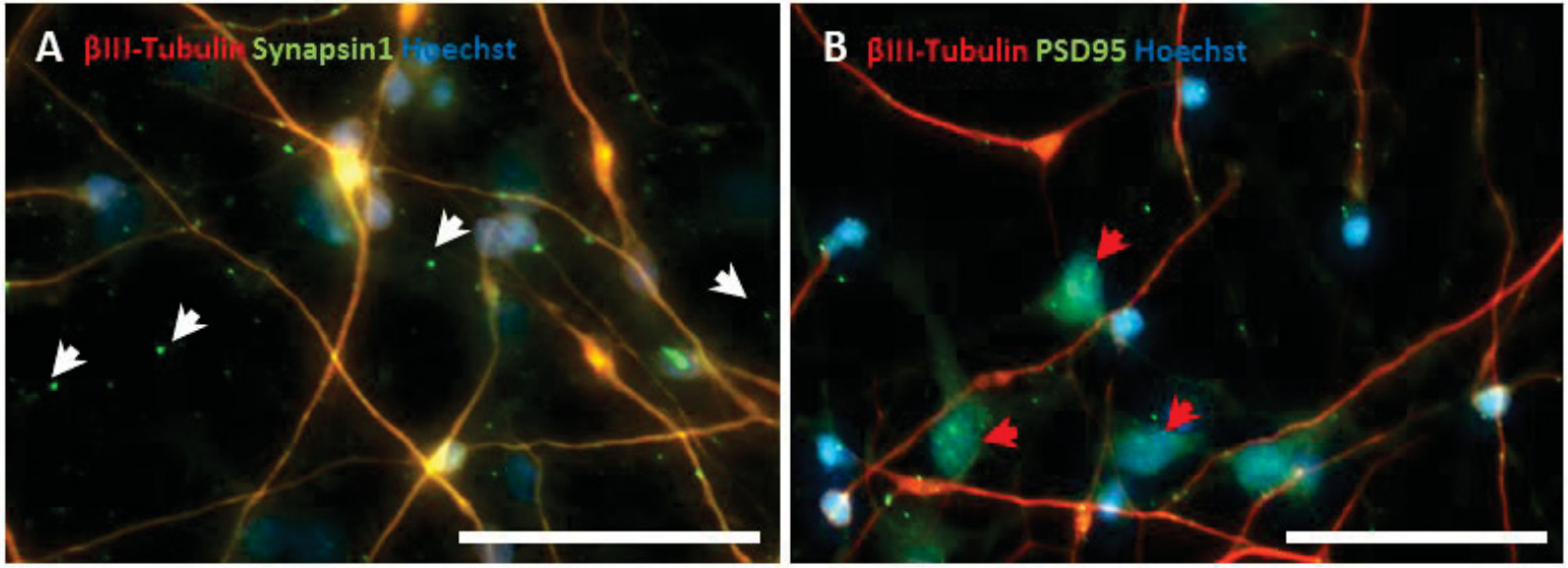

Fig. S9: Representative images of differentiating primary human neurospheres. Primary human neurospheres were differentiated for 28 days and stained for βIII-Tubulin positive neurons (red, **A-B**) and Synapsin1 (green, **A**) or PSD-95 (green, **B**). Synapsin 1 and PSD-95 show many artifacts (white arrows) and unspecific staining (red arrows) indicating low expression of synaptic markers in primary human neurons. Nuclei are counter-stained with Hoechst 33258. Scale bars **A – B** = 100 µm.

Figure S10

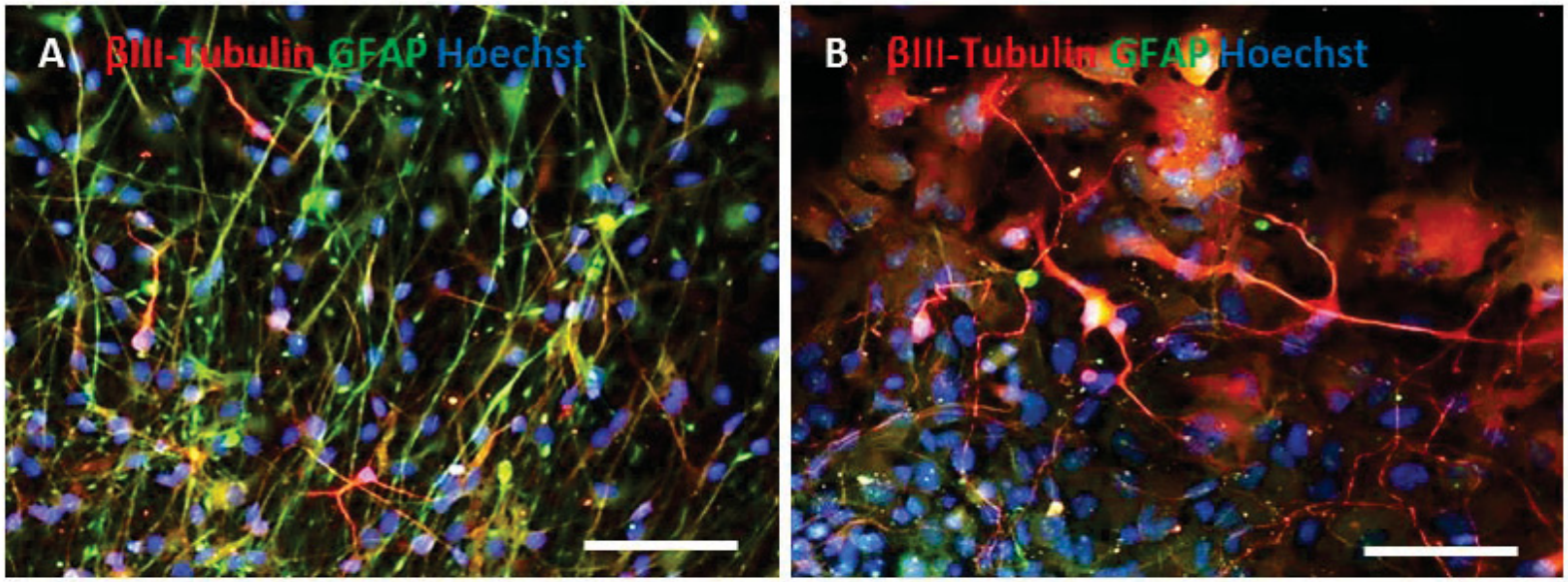

Fig. S10: Representative images of 3 days differentiated neurospheres. Primary human neurospheres **(A)** and hiPSC-neurospheres **(B)** were differentiated for 3 days and stained for βIII-Tubulin (red) and GFAP (green). Whereas βIII-Tubulin positive neurons migrate on top of GFAP+ cells in primary human neurospheres **(A)**, βIII-Tubulin positive neurons migrate on top of GFAP- cells in hiPSC-neurosoheres **(B)**. Nuclei are counter-stained with Hoechst 33258. Scale bars A – B = 100 µm.

Table S1: Oligonucleotide primers used for qRT-PCR.

Oligonucleotide primers	Sequence
hβ-ACTIN	FW: 5’ CAGGAAGTCCCTTGCCATCC 3’
	RV: 5’ ACCAAAAGCCTTCATACATCTCA 3’
hOCT4	FW: 5’ CGAGAAGGATGTGGTCCGAG 3’
	RV: 5’ AGCCTGGGGTACCAAAATGG 3’
hNANOG	FW: 5’ CAATGGTGTGACGCAGAAGG 3’
	RV: 5’ TGCACCAGGTCTGAGTGTTC 3’
hSOX2	FW: 5’ GGGAAAGTAGTTTGCTGCCTC 3’
	RV: 5’ AGAGAGGCAAACTGGAATCAGG 3’
hNestin	FW: 5’ CAGCTGGCGCACCTCAAGATG 3’
	RV: 5’ AGGGAAGTTGGGCTCAGGACTGG 3’
hPAX6	FW: 5’ ACACCGGTTTCCTCCTTCAC 3’
	RV: 5’ GGCAGCATGCAGGAGTATGA 3’
hMAP2	FW: 5’ TGCGCTGATTCTTCAGCTTG 3’
	RV: 5’ TGTGTCGTGTTCTCAAAGGGT 3’
hPDGFRα	FW: 5’ ATTAAGCCGGTCCCAACCTG 3’
	RV: 5’ AGCTCCGTGTGCTTTCATCA 3’
hNG2	FW: 5’ CCCATCCTCACTACAAACACA 3’
	RV: 5’ TGTAGACCAGATCCTCAGACC 3’
hGFAP	FW: 5’ GATCAACTCACCGCCAACAGC3’
	RV: 5’ CTCCTCCTCCAGCGACTCAATCT 3’

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Manuscripts

Comparative performance analysis of human iPSC-derived and primary neural progenitor cells (NPC) grown as neurospheres *in vitro*.

Maxi Hofrichter*, **Laura Nimtz***, Julia Tigges, Yaschar Kabiri, Friederike Schröter, Brigitte Royer-Pokora, Barbara Hildebrandt, Alexey Epanchintsev, Stephan Theiss[e], James Adjaye, Jean-Marc Egly, Jean Krutmann, Ellen Fritsche

* equal contribution

Journal:	Stem Cell Research
Impact factor:	3,902 (2017)
Contribution to the publication:	50% Partial planning and partial performance of neural inductions with several hiPSC lines, partial performance of proliferation, migration and differentiation experiments including immunocytochemical stainings and qRT-PCR analyses, complete performance, evaluation and analyses of synapse staining and electrophysiological experiments and data, in parts toxicological evaluations.
Type of authorship:	first authorship
Status of publication:	Published 26th October 2017

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

Ellen Fritsche, Marta Barenys, Jördis Klose, Stefan Masjosthusmann, **Laura Nimtz**, Martin Schmuck, Saskia Wuttke, and Julia Tigges

Toxicological Science

Die Entwicklung des menschlichen Gehirns besteht aus einer Reihe komplexer räumlicher und zeitlicher Prozesse, die durch die Einwirkung chemischer Substanzen gestört werden kann. Auswirkungen sind häufig irreversible Störungen des Nervensystems. Um eine chemische Beeinträchtigung in einem alternativen Assay bewerten zu können, muss das komplexe Zusammenspiel der Gehirnentwicklung in mehrere neurologische Entwicklungsprozesse zerlegt werden. Durch eine bestimmte Kombination verschiedener alternativer Assays können sogenannte spezifische Endpunkte abgebildet werden. In diesem Review stellen wir eine wissenschaftliche Begründung für neurologische Entwicklungsendpunkte dar, die derzeit ausgewählt werden, um Assays mit menschlichem Stamm- und Vorläuferzellen zu etablieren. Es wird angenommen, dass Assays, die diese wichtigen neurologischen Entwicklungsendpunkte abdecken, das Gerüst einer zukünftigen auf Stamm- und Vorläuferzellen beruhenden ENT-Testbatterie darstellen werden.

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TOXICOLOGICAL SCIENCES, 165(1), 2018, 21–30

doi: 10.1093/toxsci/kfy178
Advance Access Publication Date: July 5, 2018
Contemporary Review

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).

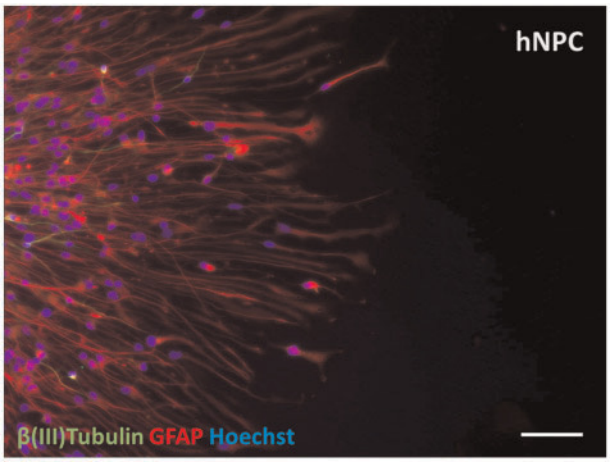

Figure 1. hNPC (Lonza, Verviers, Belgium) were plated onto poly D-lysin/laminin-coated glass slides. After 24 hours, cells were fixed with paraformaldehyde and stained with antibodies against GFAP and β(III)tubulin. Nuclei were stained with Hoechst. Scale bar = 50μm

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

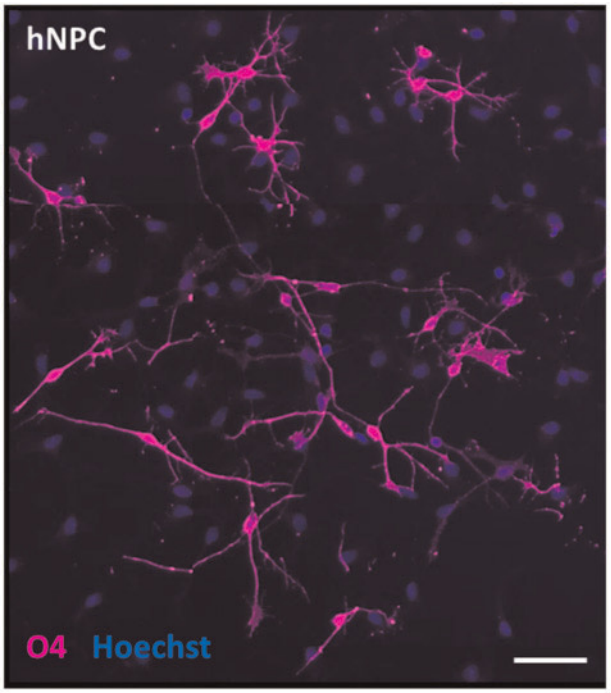

Figure 2. Oligodendrocyte differentiation of primary hNPC. Oligodendrocyte differentiation was assessed by immunocytochemical staining for the oligodendrocyte marker O4 of 5 days differentiated hNPC. Nuclei were counterstained with Hoechst. Scale bar = 50μm. The staining method was previously published 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

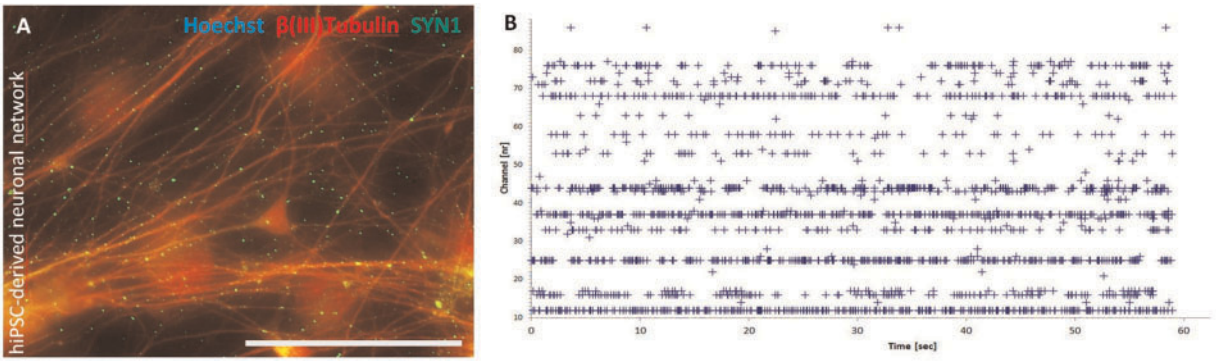

Figure 3. Synapse formation and neuronal network activity of a hiPSC-derived network. A, hiPSC (IMR-90, Wicell) were neurally induced to NPCs using the NIM-protocol (Hofrichter *et al.* 2017) and differentiated on D-lysin/laminin-coated glass slides for 28 days, fixed with paraformaldehyde and stained 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

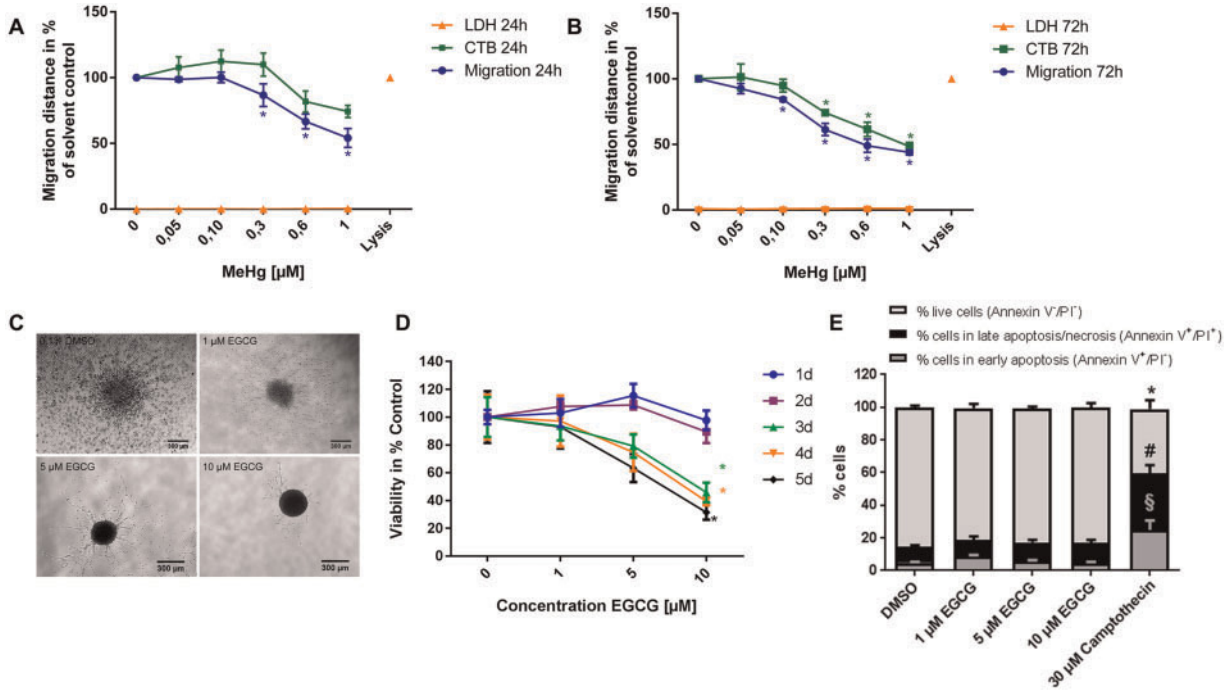

Figure 4. hNPC growing as neurospheres in proliferation culture, were plated for migration analyses onto poly D-lysin/laminin-coated glass slides in presence and absence of MeHgCl. After (A) 24 and (B) 72 h, migration distance was measured from the outer sphere rim to the furthest migrated cells at four 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.

FUNDING

This work was supported by the German Ministry of Education and Research [grant number 16V0899]; the Ministry of Innovation, Science and Research of North Rhine Westphalia; and a German Academic Exchange Service (DAAD) Research Fellowship.

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Manuscripts

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

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Journal:	Toxicological Sciences (Toxicol Sci)
Impact factor:	4,081 (2016)
Contribution to the publication:	20% Performance of experiments for the sections 'synapse formation and neuronal network activity', writing of the manuscript sections 'neuronal subtype differentiation' and 'Synaptogenesis/neuronal network formation'.
Type of authorship:	first authorship
Status of publication:	Published 5th July 2018

2.3 Development of the Concept for Stem Cell-Based Developmental Neurotoxicity Evaluation.

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Toxicological Science

Es gibt Hinweise darauf, dass die Exposition gegenüber Chemikalien während der menschlichen Entwicklung zu irreversiblen Beeinträchtigungen des Nervensystem führen kann. Daher hat das Testen von Substanzen auf das entwicklung-neurotoxische Potential eine hohe Priorität für verschiedene Interessengruppen: Wissenschaft, Industrie und Aufsichtsbehörden. Aufgrund der Ressourcenintensität der derzeitigen Entwicklungsneurotoxizität (ENT)-Richtlinien *in vivo*, sind von den Regulierungsbehörden alternative Methoden gewünscht, die wissenschaftlich valide sind und eine hohe Vorhersagbarkeit für den Menschen haben. In diesem Review überprüfen wir die Verfügbarkeit von auf neurologischen Entwicklungsprozessen basierenden *In-vitro*-Methoden mit Stamm- und Vorläuferzellen für die ENT-Evaluierung. Diese Methoden werden zu einer bislang noch lückenhaften ENT-*In-vitro*-Testbatterie zusammengefügt.

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TOXICOLOGICAL SCIENCES, 165(1), 2018, 14–20

doi: 10.1093/toxsci/kfy175
Advance Access Publication Date: June 29, 2018
Contemporary Review

CONTEMPORARY REVIEW

Development of the Concept for Stem Cell-Based Developmental Neurotoxicity Evaluation

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ABSTRACT

Human brain development consists of a series of complex spatiotemporal processes that if disturbed by chemical exposure causes irreversible impairments of the nervous system. To evaluate a chemical disturbance in an alternative assay, the concept evolved that the complex procedure of brain development can be disassembled into several neurodevelopmental endpoints which can be represented by a combination of different alternative assays. In this review article, we provide a scientific rationale for the neurodevelopmental endpoints that are currently chosen to establish assays with human stem/and progenitor cells. Assays covering these major neurodevelopmental endpoints are thought to assemble as building blocks of a DNT testing battery.

Key words: stem cells; ESC; cellular and molecular biology.

For almost 20 years there has been considerable concern that chemical exposure might contribute to the increasing incidence of neurodevelopmental diseases in children (Bennett *et al.*, 2016; Grandjean and Landrigan, 2006, 2014; Schettler, 2001). Despite this concern, most chemicals have not been evaluated for their neurodevelopmental toxicity (Crofton *et al.*, 2012; Goldman and Koduru, 2000). The main reason for this data gap lies in the resource-intensity of the current guideline studies: EPA 870.6300 developmental neurotoxicity (DNT) guideline (U.S. EPA, 1998) and the draft OECD 426 guideline (OECD, 2007). These guidelines demand significant time, money and animals (Crofton *et al.*, 2012; Lein *et al.*, 2005) and are therefore not suited for testing large number of chemicals. The DNT TestSmart initiative originated and led by Alan Goldberg from the John's Hopkins University in 2006, took this issue up by bringing international scientists into communication on how to test for DNT with alternative methods (Lein *et al.*, 2007). Since then, international researchers have been developing concepts on how to use and interpret such alternative DNT methods with the final goal of regulatory application (Bal-Price *et al.*, 2012, 2015, 2018; Crofton *et al.*, 2011; Fritsche *et al.*, 2017, 2018; Lein *et al.*, 2005). The concept evolved that the complex procedure of brain development is disassembled into spatiotemporal neurodevelopmental processes that are necessary for forming a functional brain AND can be tested for adverse effects of compounds in *in vitro* assays (Bal-Price *et al.*, 2015, 2018; Fritsche, 2016; Lein *et al.*, 2007). Here, human-based systems are preferred because species differences in toxicokinetics, e.g., due to developmental timing, and/or toxicodynamics might affect responses to compounds (Dach *et al.*, 2017; Gassmann *et al.*, 2010; Gold *et al.*, 2005; Knight, 2007; Leist and Hartung, 2013; Masjosthusmann *et al.*, 2018; Seok *et al.*, 2013).

In the following paragraphs, we will provide the scientific rationales for the endpoints that are currently chosen for assay

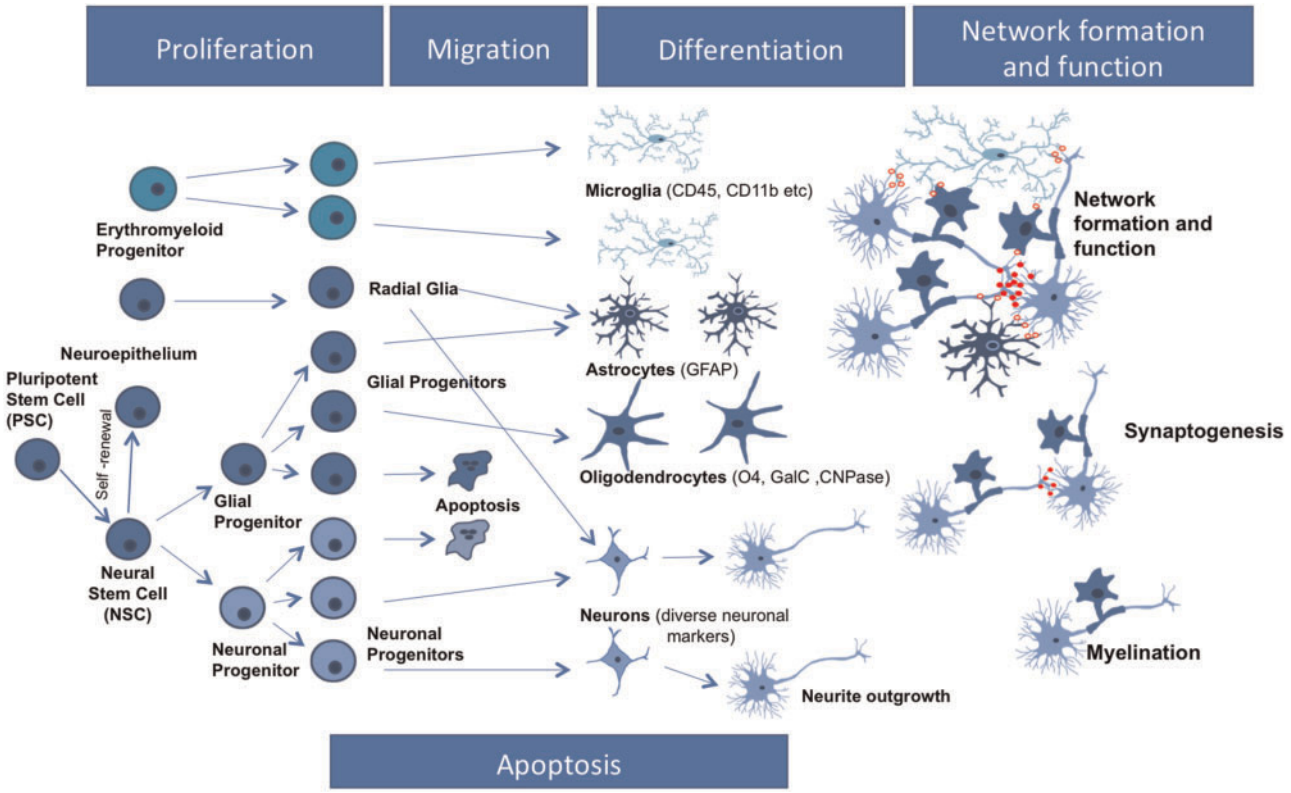

Figure 1. Neurodevelopmental processes essential for nervous system development. It is assumed that DNT toxicants exert their toxicity by disturbing at least one of these processes. Therefore, disturbances of the processes depicted here in blue boxes are key events of adverse outcome pathways relevant for DNT. From Bal-Price *et al.* (2018).

establishment with human stem/progenitor cells and depicted in Figure 1. Such assays are then thought to assemble as building blocks of a DNT testing battery covering neurodevelopmental endpoints over time.

ESC DIFFERENTIATION TO NEUROEPITHELIAL PRECURSORS/INDUCTION OF NEURONAL ROSETTES

During embryogenesis, stem cells develop into the primordium including the primordium of the central nervous system (CNS). During this neurulation, the neural plate and the neural groove form that lead to the emergence of the neural tube by fusing of the neural folds. Polarization and patterning of the neural tube ultimately develop into the 3 major vesicles of the future brain: forebrain, midbrain, and hindbrain (reviewed by Silbereis *et al.*, 2016). Neural tube and axial defects of the vertebrate embryo belong to the most common developmental malformations in man. They include neural tube defects, which are among the most prominent birth defects in the human population with a prevalence of around 35 cases of spina bifida, 20 cases of anencephaly, and 10 cases of encephalocele per 100 000 births (CDC, http://www.cdc.gov/ncbddd/birthdefects/data.html; last accessed July 16, 2019). In addition, disruption of axial development might cause diverse craniofacial, limb, and cardiac malformations. During evaluation of developmental effects of chemicals and pharmaceuticals with experimental animal studies, neural tube and axial defects are frequently observed findings (Knudsen *et al.*, 2009). Examples for such human teratogens are anticonvulsants (e.g. valproate and carbamazepine), cytostatic agents (e.g. cyclophosphamide and methotrexate), and retinoids. An adverse outcome pathway framework was recently developed linking neural tube and axial defects to modulation of retinoic acid homeostasis (Tonk *et al.*, 2015). This framework was used as one of the building blocks for generation of developmental toxicity ontology (Baker *et al.*, 2018).

Human embryonic stem cells are able to differentiate into early neuroepithelial precursor (NEP) in the form of neural rosettes. These peculiar structures represent an *in vitro* primitive neural stem cell state with all the properties of neural plate cells and recapitulate the early neurulation events that bring the formation and closure of the neural tube (Elkabetz *et al.*, 2008; Lazzari *et al.*, 2006; Pankratz *et al.*, 2007). Such cells have a default anterior-dorsal pattern that is reverted by exposure to ventralizing signals such as sonic hedgehog and fibroblast growth factor 8 (Cowan *et al.*, 2004).

NEURAL PROGENITOR CELL PROLIFERATION

The brain is a highly organized structure and its development depends on the proliferation of a variety of progenitor cell types. When compared with lissencephalic species like mice and rats, brains of gyrencephalic species, like humans and ferrets, contain a larger variety of neural progenitor cells (NPCs) involved in e.g., the formation of the 6-layered neocortex. Also duration of proliferative NPC activity correlates with brain complexity, i.e. the phase of extensive progenitor self-renewal takes 2 weeks in mouse and 3 months in human developing brains. Such proliferative activity is directly coupled to the number of produced neural cells including neurons during corticogenesis, thus determining brain size, as well as to the formation of cortical gyri

and sulci. Here, centrosome-related proteins that determine spindle orientation and centrosome biosynthesis, the primary cilium, junctional adhesion molecules, and cell cycle length, determine NPC proliferation (comprehensively reviewed in Uzquiano *et al.*, 2018). A disturbance of NPC proliferation during brain development leads to significant alterations of brain morphology like a reduction of size, weight, or volume of the entire brain (microcephaly; de Groot *et al.*, 2005) or of individual brain structures (Moore *et al.*, 2006) having detrimental effects on the neurological outcome (Lang and Gershon, 2018; Ostergaard *et al.*, 2012). Besides genetic factors (Uzquiano *et al.*, 2018), also environmental elements can cause microcephaly. One recent example is Zika virus infection causing microcephaly in children (Devakumar *et al.*, 2018; Tang *et al.*, 2016).

NPC APOPTOSIS

Apoptosis is a crucial and strictly regulated event during brain development. Too much apoptosis can deplete the NPC pool in the developing brain. Here, loss of centrosome biogenesis in NPC, e.g., by deletion of *Cenpj* (*Sas4*), causes mitotic delay and an elimination of a subtype of NPC, apical radial glia (aRG), from the ventricular zone (VZ). This results in a thinning of upper cortical layers and microcephaly (reviewed in Uzquiano *et al.*, 2018). On the contrary, reduction in apoptosis due to inactivation of caspases or their copartners like *Apaf* can lead to morphological defects like hyperplastic brains. An apoptosis pathway involving caspases-3 and -9 is of particular importance in the developing brain. A reduction in apoptosis observed in Casp9$^{-/-}$ knockouts is thought to account for increased numbers of Bromodeoxyuridine-positive cells in the germinal zones of the brain that is consistent with an increased survival of NEP. As a consequence, both the VZ and forebrain cortical structures are expanded in these knockout mice, disrupting cortical organization and ultimately resulting in intracranial hemorrhage and death (Hakem *et al.*, 1998; Yoshida *et al.*, 1998). Apoptosis is therefore a well-balanced procedure where disturbance in either direction has detrimental outcomes.

RADIAL GLIA PROLIFERATION

Radial glia cells play key roles during cerebral cortex development. They are not a uniform cell type but are specified into different radial glia types with different functions across species. Describing the detailed functions of radial glia types extends beyond the scope of this article, for more in-depth information the interested reader is referred to excellent review articles (Gotz and Huttner, 2005; Uzquiano *et al.*, 2018). Briefly, aRG cells comprise the predominant neuronal progenitor cell type within the developing neocortex. They are highly polarized cells, exhibiting basal processes attached to the basement membrane, and apical processes linked by adhesion with cerebrospinal fluid in the ventricles. aRGs undergo asymmetric proliferative division into postmitotic neurons and neurogenic progenitors. In gyrencephalic species including humans, the initial pool of aRGs is greater than in lissencephalic species mainly contributing to neurogenesis through the production of a variety of basally located progenitors including basal radial glia-like cells (bRG), which are mainly intermediate progenitors (IPs). In primates including humans, IPs undergo several rounds of self-renewing before terminal differentiation. This higher neuronal production in gyrencephalic species impacts cortical size and folding (Borrell and Gotz, 2014; Fish *et al.*, 2008; Uzquiano *et al.*, 2018). Besides radial glia function as primary stem and progenitor cells that proliferate (see above) and give rise to neurons and glia, they also act as scaffolds for migrating neurons building the cerebral cortex architecture (Borrell and Gotz, 2014; Gotz and Huttner, 2005; Malatesta and Gotz, 2013). Due to the fundamental role of radial glia cells in brain development, disturbance of their biology will have detrimental results. For example, induced proliferation of the gyrencephalic ferret bRG leads to an expansion of the cortical surface area and the formation of new folds and fissures, while it increases surface area without creating new folds and fissures in the mouse (Nonaka-Kinoshita *et al.*, 2013). The instance that there are primate-human specific traits in brain ontogenesis that are targets of brain diseases and cortical malformations, like in the Miller-Dieker Syndrome, might explain why mouse models often fail to recapitulate patients' phenotypes (reviewed in Uzquiano *et al.*, 2018).

NEURAL CREST CELL/RADIAL GLIA/NEURONAL MIGRATION

Different neural cell types need proper migration during development. During embryogenesis, neural crest cells (NCC) migrate to distinct parts of the embryo developing into e.g. sensory and enteric neurons, Schwann cells, melanocytes, craniofacial structures like bone and cartilage, and chromaffine cells of the adrenal gland. Defective NCC migration and differentiation can cause a variety of diseases like cleft palate, hearing loss, Morbus Hirschsprung or CHARGE syndrome (Dupin and Sommer, 2012; Mayor and Theveneau, 2013).

Cortex development takes place during the fetal phase of brain development involving radial glia as well as neuronal migration (Borrell and Gotz, 2014). This neuronal migration process on scaffolds generated by radial glia migration is a fundamental neurodevelopmental key event, because radial glia as well as postmitotic differentiating cells migrate and differentiate over time into the main effector cells neurons, astrocytes and oligodendrocytes thereby ensuring normal brain structure and function (Carpenter *et al.*, 1999). Developmental brain disorders such as heterotopia and lissencephaly or diseases such as schizophrenia and epilepsy have been associated with disruptions of this cortical migratory process (Barkovich *et al.*, 2005; Bozzi *et al.*, 2012; Volk *et al.*, 2012).

ASTROCYTE DIFFERENTIATION/MATURATION

During the last decades, the view on astrocytes' physiology and their contribution to toxicity and disease has fundamentally changed. Although they were initially thought to play only a supporting and scaffolding role in brain, an increasing diversity of functions—also in a brain region-specific context—have now been appointed to this diverse cell type (reviewed in Hu *et al.*, 2016; Volterra and Meldolesi, 2005). Astrocytes create the brain environment, build up the microarchitecture of the brain parenchyma, maintain brain homeostasis, store and distribute energy substrates, control the development of neural cells, contribute to synaptogenesis, and synaptic maintenance, regulate cerebral blood flow, maintain the blood-brain barrier, and provide brain defense. Therefore, astroglia differentiation is a crucial event during brain development. Astrocytes express astroglial intermediate filament proteins like glial fibrillary acidic protein (GFAP) and vimentin, thus expression of GFAP is commonly used as a specific marker for astrocyte identification. However, it is to consider that *in situ* the levels of GFAP expression vary

quite considerably. For example, GFAP is expressed by virtually every Bergmann glial cell in the cerebellum whereas only about 15%–20% of astrocytes in the cortex of mature animals express GFAP. The same heterogeneity of astrocyte marker expression is also seen in different astrocyte *in vitro* methods (Lundin *et al.*, 2018). There is a large variety of different astrocytes present in brains probably conferring to the heterogeneity of astrocyte marker expression. For example, protoplasmic astrocytes are present in gray matter, while fibrous astrocytes are present in white matter yet with region-specific functions (Hu *et al.*, 2016). Another large class of astroglial cells are the radial glia, which are bipolar cells each with an ovoid cell body and elongated processes (described earlier Uzquiano *et al.*, 2018). After maturation, radial glia disappear from many brain regions and transform into stellate astrocytes (Adapted from Kettenmann and Verkhratsky, 2011).

Astrocytes seem to play a "yin-and-yang" role in health maintenance and disease of the brain. Their responses and roles in brain pathologies range from beneficial to adverse. Such astrocyte responses to a variety of stimuli are called reactive astrogliosis, a context-dependent progress undergoing a mild, moderate or severe substantial alteration of morphology and molecular function, i.e. releasing inflammatory factors. Reactive astrocyte responses might also be involved in the pathogenesis of neurodegenerative diseases like Parkinson's and Alzheimer's disease as well as amyothrophic lateral sclerosis. In addition, they seem to contribute to the pathogenesis of demyelinating diseases as well as brain aging (reviewed in Hu *et al.*, 2016). Despite just astrocyte marker expression, astrocyte function is crucial when studying astrocyte development. Four key astrocytic features of importance are (Lundin *et al.*, 2018): the uptake of the neurotransmitter glutamate, essential for synapse dynamics; inflammatory response to trauma; calcium signaling response to neurotransmitters; and the secretion of apolipoprotein E, a lipid and cholesterol transporter in the brain (Bazargani and Attwell, 2016; Khakh and Sofroniew, 2015; Yu *et al.*, 2014).

OLIGODENDROCYTE DIFFERENTIATION/ MATURATION

Oligodendrogenesis is necessary for proper brain function, as oligodendrocytes form and keep myelin sheaths around axons, a necessity for nerve cell function by enabling salutatory conduction. The peak of this process starts during the late fetal period and continues until the child's third year of age. Because myelin inhibits synaptogenesis and neuronal plasticity, this extended myelin production in humans prolongs the phase for learning capacities and memory (reviewed in Silbereis *et al.*, 2016). Several processes are involved in the generation of a sufficient number and proper functioning of oligodendrocytes. These include oligodendrocyte formation from oligodendrocyte progenitor cells (OPCs), maturation of OPCs, generation of myelin from matured oligodendrocytes and finally correct myelin sheet enclosure around axons. Disturbance of oligodendrocyte development may result in demyelination diseases that severely affect neuronal functioning and can be accompanied by impaired e.g. sensory, motor, or vegetative functions as well as memory (Baumann and Pham-Dinh, 2001; Nawaz *et al.*, 2015).

Oligodendrocyte development is linked to thyroid hormone (TH) action. Children suffering from the Allan-Herndon-Dudley Syndrome experience intrauterine brain hypothyroidism due to a mutation in the TH transporter monocarboxylate transporter 8 (MCT-8) resulting in delayed CNS myelination (Rodrigues *et al.*, 2014; Tonduti *et al.*, 2013). From the clinical data it is not clear if this myelination delay is due to less oligodendrocyte formation or maturation or a combination of both. However, *in vitro* studies using primary human neurospheres differentiating into oligodendrocytes suggest that TH induces oligodendrocyte maturation, but not formation, while in mouse neurospheres both endpoints are TH-dependent (Dach *et al.*, 2017). Thus, the oligodendrocyte maturation assay seems to be well suited to study TH disruption in developing human and mouse NPC.

NEUROGENESIS AND NEURONAL MATURATION

Neurogenesis in the human CNS begins shortly after the fusion of neural folds. Here, motor neurons in the ventral horn of the cervical spinal cord and neurons of certain cranial nerve nuclei in the brainstem appear first at gestational week 4 (Bayer and Altman, 2007; O'Rahilly and Muller, 2006). Prenatal neurogenesis continues throughout embryonic and fetal development mainly in the neocortex and the cerebellum. The cerebral cortex of a middle-aged male is estimated to be comprised of 16.34 billion neurons (Azevedo *et al.*, 2009), which are produced from neural stem or progenitor cells within the VZ and subventricular zone (SVZ) of the developing cerebral cortical wall. Of those, approximately 80% (13.07 billion) are estimated to be excitatory glutamatergic projection neurons (i.e. pyramidal and modified pyramidal neurons; DeFelipe *et al.*, 2002) and the rest are GABAergic inhibitory interneurons (reviewed by Silbereis *et al.*, 2016). Neurons are indispensable for life and their differentiation patterns are tightly regulated. Thus, modulation of neuronal differentiation into both directions (promotion or inhibition of neurogenesis) is considered as adverse. For example, reduced neurogenesis is thought to be involved in the pathogenesis of depressive mood disorders (Song and Wang, 2011) or the intellectual disabilities in Down Syndrome patients initiated during the fetal period (Guidi *et al.*, 2018; Stagni *et al.*, 2018).

Subsequent to neuronal differentiation, dendritic, and axonal (neurite) outgrowth followed by the formation of synapses are key cellular features associated with the functional maturation of the CNS. At midgestation, immature neocortical neurons have spread axons and instigated to expand dendrites that initiate an extended period of axon outgrowth, dendritic arborization and synaptogenesis extending into early childhood. Despite this general developmental concept, these processes vary substantially between brain layers, areas, and human neocortical neuronal subtypes (reviewed by Silbereis *et al.*, 2016).

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. For example, glutamatergic neurons are generated from the VZ and SVZ of the dorsal mesencephalon (Fode *et al.*, 2000; Guillemot, 2007), GABAergic neurons from the ventral telencephalon (Casarosa *et al.*, 1999; Hansen *et al.*, 2010, 2013; Poitras *et al.*, 2007), while dopaminergic neurons are generated from several brain regions like the mesencephalon, hypothalamus, and retinal and olfactory bulbs (Alizadeh *et al.*, 2015; Arenas *et al.*, 2015; Zhang and van den Pol, 2015). Occurrence of neuronal subtype differentiation during early neurogenesis into 80% excitatory and 20% inhibitory neurons and their interactions are essential for the neuronal function of the CNS (DeFelipe *et al.*, 2002; Hansen *et al.*, 2010, 2013; Workman *et al.*, 2013).

SYNAPTOGENESIS/NEURONAL NETWORK FORMATION

During early neurogenesis neurons start to mature, connect, and become electrically active in the embryonic phase of neurodevelopment—between the fourth and fifth postconceptional week (Okado *et al.*, 1979; Zecevic and Antic, 1998). For the function of the CNS this neuronal maturation and the formation of synapses is crucial. The neuronal intercellular communication takes place at synaptic connections, which is associated with learning and memory through synaptic plasticity (Lundin *et al.*, 2018). When synaptogenesis is disrupted by a compound, neuronal network activity is altered (Robinette *et al.*, 2011). Here, synaptogenesis might converge on a variety of neurodevelopmental processes, i.e. key events, converging a compounds' effects on neuronal (subtype) differentiation, neurite outgrowth, axon or dendrite formation, dendritic spine development or synaptogenesis itself. Thus, this functional endpoint is a crucial readout for integration of neuronal function of the human brain.

CONCLUSION

Taken together, these neurodevelopmental processes have been identified somewhat as a minimum requirement for a proposed DNT testing battery, covering the complexity of neurodevelopmental processes as well as timing aspects of brain development. Moreover, they are considered for a DNT testing battery because there are stem/progenitor cell methods available allowing the set up of test methods for DNT evaluation (Bal-Price *et al.*, 2018). However, the current state of the science concerning identified endpoints is probably still at an early, immature state. The key events discussed here have clear rationales for their crucial function during brain development. Other key events like astrocyte differentiation and maturation, especially with regards to astrocyte heterogeneity, astroglia function, neuronal maturation, and neuronal network formation; however, are less well characterized. Moreover, the complexity of brain region-specific neural differentiation and function is not well understood, especially in the human context. Hormonal contributions to brain development are manifold and complex and chemicals with endocrine activities are 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). As basic scientific knowledge on these neurodevelopmental key events deepens, implications for additional toxicity testing will arise and with this an evolvement of stem/progenitor cell-based methods for studying additional endpoints. However, the current state-of-knowledge is a satisfying start to put a DNT testing battery into action that covers neurodevelopmental endpoints across time.

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Manuscripts

Development of the Concept for Stem Cell-Based Developmental Neurotoxicity Evaluation.

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Journal:	Toxicological Sciences (Toxicol Sci)
Impact factor:	4,081 (2016)
Contribution to the publication:	20% Writing of the manuscript sections 'neuronal subtype differentiation' and 'Synaptogenesis/neuronal network formation'.
Type of authorship:	first authorship
Status of publication:	Published 29th June 2018

2.4 Characterization and application of electrically active neuronal networks established from human induced pluripotent stem cell-derived neural progenitor cells for neurotoxicity evaluation.

Laura Nimtz, Julia Tigges, Stefan Masjosthusmann, Martin Schmuck , Eike Keßel, Julia Hartmann, Stephan Theiss, Karl Köhrer, Patrick Petzsch, James Adjaye, Claudia Wigmann, Dagmar Wieczorek, Barbara Hildebrandt, Farina Bendt, Ulrike Hübenthal, Gabriele Brockerhoff, and Ellen Fritsche

Stem Cell Research

Neurotoxizität wird durch eine Vielzahl von Wirkmechanismen vermittelt, die zu einer Störung der neuronalen Funktion führen. Für die Überpprüfung einer größeren Anzahl von Substanzen auf ihr neurotoxisches Potenzial können funktionelle neuronale Netzwerke (NN) *in vitro* hilfreich sein. Wir haben humane NN (hNN) aus von hiPSC abgeleiteten neuralen Vorläuferzellen (hiNPC) generiert und durch Vergleiche der hNN-Bildung mittels zwei unterschiedlicher Differenzierungsmedien charakterisiert: in Gegenwart (CINDA) und Abwesenheit (NDM) von Reifungsfördernden Faktoren. Als NN-Kontrolle haben wir differenzierende Ratten-NN (rNN) in die Studie aufgenommen. Die Gen-/ Protein-Expressionen und die elektrische Aktivität von sich entwickelndem NN *in vitro* wurden zu mehreren Zeitpunkten analysiert. Transkriptomanalysen von 5, 14 und 28 Tagen *in vitro* innerhalb CINDA-differenzierten hNN wurden zudem mit Genexpressionsprofilen von sich entwickelnden menschlichen Gehirnen *in vivo* verglichen. Molekulare Expressionsanalysen sowie Messungen der elektrischen Aktivität weisen darauf hin, dass NN im Laufe der Zeit zu Neuronen und Astrozyten reifen. Im Gegensatz zu rNN sind hNN innerhalb des gleichen Zeitraums der Differenzierung weniger elektrisch aktiv. HNN, die in CINDA-Medium differenziert wurden, übertreffen hNN ohne Zusätze. Die Exposition von NN mit neuronalen Rezeptorstimulatoren und -inhibitoren zeigen das Vorhandensein inhibitorischer GABAerger Neuronen. In CINDA-Medium differenzierte hiPSC-abgeleitetes GABAerge hNN könnten als Teil einer *In-vitro*-Test-Batterie zur Beurteilung der Neurotoxizität nützlich sein.

Elsevier Editorial System(tm) for Stem Cell Research

Manuscript Draft

Manuscript Number:

Title: Characterization and application of electrically active neuronal networks established from human induced pluripotent stem cell-derived neural progenitor cells for neurotoxicity evaluation

Article Type: Research paper

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Abstract: Neurotoxicity is mediated by a variety of modes-of-actions (MoA) leading to disturbance of neuronal function. For screening larger numbers of compounds for their neurotoxic potential, in vitro functional neuronal networks (NN) might be helpful tools. We established and characterized human NN (hNN) from hiPSC-derived neural progenitor cells (hiNPC) by comparing hNN formation with two different differentiation media: in presence (CINDA) and absence (NDM) of maturation-supporting factors. As a NN control we included differentiating rat NN (rNN) in the study. Gene/protein expression and electrical activity from in vitro developing NN were assessed at multiple time points. Transcriptomes of 5, 14 and 28 days in vitro CINDA-grown hNN were compared to gene expression profiles of in vivo human developing brains. Molecular expression analyses as well as measures of electrical activity indicate that NN mature into neurons and astrocytes over time. In contrast to rNN, hNN are less electrically active within the same period of differentiation time, yet hNN grown in CINDA medium outperform hNN without supplements. Challenge of NN with neuronal receptor stimulators and inhibitors demonstrate presence of inhibitory, GABAergic neurons. HiPSC-derived GABAergic hNN grown in CINDA medium might be useful as part of an in vitro battery for assessing neurotoxicity.

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Thomas Zwaka, Editor-In-Chief

STEM CELL RESEARCH

IUF
LEIBNIZ-INSTITUT FÜR UMWELT-MEDIZINISCHE FORSCHUNG

Dear Prof. Zwaka,

attached please find our manuscript entitled 'Characterization and application of electrically active neuronal networks established from human induced pluripotent stem cell-derived neural progenitor cells for neurotoxicity evaluation' that we would like to publish in Stem Cell Research.

The field of human induced pluripotent stem cells (hiPSC) for a variety of bio-applications is rapidly growing. Usage of alternative human-based in vitro methods for efficacy and toxicity testing is currently increasing to gain more human relevance in cell-based assays, to overcome source limitations and ethical concerns. For testing compounds' neurotoxic potential, hiPSC-derived, functionally active neuronal networks are thought to increase predictive capacity for humans. Therefore, we here continue our previously published work on generating in vitro neuronal networks from hiPSC-derived neural progenitor cells (NPC) cultured as neurospheres consisting of a co-culture of neurons and astrocytes (Hofrichter et al. Stem Cell Res 2017) by

- Molecularly characterizing neuronal networks for synapse-specific proteins, neuronal subtype-specific receptors and enzymes.
- Establishing neuronal networks from rat NPC as control cultures.
- Demonstrating functional activity performance across human and rat neuronal networks on microelectrode arrays (MEA).
- Establishing 24-multi-well-MEAs for compound testing purposes in addition to the single well-MEAs.
- Adding microarray data from differentiated neuronal networks to the data set that is compared to gene expression data from developing brain *in vivo*.

Hence, the manuscript describes a method for the generation of neuronal networks from hiPSC-derived 3D-NPC, which might be suitable for neurotoxicity evaluation or disease modeling and thus we expect our work to be attractive for a broad readership.

I hope that you positively consider our work and remain with

kind regards,

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Highlights:

- Establishment of electrically active neuronal networks from human iPSC
- Molecular characterization of hiPSC-derived neuronal networks
- Functional analysis of electrical activities on microelectrode arrays
- Activity modification by pharmacological compound applications
- Transcriptome comparison of *in vitro* and *in vivo* developing neural cells

Title: Characterization and application of electrically active neuronal networks established from human induced pluripotent stem cell-derived neural progenitor cells for neurotoxicity evaluation.

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ABSTRACT:

Neurotoxicity is mediated by a variety of modes-of-actions (MoA) leading to disturbance of neuronal function. For screening larger numbers of compounds for their neurotoxic potential, *in vitro* functional neuronal networks (NN) might be helpful tools. We established and characterized human NN (hNN) from hiPSC-derived neural progenitor cells (hiNPC) by comparing hNN formation with two different differentiation media: in presence (CINDA) and absence (NDM) of maturation-supporting factors. As a NN control we included differentiating rat NN (rNN) in the study. Gene/protein expression and electrical activity from *in vitro* developing NN were assessed at multiple time points. Transcriptomes of 5, 14 and 28 days *in vitro* CINDA-grown hNN were compared to gene expression profiles of *in vivo* human developing brains. Molecular expression analyses as well as measures of electrical activity indicate that NN mature into neurons and astrocytes over time. In contrast to rNN, hNN are less electrically active within the same period of differentiation time, yet hNN grown in CINDA medium outperform hNN without supplements. Challenge of NN with neuronal receptor stimulators and inhibitors demonstrate presence of

MoA:mode(s)-of-action(s); NN:neuronal networks; hNN:human NN; hiNPC:hiPSC-derived neural progenitor cells; CINDA:self-made neuronal differentiation medium; NDM:neural differentiation medium; rNN: rat NN

inhibitory, GABAergic neurons. HiPSC-derived GABAergic hNN grown in CINDA medium might be useful as part of an *in vitro* battery for assessing neurotoxicity.

MoA:mode(s)-of-action(s); NN:neuronal networks; hNN:human NN; hiNPC:hiPSC-derived neural progenitor cells; CINDA:self-made neuronal differentiation medium; NDM:neural differentiation medium; rNN: rat NN

KEYWORDS:

neurotoxicology, stem cell, hiPSC-NPC, MEA, neuronal network, electrical activity, transcriptome, *in vitro in vivo* comparison

1. INTRODUCTION:

For protecting human and environmental health, industrial, agricultural and consumer products must be registered and approved by the European Food Safety Authority (EFSA) or the European Chemical Agency (ECHA) before entering the market. Especially neurotoxic effects are of major scientific and socio-political concern, because they often result in irreversible adverse outcomes [Costa, 2008; Aschner, 2017]. Neurotoxicity guideline studies [OECD TG 424; US EPA OPPTS 870.6200] are currently performed *in vivo*. These are resource-intensive regarding the time and costs required [Bal-Price, 2008] and might not well reflect the human situation because of inter-species variations [Matthews, 2008; Leist, 2013]. Therefore, alternative *in vitro* testing strategies based on human cells that reduce or replace animal experiments [Russell, 1959] are of high interest for neurotoxicity research [Coecke, 2006; Zuang, 2018]. Medium- to high-throughput *in vitro* testing requires a large amount of cell material. In contrast to the use of human embryonic stem cells, which bear ethical concerns [Kao, 2008; Singh, 2015; Mayer, 2018] human induced pluripotent stem cells (hiPSC) are ideal for providing an ethically inoffensive and unlimited supply of material for *in vitro* neurotoxicological evaluations [Takahashi, 2007; Robinton, 2012; Zagoura, 2017]. Neurotoxicity is mediated by modes-of-actions (MoA) leading to disturbance of neuronal function [Masjosthusmann, 2018a]. For screening larger numbers of compounds for their neurotoxic potential, *in vitro* functional neuronal networks (NN) derived from hiPSC might be helpful tools. Neural differentiation, NN formation and establishment of functional signal transmissions for neurotoxicity assessment based on hiPSC is thus very auspicious, yet still barely studied [Odawara, 2014; Cotterill, 2016; Kasteel, 2017; Pistollato, 2017; Paavilainen, 2018; Tukker, 2018; Izsak, 2019].

One method for studying electrophysiology of neurons and NN is the microelectrode array (MEA) technology. MEAs record extracellular local field potentials at different locations of connected neurons on network-level and provide data about their activity properties and patterns [Johnstone, 2010]. The MEA technology allows assessment of NN electrical activity in real-time and evaluation of the dynamics of network behavior under chemical manipulations [Odawara, 2014; Odawara, 2016; Tukker, 2016; Tukker, 2018]. The use of MEAs in toxicological testing is relatively new and has so far been mainly applied for rat NN (rNN) [Hogberg, 2011; McConnell, 2012; Valdivia, 2014; Brown, 2016; Cotterill, 2016; Frank, 2017; Vassallo, 2017; Shafer, 2019].

In this study, we continue our previous work on neural induction of hiPSC [Hofrichter, 2017] by establishing and characterizing human NN (hNN) from hiPSC-derived neural progenitor cells (hiNPC) by comparing hNN formation with two different differentiation media: in presence (CINDA) and absence (NDM) of maturation-supporting factors. As a NN control we included differentiating rNN. Gene and protein expression and electrical activity from *in vitro* developing NN were assessed at multiple time points and in presence and absence of pharmacological

compounds. Microarrays were performed for transcriptome analyses of hNN, which were compared to *in vivo* transcriptomes of developing brains.

2. MATERIAL AND METHODS:

2.1. Compounds used

γ-Aminobutyric acid (GABA), Glutamate and Domoic acid were obtained from Sigma Aldrich (Saint Louis, USA). NBQX disodium salt, DL-AP5 sodium salt and Bicuculline were obtained from Santa Cruz Biotechnologies (Texas, USA). CytoToxOne Cytotoxicity Assay Kit was obtained from Promega Corporation (Madison, USA). For detailed information on stock solutions and solvents see Supplementary Material.

2.2. Cell culture and neural induction

The hiPSC lines A4 (Wang and Adjaye, 2011) and IMR-90 (Clone-4, WiCell,USA) were cultured in mTeSR1 medium (Stemcell Technologies,Germany) on Matrigel (BD Bioscience,Germany). Medium was changed every day and cells where passaged chemically in colonies with 0.5 mM EDTA. hiPSC lines were regularly tested for their pluripotency and their chromosomal integrity.

Neural induction of hiPSC lines was performed using the neural induction medium (NIM) protocol according to [Hofrichter, 2017]. Briefly, hiPSC colonies were cut in 200x200µm squares using a passaging tool (STEMPRO EZPassage,Thermo Fisher Scientific) and cultured on polyhema (Sigma Aldrich) coated dishes with NIM medium (for detailed medium composition see Supplementary Material) for 7 days. Generated free-floating 3D-Spheres were transferred into new polyhema dishes with NIM containing 10ng/mL bFGF (R&D Systems, Germany) for another 14 days. Afterwards they were referred to as hiNPC and cultured in polyhema dishes with neural proliferation medium (NPM, see Supplementary Material).

Primary rat NPC (rNPC) were prepared from full brains of Wistar rats on postnatal day 1 as previously described [Baumann, 2014]. 3D neurospheres were cultured free-floating and medium was changed every 2-3 days.

Proliferating NPC were expanded using a McIlwaine tissue chopper, which cuts them into small squares with a diameter of 200µm (Mickle Laboratory, UK) [Fritsche, 2011; Baumann, 2014]. For experimental use, spheres were chopped 2 days before plating.

2.3. Neuronal differentiation and Immunocytochemistry

Neuronal differentiation and immunocytochemistry were performed as described previously Hofrichter, 2017 using NDM and CINDA medium. For detailed information and medium composition see Supplementary Material.

Quantification of synapses and receptors was performed by analyzing neurite mass in μm^2 and number of synapses (synapses/neurite area [μm^2]) using the Omnisphero software as previously described [Hofrichter, 2017]. To receive brighter TUBB3 stainings structuring elements to

eliminate uneven backgrounds were adjusted as well as thresholding of the resulting images with the Otsu method was applied (for detailed information see Supplementary Material).

2.4. Quantitative reverse-transcription PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen,Germany) according to manufacturer's protocol. RNA of hiPSC (undifferentiated controls), proliferating hiNPC and rNPC (30 neurospheres of 300µm diameter each) and human and rat NN after 7, 14, 21 and 28 days *in vitro* (DIV) were prepared. For the latter, cells were chopped to 100µm aggregates and plated on PDL/laminin-coated 24-well-plates in NDM or CINDA (hiNPC) or NDM (rNPC). For reverse transcription, 500 ng RNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen,Germany). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using the QuantiFast SYBR Green PCR Kit (Qiagen,Germany) in the Rotor Gene Q Cycler (Qiagen,Germany) following manufacturer's instructions. Analysis was performed using standards of the gene of interest, allowing to calculate copy numbers and expression was normalized to β-actin [Dach, 2017]. Each experiment was performed at least three times with three independent neural inductions. For primer sequences see Supplementary Material.

2.5. NN differentiation on microelectrode arrays (MEA)

Electrical activities of NN were recorded as described in Hofrichter, 2017. Briefly, we used 200 hiNPC or rNPC neurospheres, seeded onto a PDL/laminin pre-coated single-well MEAs (Multichannelsystems (MCS)) either in NDM or CINDA medium. Recordings were performed with the MC-Rack (MCS) from 2 to 15 weeks *in vitro*. After approximately 5 min of equilibration, each recording consisted of a 5 min baseline recording of spontaneous activity. For data analyses the first min was cut off and mean values of the mean firing rate (MFR), mean bursting rate (MBR), spikes per burst and active electrodes (AE) of the last 4 min were calculated. For statistical analyses see Supplementary Material.

2.6. NN characterization with pharmaceuticals

To characterize NN regarding their receptor constitutions MEA chips were equilibrated in the MEA-headstage 2 min prior to recording. To be regarded as active, NN had to have a minimum of 3 AE. These were defined by the detection of a minimum of 5 spikes/min. For treatment analyses, a baseline measurement of active MEAs for 5 min was recorded. Afterwards, the respective receptor agonist/antagonist was added to the well and allowed to equilibrate for 5 min (wash-in-phase), followed by 5 min recording. Afterwards the MEAs were washed twice with medium (wash-out-phase) and further cultivated in fresh medium. Measurements of receptor treatment were performed twice a week starting at day 7. The recordings and data analyses were done as described in 2.5.

2.7. Domoic Acid (DA) treatment on multi-well-MEAs

MEA 24-well plates, each well containing 12 gold electrodes (MCS) were used. Wells were coated with PDL (0.1 mg/mL, 50 µL for 48 h at 4 °C; Sigma Aldrich) washed with PBS and recoated with Laminin (0.01 mg/mL, 48 h at 4 °C, L2020, Sigma Aldrich). Afterwards, 50 hiNPC or rNPC spheres (100µm diameter) were seeded into the wells and incubated either with NDM

or CINDA medium. MEAs were recorded with the Multiwell-Screen (MCS) and analyzed with Multiwell-Analyzer (MCS, for details on hardware settings and spike/burst detection parameters see Supplementary Material). Active networks were defined as stated in 2.5. Individual recordings were performed in week 2-6, in the absence (baseline) or presence of the indicated concentrations of DA in NDM or CINDA for 15 min.

2.8. Affymetrix Microarrays

For hiPSC and hiNPC (30 neurospheres of 300µm diameter each) isolation of RNA was performed according to manufacturer's protocol using the RNeasy Mini Kit (Qiagen, Germany). Therefore, cells were chopped to 100µm aggregates and plated on PDL/laminin-coated 6-well-plates. After 5, 14 and 28 days of cultivation in CINDA medium cells were harvested and RNA prepared. cDNA synthesis and biotin labeling of cRNA was performed according to the manufacturer's protocol (3´ IVT Plus Kit; Affymetrix, Inc.) and as previously described [Masjosthusmann, 2018b]. For details of analysis of Affymetrix CEL files see Supplementary Material.

2.9. Data analysis and statistics

Unless otherwise stated all statistical analyses were performed using GraphPad Prism 6.00 for Windows (GraphPad, USA). Immunocytochemical quantification and pharmaceutical data were analyzed using one-way ANOVA, qRT-PCR and DA data were analyzed using a two-way ANOVA followed by Bonferroni test to correct for multiple testing. The significance cut-off was set to $p \leq 0.05$.

3. RESULTS

3.1. Molecular characterization of hiNPC-differentiated cultures

Differentiation of hiNPC was performed with neural differentiation medium (NDM) and maturation supporting CINDA medium. Before plating, hiNPC stained positive for the markers PAX6, NESTIN and SOX2 (Fig. S1). After 28DIV hiNPC grown in NDM or CINDA medium, and rNPC differentiated into $TUBB3^+$ neurons and $GFAP^+$ astrocytes (Fig.1, 2A, S2). Human neurons express the pre- and postsynaptic proteins SYN1 and PSD95 as well as the receptor-specific proteins GABAARβ, GluR1 and NMDAR1 with no significant differences between both medium conditions (Fig.2A,B). To further characterize the networks, mRNA expression analyses were performed and gene copy numbers determined by product-specific copy number standards. This procedure enables comparison of gene expression across species and has the advantage of giving information on magnitude of absolute gene expression rather than relative gene changes as by using the ΔΔCT method. Gene expression of the NPC marker *NESTIN* was stably expressed in human cultures over time, whereas its expression significantly decreased in rat cultures starting at DIV14 (Fig.3A). The expression of the mature astrocytic marker *AQP4* was significantly upregulated on DIV28 in CINDA- compared to NDM-cultures, whereas *Aqp4* expression in rat cultures increased early starting at DIV7 (Fig.3C). Expression of the glial fibrillar astrocytic protein *gfap/GFAP* significantly increased from DIV7 in rNN but exhibited only marginal changes in human cells (Fig.3D). The neuronal marker *MAP2* increased in hNN from DIV7 and low expression values for *map2* were observed in rNN until DIV28 (Fig.3E). To

analyze the time of synapse formation we used the pre- and post-synaptic markers *SYN1* and *DLG4,* respectively, which are expressed after 7DIV in both species (Fig.3F-G). Expressions of *SLC17A7* (glutamate transporter) and *GAD1* (glutamic acid decarboxylase) differ remarkably. While *SLC17A7* copy numbers are extremely low (<1-5 copy numbers/10,000 copies *β-ACTIN*), *GAD1* is well expressed (>100 copy numbers/10,000 copies *β-ACTIN*) suggesting predominantly GABAergic neurotransmission (Fig.3H,I). Concerning neuronal suptypes, hNN increase expression of *ACHE* (acetylcholinesterase), *GRIA1* (AMPA receptor), and *TH* (tyrosine hydroxylase) over time with *TH* expression significantly induced on 14DIV in CINDA-NN compared to NDM-NN (Fig.3J), while similar to *SLC17A7*, *GRIN1* (NMDAR, Fig. 3K) and *SLC6A4 (*serotonergic neurons*,* Fig.S3*)* copy numbers are very low. In contrast, rNN revealed very low expression of *ache* and *th* (<1 copy numbers/10,000 copies *β-actin*) compared to human cells (Fig.3J,M).

3.2. Electrical activity of hiNPC-CINDA- and hiNPC-NDM-NN over time

We studied whether differentiation of neural cultures resulted in generation of functional NN. Therefore, we examined multiple electrophysiological parameters on MEA chips dual cells as well as the entire network. Human NN exhibited spontaneous electrical activities after 2 weeks in culture. Starting with the same number of MEA-Chips for both medium conditions, hNN grown in CINDA medium (CINDA-NN) produced more active chips (Fig.4+Fig.S4) and higher electrical activities in terms of mean bursting rate (MBR), spikes per bursts and the number of active electrodes (AE) than NDM-NN (Fig.4B-D). Here, in week 3 the mean value of all recorded activities (except for the mean firing rate (MFR)) was significantly higher in CINDA-NN compared to NDM-NN. NN generated from rNPC serve as positive controls [Mack, 2014; Alloisio, 2015; Wallace, 2015] and exhibited earlier and higher activity levels than hNN (Fig.4A-D). Over the entire differentiation time of 15 weeks (Fig.S4), CINDA- and NDM-NN reached highest activity levels of all measured parameters within the first 6 weeks with CINDA-NN exhibiting higher MBR, spikes/burst and number of active electrodes than NDM-NN. In contrast, rNN reached their maximum activity across all parameters measured within 7 to 11 weeks of differentiation.

3.3. Characterization of NNs by using agonists and antagonists of neuronal receptors

To study if differentiated NN contain functional GABAergic and glutamatergic neurons we treated NN with the GABA receptor (GABAR) agonist GABA or the glutamate receptor agonist glutamate (Fig.5). NN of both species responded to GABA with a reduction in MFR, MBR and number of spikes/burst (Fig.5A-C,D) suggesting presence of functional GABA receptors. Glutamate also decreased these parameters, yet to a lower extent indicating no functional excitatory glutamatergic receptors. Treatment of NN with the GABAR inhibitor bicuculline also reduced MFR and spikes/burst in NN not treated with external GABA (Fig.5A,C,E) implying a lack of glutamatergic neurons in the networks. If the NN consisted of a comparable amount of glutamatergic and GABAergic neurons, one would expect a strong increase in network activity after bicuculline exposure [Xiang, 2007; Mack, 2014] that we did not see in our cultures. In addition, the NMDAR and AMPAR antagonists, AP5 and NBQX, respectively, exhibit no effects on NN except for AP5 reducing spike frequency in the rNN that suggests possible NMDAR presence in rNN (Fig.5A).

3.4. NN response to the shellfish toxin domoic acid (DA)

To determine if the NN assay based on hiNPC differentiated in CINDA medium is a useful tool for acute neurotoxicity testing *in vitro*, we performed a proof-of-concept study by treating CINDA- as well as rNN with the shellfish toxin and glutamate analogue DA [Chandrasekaran, 2004; Watanabe, 2011; Vassallo, 2017]. DA binds to postsynaptic glutamate receptors with a 100-fold higher affinity than glutamate, causing receptor overactivation, which leads to neuronal excitotoxicity, neuronal degeneration and ultimately cell death [Watanabe, 2011; Magdalini, 2019]. For concentration-response analyses we used 24-well multiwell- (mwMEAs) instead of single-well MEAs as a medium throughput setup for compound testing. One well of a mwMEA contains 12 electrodes, compared to 59 electrodes in a single well MEA (Fig 6A). The distances between electrodes are 200 µm. Of the total of 72 and 96 wells measured with human and rat NN, respectively, 33 (45.8%) and 32 wells (44.5%) had 9-12 AE/well, 22 (30.6%) and 30 wells (31.3%) had 5-8 AE/well, 13 (18.1%) and 28 wells (29.2%) had 1-4 AE/well, and only 4 (5.6%) and 6 wells (6.3%) had no AE/well (Fig.6B) demonstrating the suitability of mwMEAs for testing purposes. Acute exposure to increasing concentrations of DA for 15min increased spontaneous activity of hNN and rNN with significant results only for the total burst count in rNN (Fig.6C) but did not cause cytotoxicity in either of the networks up to 24 hours after exposure (Fig.S5). Although not statistically quantifiable, the firing pattern visualized in representative SRPs reveals changes in activity pattern in both species after treatment with 1µM DA compared to baseline activity (Fig.6D). Additional activity parameters exhibited no changes (Fig.S5).

3.5. Microarray analyses of CINDA-NN compared to published *in vivo* data

To monitor differentiation and maturation processes of hNN on the transcriptome level, we analyzed mRNA expression profiles of hiPSC, proliferating hiNPC, and hiNPC-derived NN differentiated for 5, 14, and 28DIV using Human PrimeViewArrays from Affymetrix. Microarray data was validated via qRT-PCR analyses for representative genes (Fig.S6). Only genes that were at least 2-fold significantly regulated ($p \leq 0.01$) were used for subsequent analysis. In differentiating hiNPC a total of 2619 genes are differentially expressed (DEX) across the three time points (Fig.7C). The number of DEX genes increased over differentiation time, from 1370 (DIV5) to 1793 (DIV14) and to 2368 (DIV28). Moreover, the overlap of all conditions was 43,3% (1139 genes), indicating that these genes are generally important for differentiation. Gene ontology (GO) analyses reveal gene changes in neurodevelopmental GO terms including neural proliferation, astrocytic-, general glia cell- and neuronal differentiation, synapse formation and synapse plasticity (Appendix II). These data support the *in vitro* development of hNN consisting of neurons and astroglia cells, forming synapses into a NN. Cluster- and PCA analyses support the strong differentiation potential of hiPSC (turquoise) into proliferating hiNPC (green) and further differentiating hiNPC at DIV5 (blue), DIV14 (red) and DIV28 (purple) with very small replicate variations (Fig.7A). To determine to which extent the *in vitro* gene changes are represented in developing human brains *in vivo* and vice versa, *in vitro* DEX genes (0vs28DIV) were compared to published DEX genes of *in vivo* transcription profiles of the prefrontal cortex between post-conceptional week 6 (embryonic) and 12 (fetal; [Kang, 2011]). These timepoints were chosen based on a recent publication mapping developing brain organoids to *in vivo* fetal brain samples [Amiri, 2018].The comparison of *in vivo* vs. *in vitro* DEX genes revealed that from more than 2837 *in vivo* DEX genes, 868 (31% and 37%, respectively) are commonly regulated (Fig.7D). DEX gene groups over-represented in these data sets were identified by gene set enrichment analyses (Appendix I). DEX genes within a selection of manually selected GO-terms

including the neural/neuronal specific terms, i.e. neural proliferation (NP), astrocytic-, general glia cell- and neuronal differentiation, synapse formation/plasticity, glutamatergic neurons, GABAergic neurons, and radial glia (RG) differentiation (Appendix II) was compared between the *in vitro* and *in vivo* data sets. These data show that *in vivo* more GO-terms are enriched, more genes are generally regulated in each GO term and respective genes are mostly stronger regulated *in vivo* than *in vitro,* yet transcriptomes of the *in vitro* cultures reveal well-defined cellular differentiation processes (Fig.7E, see Appendix II).

4. DISCUSSION:

Human hazard and risk assessment of potentially neurotoxic chemicals is based on *in vivo* animal experiments [Bal-Price, 2008]. Those experiments are time and cost intensive and might not well reflect the human situation because of inter-species variations [Leist, 2013]. Thus, alternative *in vitro* testing strategies using human-based material are of special interest for neurotoxicity testing [Coecke, 2006; Zuang, 2017]. We previously established the neural induction of hiPSC to hiNPC, cultured as 3D neurospheres [Hofrichter, 2017] and now continued the work by characterizing differentiating hiNPC for their NN formation. We assembled the CINDA medium by adding creatine monohydrate, interferon-γ, neurotrophin-3, dibutyryl-cAMP and ascorbic acid to NDM, which supports (i) synapse formation, (ii) maturation of different neuronal subtypes and (iii) spontaneous NN activity. Human cultures differentiating over a time course of 28DIV into neurons and astrocytes express *NESTIN* during the whole differentiation time. Although it is down-regulated and replaced by neurofilaments and *GFAP* during neuro- or gliogenesis *in viv*o [Michalczyk, 2005], as well as in the differentiating rat cultures, others reported earlier that neurally differentiated hiNPC do not down-regulate *NESTIN* over time [Pistollato, 2014; Zagoura, 2017]. In human stem cell-derived culture neurogenesis occurs before astrogenesis [Grandjean, 2006; Nat, 2007; Liu, 2011; Yuan, 2011]. Thus, the neuronal marker *MAP2* already plateaus after 7DIV with no differences between CINDA medium and NDM, while it takes until 21DIV for the astrocyte marker *AQP4* to reach maximum expression. CINDA-NN seem to favor astrocyte differentiation compared to NDM-NN as *AQP4* is significantly higher expressed in CINDA-NN compared to NDM-NN. In contrast, *gfap* expression already levels after 7DIV in rNN followed by a continuous up-regulation of *map2* over the 28DIV. RNN are not produced from iPSC-derived NPC, but are primary NPC prepared from rat PND1 pups. These resemble a later developmental time that probably explains their different cell behavior [Baumann, 2014, Baumann, 2016, Masjosthusmann, 2018a]. Compared to the rat, human NDM-NN express 25-100-fold less *GFAP* and CINDA-NN hardly any *GFAP* on mRNA level, while the immunostainings suggest presence of GFAP protein. One reason for this could be that astrocytes in hNN are protoplasmic, which express far less GFAP than fibrous astrocytes [Molofsky, 2012; Molofsky, 2015]. The significantly stronger *gfap* expression in rNN is likely due to presence of radial glia in these cultures, which is suggested by their morphology. Molecular analyses reveal the presence of pre- (*SYN1*) and post-synaptic (*DLG4*/PSD95) structures as well as glutamate NMDA and AMPA receptors and the GABAA receptor in developing NN with no differences between NDM and CINDA medium. Despite receptor expression, glutamatergic neurons seem to be absent in the cultures and neurons mainly seem to be of GABAergic nature with presence of dopaminergic and cholinergic neurons. Here, only *TH* expression was significantly higher expressed in CINDA-NN on DIV14 compared to NDM-NN. Dopaminergic differentiation might be promoted by cAMP in the CINDA medium [Belinsky, 2013].

Only few groups have been analyzing electrical activity of hiPSC-derived neural cultures on MEAs [Toivonen, 2013; Odawara, 2014; Odawara, 2016; Tukker, 2016; Hofrichter, 2017; Seidel, 2017; Tukker, 2018; Izsak, 2019; Shimba, 2019]. Some studied spontaneous firing in hiPSC-derived pure neuronal cultures [Toivonen, 2013; Tukker, 2016; Tukker, 2018] or in co-cultures with rat astrocytes [Odawara, 2014; Seidel, 2017]. Astrocytes contribute to neuronal signal transmission and maturation and should therefore be advantageous in *in vitro* NN [Clarke, 2013]. However, instead of rat astrocytes, the use of human astrocytes in neuronal co-culture is preferred [Oberheim, 2006; Tjarnlund-Wolf, 2014]. The differentiation protocol used in this study produces hiPSC-derived neurons and astrocytes simultaneously. NN differentiated in medium with CINDA and NDM suggest a promotion of astrocyte maturation and overall electrical network activity with a higher number of AE, MBR and spikes/burst when cells were grown in CINDA medium. Bursting activity is associated with enhanced synapse formation and long-term potentiation of neuronal connections [Maeda, 1995; Lisman, 1997] indicating a certain degree of network maturity. RNN were more active than hNN during week 2-6, possibly due to a faster development of this species [Semple, 2013].

To test for the functionality of neuronal receptors, we treated CINDA- and rNN with GABA or glutamate receptor agonists and antagonists. Network data indicates presence of functionally active GABAR that respond to GABA with NN inhibition in both species. In contrast, glutamate receptors seem to be non-functional, as the excitatory neurotransmitter glutamate did not increase spike frequency. The small decrease in MFR upon glutamate treatment might be owed to astrocyte glutamate metabolism [Gegelashvili, 1998] to glutamine that is then used by GABAergic neurons to synthesize GABA in absence of glutamatergic neurons [Lujan, 2005; Walls, 2015]. That glutamatergic neurons are missing in the NN becomes obvious upon bicucculline treatment: GABAR inhibition by bicucculline is not increasing the NN firing, which is expected in presence of active glutamatergic neurons [Fukushima, 2016; Odawara, 2014; Odawara, 2016]. Very low mRNA expression of *SLC17A7* (vGLUT1) reinforces these functional findings. Supporting differentiation of NPC into glutamatergic neurons might be achieved by addition of the neurotrophic factors BDNF and GDNF [Izsak, 2019] or retinoic acid [Zhang, 2013] to the CINDA medium. This will be subject of further studies. Antagonization of the glutamate receptors NMDA and AMPA with AP5 and NBQX, respectively, cause only small changes in hNN activity levels. Presence of their gene products *GRIN1* and *GRIA1* as well as their receptor proteins in combination with the functional data is indicative of not fully matured glutamate receptors. Similar results were reported from Tang, 2013. Plating hiPSC-derived NPCs on laminin for patch-clamp analyses revealed much larger GABA receptor currents than glutamate receptor currents with NMDA receptor currents not appearing after 60DIV. This was similar to previous findings on rat embryonic neurons [Deng, 2007] making the authors suggest an evolutionarily conserved role of GABA during early neural development [Tang, 2013]. However, rNN seem to possess some functional NMDAR, as AP5 strongly decreases network activity.
It has been proposed that iPSC-derived cell models might be suitable alternative models for future *in vitro* toxicological testings [Jennings, 2015; Suter-Dick, 2015]. We adapted the NN to a 24-well plate MEA testing format that allows higher throughput testing than single well MEAs. Our data suggests that stability and reproducibility of the multi-well MEAs is higher compared to single well MEAs because the number of active chips and the percentage of AE is higher in multi-well compared to single well MEAs. Here we used the shellfish-toxin DA, which is known to hyperstimulate excitatory glutamatergic neurons and leads to cell death [Chandrasekaran, 2004;

Watanabe, 2011]. SRP of both species indicate pattern changes after acute DA exposure, yet only rNN display a significant alteration of the total burst count at 1µM. NN characterization suggests an immature hNN glutamate receptor system, while rNN seem to possess at least some functional NMDAR that probably mediate the DA effect on the burst count. In previous studies rNN from cortical cultures responded to an acute treatment of 500nM DA with a decrease of network activity parameters [Hogberg, 2011; Wallace, 2015; Vassallo, 2017], suggesting cytotoxicity of neurons due to hyperstimulation. The higher sensitivity of these cultures is probably due to a higher maturity than our rNN that were differentiated from NPC. So far no data on DA treatment of hNN *in vitro* exists. RNN grown on MEAs are well suited for screening large amounts of neurotoxic compounds has recently been impressively shown. This is especially due to their low inter-experimental variability [Frank, 2017; Shafer, 2019].

To compare the hNN *in vitro* system to developing brains *in vivo*, we performed transcriptome analyses of hiPSC, hiNPC and further differentiated NN at DIV5, 14 and 28. Different *in vitro* differentiation stages clearly separate in the PCA analyses showing hiPSC differentiation potential on a global gene expression level. Similar results were e.g. published earlier for generating GMP grade hiNPC for therapeutic purposes [Rosati, 2018] as well as for hiPSC-derived brain organoids [Amiri, 2018]. The *in vitro-in vivo* comparison relates DEX genes between 0 and 28DIV with DEX genes between 6 and 12 weeks post conception from the previously published data of *in vivo* prefrontal cortex samples [Kang, 2011]. Of the 2837 DEX genes during early brain development *in vivo*, 868 are also regulated *in vitro* indicating that as expected only a fraction of the neurodevelopmental processes that happen in the intact organ also take place *in vitro*.

Yet, enriched GO-terms of the *in vitro* cultures reveal presence of well-defined cellular differentiation processes. However, it is striking that for the GABAergic GO-terms, no genes are enriched for the human *in vitro* system despite presence and functionality of GABAR. This might for some genes, like *GAD1*, be due to the lower sensitivity of microarrays compared to qRT-PCR [Draghici, 2006]. In addition, genes related to GABAergic signaling are not necessarily annotated in the GABAergic GO-terms; i.e., the *GABRB3* gene that encodes via alternative splicing for a multitude of $GABA_A$ receptor subunit isoforms, is enriched in synapse-related GO-terms

5. SUMMARY AND CONCLUSION

NN from hiNPC cultured as neurospheres consist of a co-culture of neurons and astrocytes. Synapse-specific proteins, neuronal subtype-specific receptors and enzymes are expressed and respective proteins are present. While GABAR are shown to be functional, glutamate related receptors seem to lack presence or maturity. In contrast, at least the NMDAR seems to be functional in rNN that are also responsive to GABA. The neuronal maturation medium CINDA resulted in higher NN activity levels compared to NDM. Formation of NN in this work relied on spontaneous, self-organized hiNPC differentiation, maturation and synapse formation and is therefore highly variable. This is a critical hurdle for pharmacological or toxicological applications. Use of multi-well MEAs compared to single well MEAs seems to improve variability of MEA measurements, but more optimizing work is needed before this system is ready for testing application. Yet in the future GABAergic hNN grown in CINDA medium might be useful as part of an *in vitro* battery for assessing neurotoxicity.

6. ACKNOWLEDGEMENTS

This work was supported by the project CERST (Center for Alternatives to Animal Testing) of the Ministry for innovation, science and research of the State of North-Rhine Westphalia, Germany [Aktenzeichen 233-1.08.03.03-121972].

7. DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

None.

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9. Figure legends:

Fig. 1. Experimental set up. hiNPC were neurally induced from hiPSC or generated from rat brain (PND1) and cultivated as floating neurospheres. qRT-PCR analyses and MEA recordings were performed weekly from day 0 until 15 weeks *in vitro* (WIV). Cells were fixed for immunocytochemistry stainings after 28 days of differentiation. For MEA plating spheres were cut into 0,1mm aggregates and 200 were seeded on PDL/laminin pre-coated MEA recording fields.

Fig.2. Immunocytochemical stainings of hNN. A) Representative pictures of hNN differentiated with NDM or CINDA medium for 28 days: neurons (TUBB3; red), astrocytes (GFAP; green), pre- (SYN1) and postsynapses (PSD95; green dots), GABA receptor (GABAARβ), glutamate receptor (GluR1) and NMDA receptor (NMDAR1; green dots). Nuclei were stained with Hoechst. Scale bars=100 µm. **B**) Shown are quantifications of synapses/receptors (mean+SEM) of the ratio to total neurite area (n=3-6).

Fig.3. mRNA expression profiles of hiPSC, hiNPC and hNN compared to rNPC and rNN. qRT-PCR analysis of different cell types for neural progenitor (*NESTIN*), astrocyte (*MAP2, S100β, AQP4, GFAP*), synaptic (*SYN1, DLG4*) and neuronal subtype specific markers (*SLC17A7, ACHE, GRIA1, GRIN1, GAD1, TH*) differentiated for 28 days *in vitro* (DIV) in NDM or CINDA medium. RNN serve as positive control. Data are presented as mean+SEM. *****=significant to NDM, **#**=significant to NPC ($n = 3$, $p < 0.05$).

Fig.4. Spontaneous electrical activity of hNN- and rNN on MEA. Shown are scatter plots of hNN (NDM/CINDA) and rNN differentiated for six weeks on MEAs. Plotted are the **A**) mean firing rate (MFR), **B**) mean bursting rate (MBR), **C**) spikes per burst and **D**) active electrodes, one dot represents the mean of one chip. Both hiNPC: n=40 and rat: n=43 at start of experiment. Black lines represent the mean of all data points. *****=significant to NDM, **#**=significant within condition ($p < 0.05$).

Fig.5. Modifications of electrical activities by acute pharmacological treatments. Analyzed were the ratio of treatment/control of CINDA-NN and rNN treated with GABA [1mM], bicuculline [10µM], glutamate [100µM], AP5 [20µM] and NBQX [10µM]. Results are depicted as **A**) MFR, **B**) MBR and **C**) Spikes/Burst. hiNPC: n=7-10, rat: n= 6-10. **D**) Representative 60sec spike raster plots (SRP) of hNN and rNN of baseline- and GABA and **E**) bicuculline treatment.

Fig.6. Acute treatment of NN in mwMEAs with the shellfish toxin domoic acid (DA). A) Representative phase-contrast images of hiNN on a singlewell- and multiwell-MEA recording field. **B**) Pie-diagram of the total wells of hiNN (blue, 72 wells) and rNN (red, 96 wells) indicating the colour-coded numbers of active electrodes per well. **C**) Acute treatment of hiNPC and rNN with DA. Mean+SEM of the spike rate [Hz] and the burst count (number of total bursts) are plotted in percent of solvent control for the acute exposure time of 15min, *****=significant to control ($n = 3$, $p < 0.05$). **D**) Representative 15min SRP of 1µM DA of rNN and hiNN.

Fig.7. Transcriptome analyses of hiPSC, hiNPC and hNN and overrepresentation analyses (ORA) of gene ontology (GO) terms compared to *in vivo* data. A) Cluster analysis of hiPSC, hiNPC and hNN. Turquoise=hiPSCs; green=hiNPC; blue=hiNN (DIV5); red=hiNN (DIV14); violet=hiNN (DIV28), 4 replicates each. In the heatmap blue genes are down-regulated, red upregulated. The intensity of the colors serves as a measure of the strength of the regulation ($P \leq 0.05$). **B**) Principal component analysis (PCA) of A. **C**) Venn-diagram of regulated genes. Plotted are all genes that are at least 2-fold up or down regulated between the following treatments: red=0vs5DIV, blue=0vs14DIV and green=0vs28DIV ($P \leq 0.05$). **D**) Venn-diagram of regulated genes comparing *in vitro* to *in vivo*. Plotted are all genes that are at least 2-fold up or down regulated between the following conditions: violet: *in vitro* hiNPC (0vs28DIV) and gray: *in vivo* samples of human prefrontal cortex week 6vs12 [Kang, 2011]. **E**) Comparative analyses of

genes (at least regulated 2-fold, D) of respective GO-terms, which code for neural and synaptic markers *in vitro* (violet, 0vs28DIV) and *in vivo (*grey, prefrontal cortex, week 6vs12, [Kang, 2011]).

Figure 01:

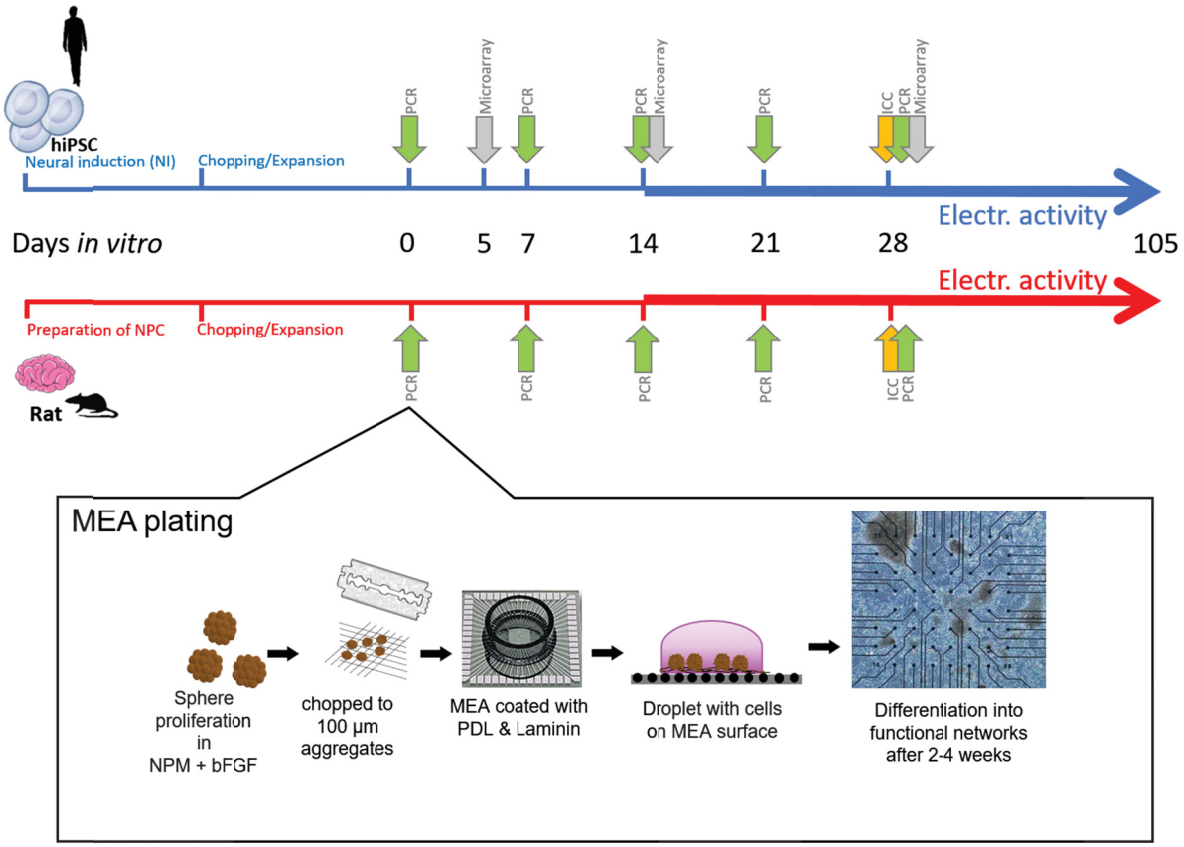
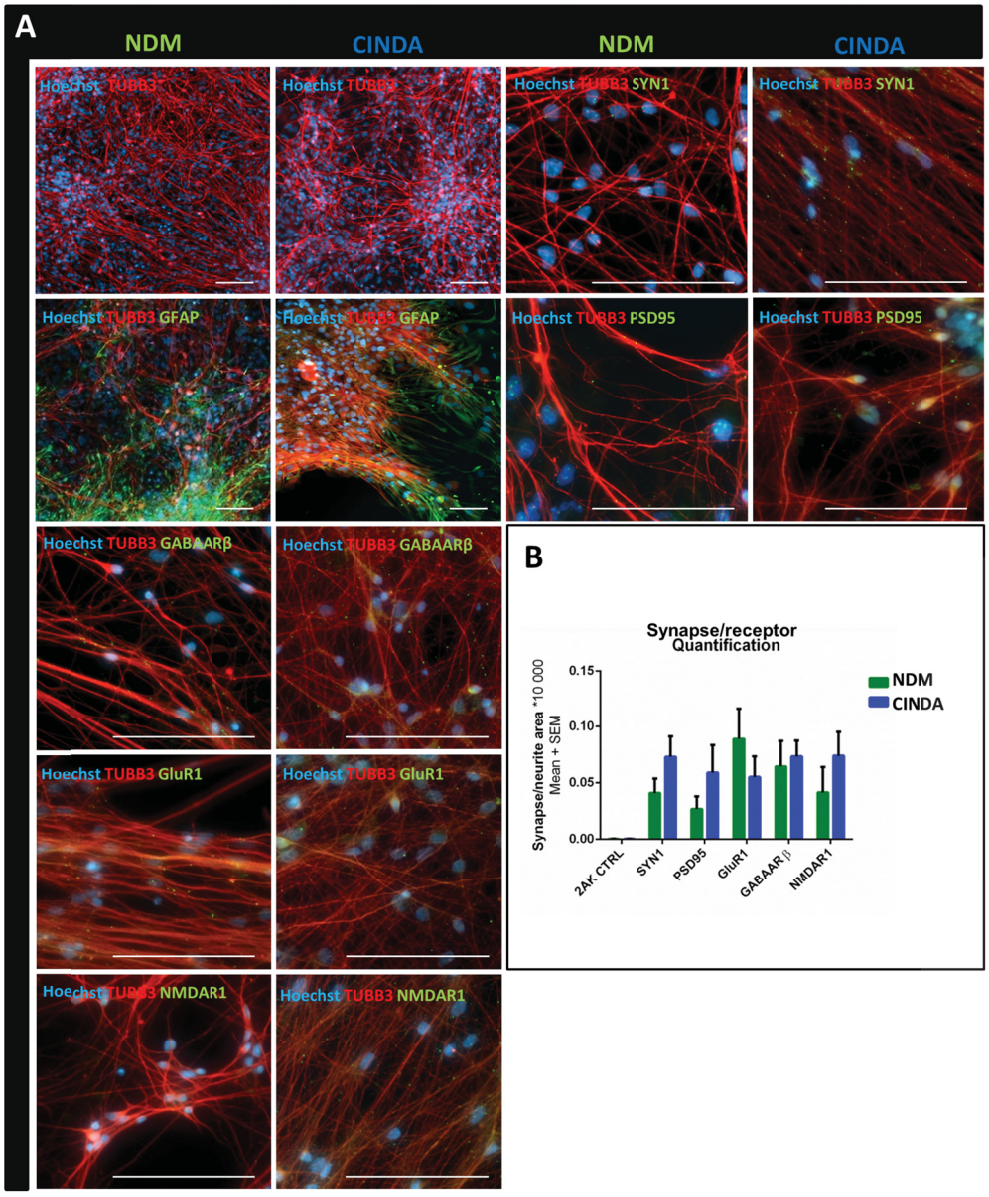

hiPSC
Neural induction (NI)
Chopping/Expansion
PCR
Microarray
PCR
PCR
Microarray
PCR
ICC
PCR
Microarray
Electr. activity
Days in vitro
0
5
7
14
21
28
105
Electr. activity
Preparation of NPC
Chopping/Expansion
Rat
PCR
PCR
PCR
PCR
ICC
PCR
MEA plating
Sphere proliferation in NPM + bFGF
chopped to 100 µm aggregates
MEA coated with PDL & Laminin
Droplet with cells on MEA surface
Differentiation into functional networks after 2-4 weeks

Figure 02:


A
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NDM
CINDA
Hoechst TUBB3
Hoechst TUBB3
Hoechst TUBB3 SYN1
Hoechst TUBB3 SYN1
Hoechst TUBB3 GFAP
Hoechst TUBB3 GFAP
Hoechst TUBB3 PSD95
Hoechst TUBB3 PSD95
Hoechst TUBB3 GABAARβ
Hoechst TUBB3 GABAARβ
Hoechst TUBB3 GluR1
Hoechst TUBB3 GluR1
Hoechst TUBB3 NMDAR1
Hoechst TUBB3 NMDAR1
B
Synapse/receptor Quantification
Synapse/neurite area *10 000 Mean + SEM
0.15
0.10
0.05
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NDM
CINDA
2AK CTRL
SYN1
PSD95
GluR1
GABAARβ
NMDAR1

Figure 03:

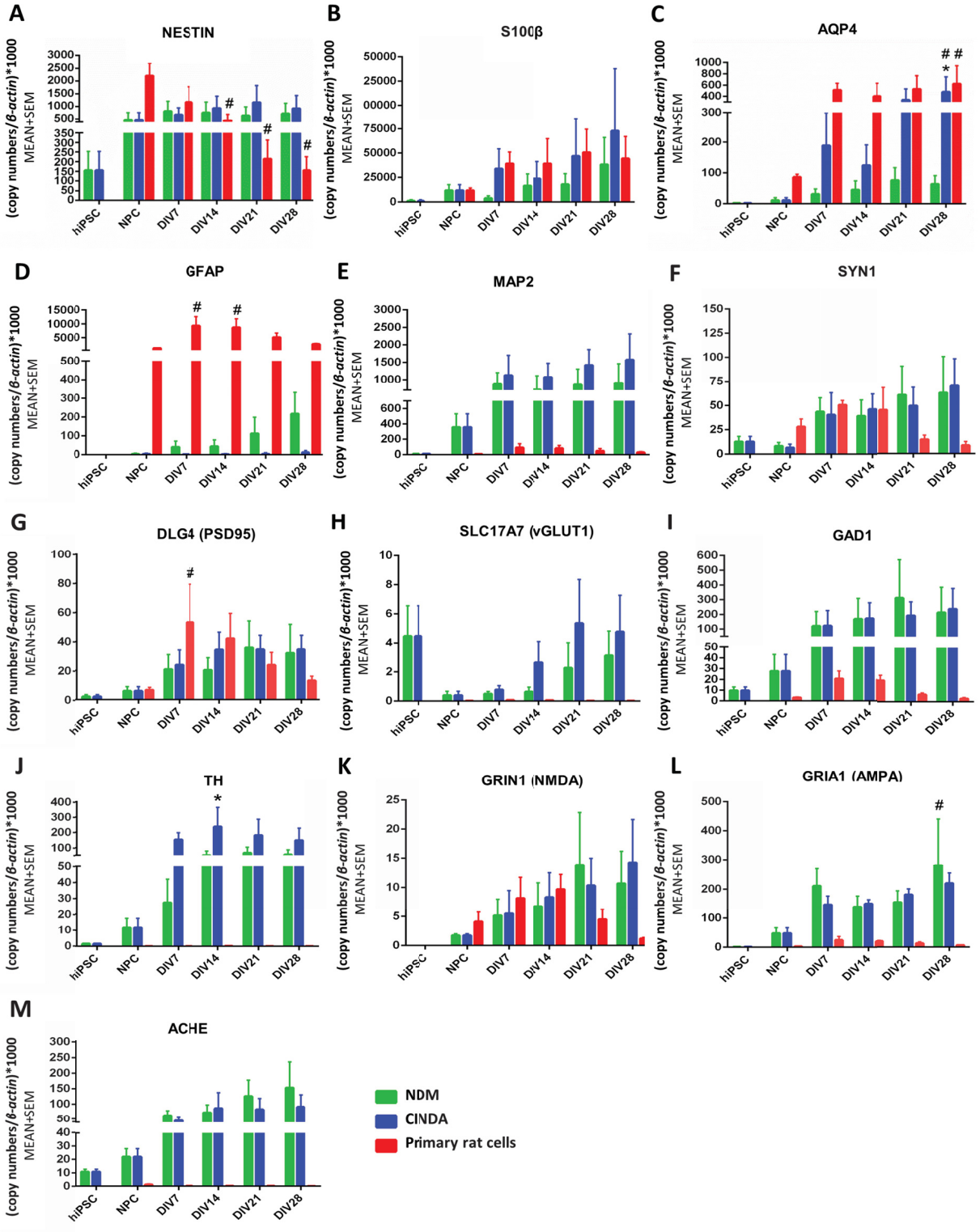
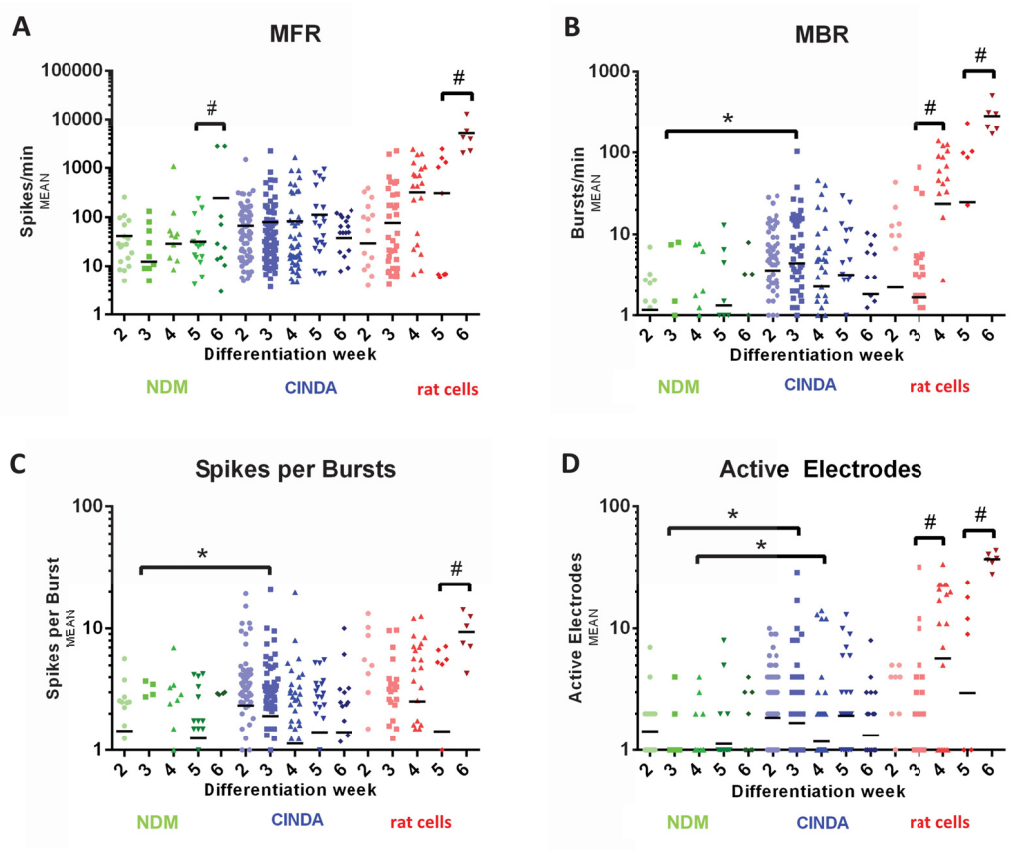

A
NESTIN
B
S100β
C
AQP4
D
GFAP
E
MAP2
F
SYN1
G
DLG4 (PSD95)
H
SLC17A7 (vGLUT1)
I
GAD1
J
TH
K
GRIN1 (NMDA)
L
GRIA1 (AMPA)
M
ACHE
(copy numbers/β-actin)*1000
MEAN+SEM
hiPSC
NPC
DIV7
DIV14
DIV21
DIV28
NDM
CINDA
Primary rat cells

Figure 04:


A
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Spikes/min
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Differentiation week
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Differentiation week
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Spikes per Burst
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Differentiation week
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Differentiation week
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Figure 05:

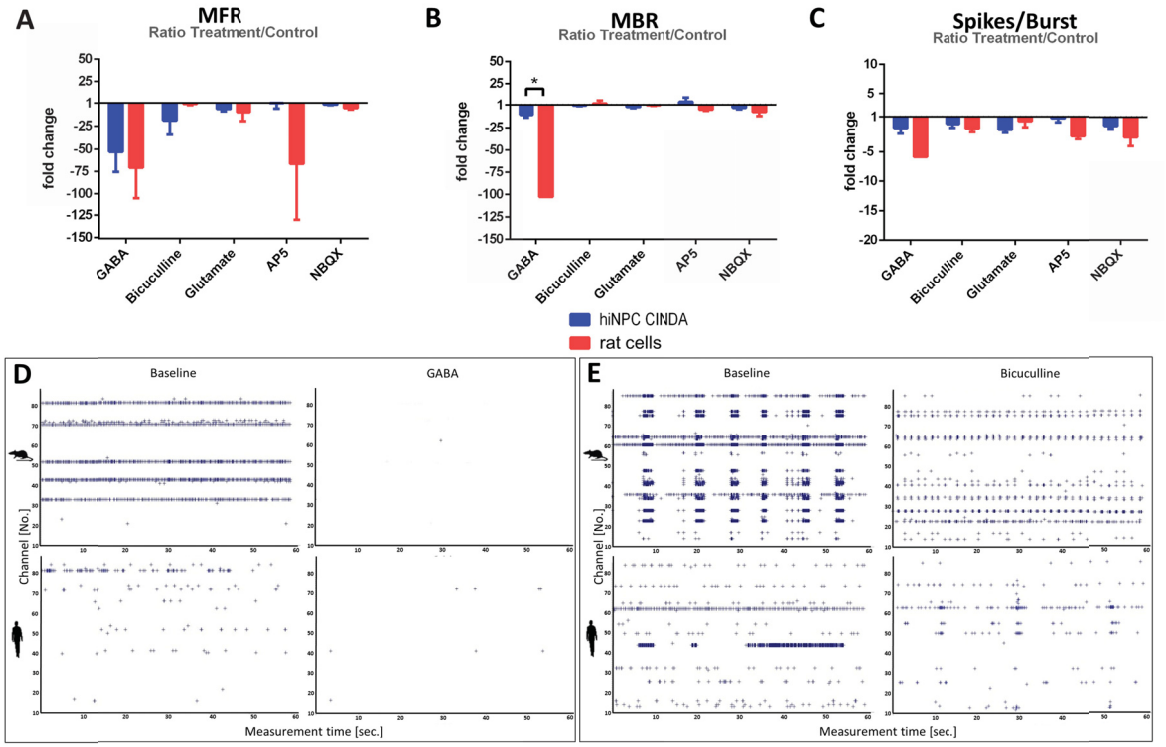

A
MFR
Ratio Treatment/Control
fold change
50
25
1
-25
-50
-75
-100
-125
-150
GABA
Bicuculline
Glutamate
AP5
NBQX
B
MBR
Ratio Treatment/Control
*
C
Spikes/Burst
Ratio Treatment/Control
10
5
1
-5
-10
-15
-20
hiNPC CINDA
rat cells
D
Baseline
GABA
Channel [No.]
Measurement time [sec.]
E
Baseline
Bicuculline

Figure 06:

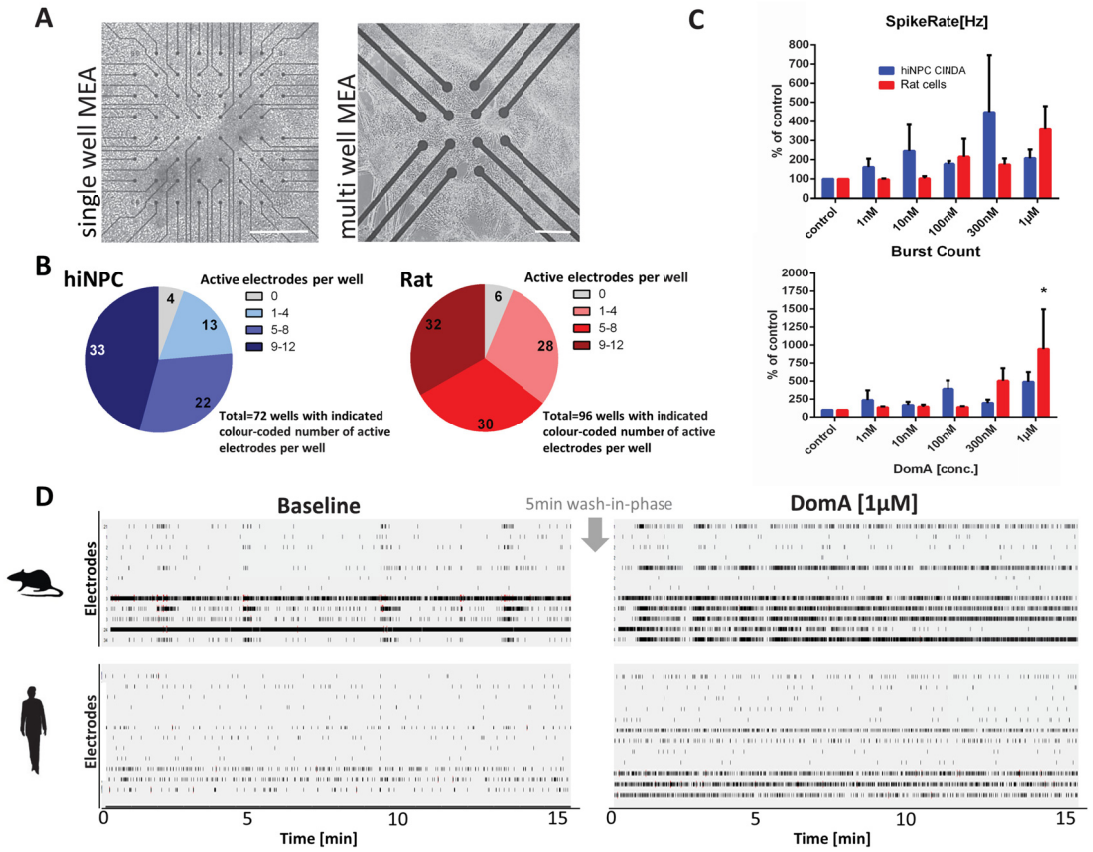
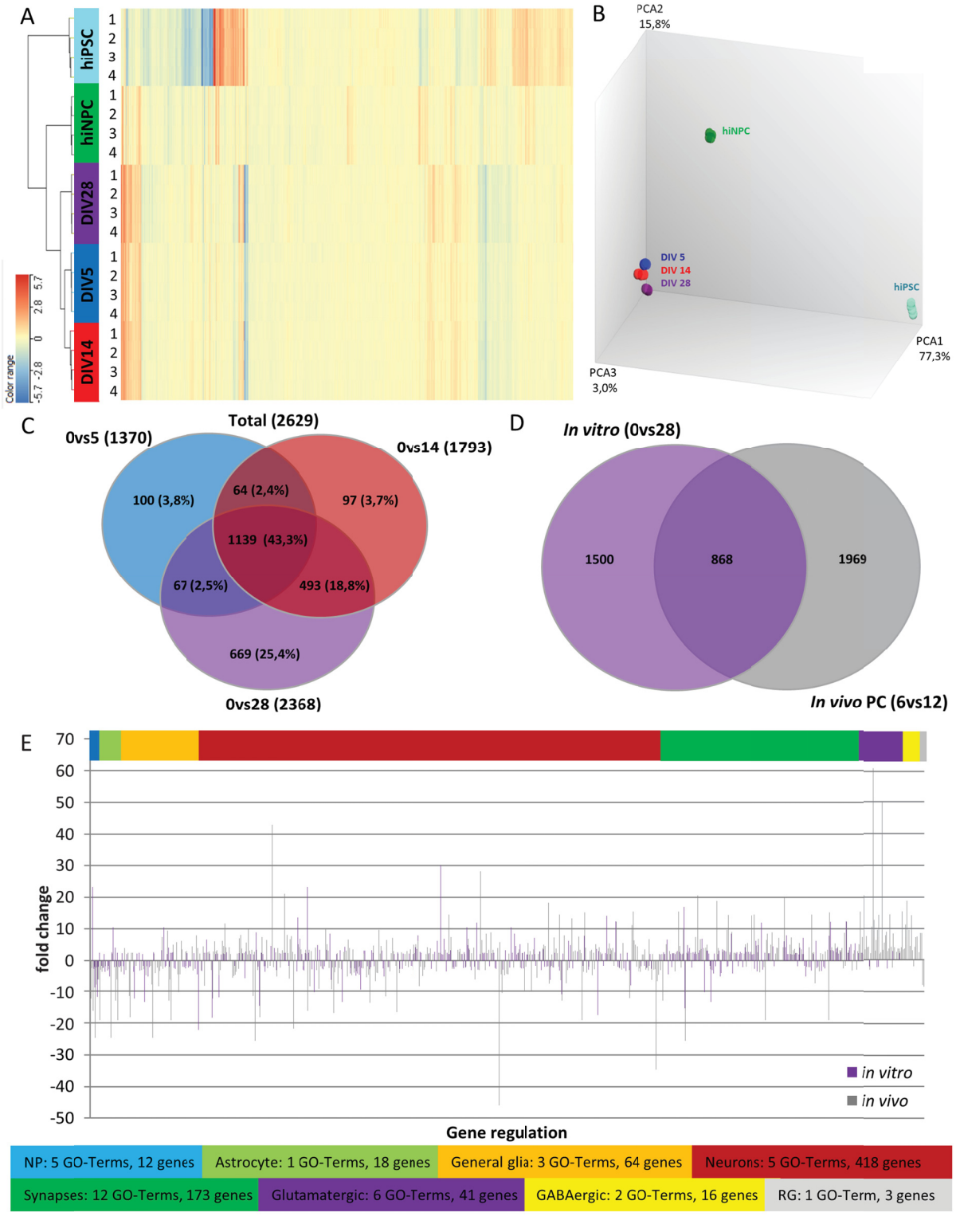
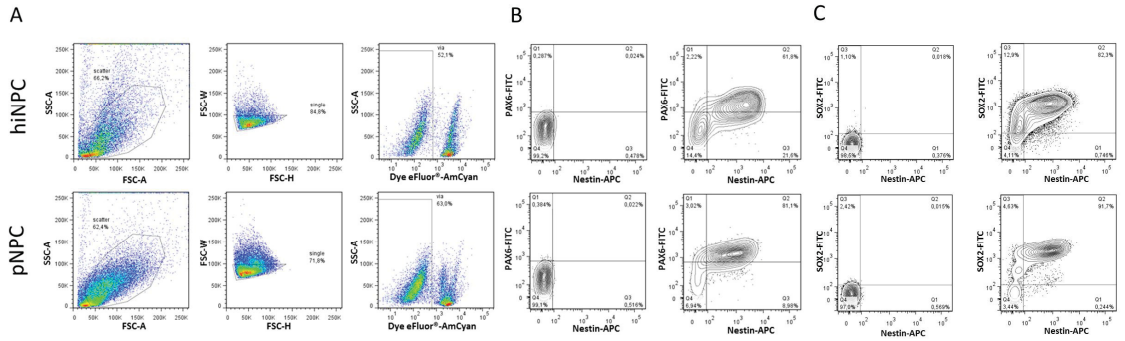

A
single well MEA
multi well MEA
B
hiNPC
Active electrodes per well
0
1-4
5-8
9-12
4
13
33
22
Total=72 wells with indicated colour-coded number of active electrodes per well
Rat
Active electrodes per well
0
1-4
5-8
9-12
6
32
28
30
Total=96 wells with indicated colour-coded number of active electrodes per well
C
SpikeRate[Hz]
hiNPC CINDA
Rat cells
% of control
control
1nM
10nM
100nM
300nM
1µM
Burst Count
% of control
DomA [conc.]
D
Baseline
5min wash-in-phase
DomA [1µM]
Electrodes
Electrodes
Time [min]
Time [min]

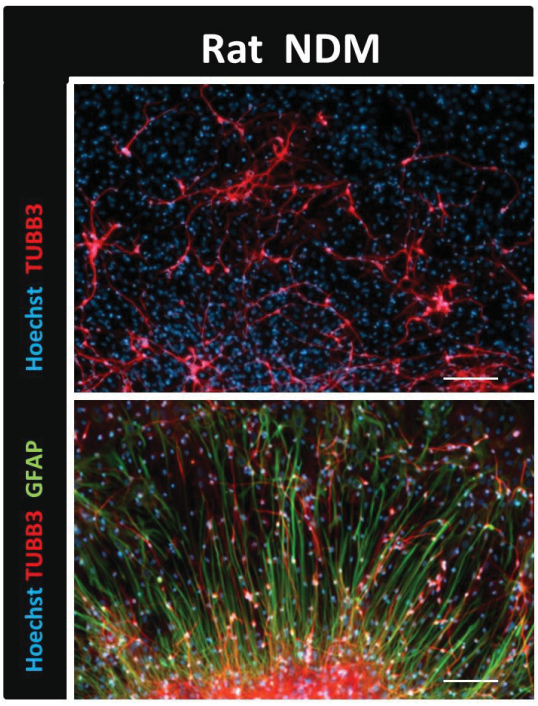
Figure 07:


A
hiPSC
hiNPC
DIV28
DIV5
DIV14
Color range
5.7
2.8
0
-2.8
-5.7
B
PCA2
15,8%
hiNPC
DIV 5
DIV 14
DIV 28
hiPSC
PCA1
77,3%
PCA3
3,0%
C
Total (2629)
0vs5 (1370)
0vs14 (1793)
0vs28 (2368)
100 (3,8%)
64 (2,4%)
97 (3,7%)
1139 (43,3%)
67 (2,5%)
493 (18,8%)
669 (25,4%)
D
In vitro (0vs28)
In vivo PC (6vs12)
1500
868
1969
E
fold change
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
in vitro
in vivo
Gene regulation
NP: 5 GO-Terms, 12 genes
Astrocyte: 1 GO-Terms, 18 genes
General glia: 3 GO-Terms, 64 genes
Neurons: 5 GO-Terms, 418 genes
Synapses: 12 GO-Terms, 173 genes
Glutamatergic: 6 GO-Terms, 41 genes
GABAergic: 2 GO-Terms, 16 genes
RG: 1 GO-Term, 3 genes

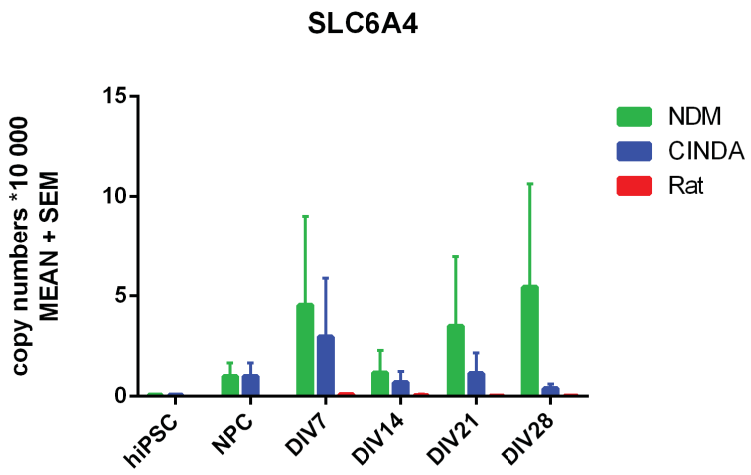
Supplementary Figure 01:



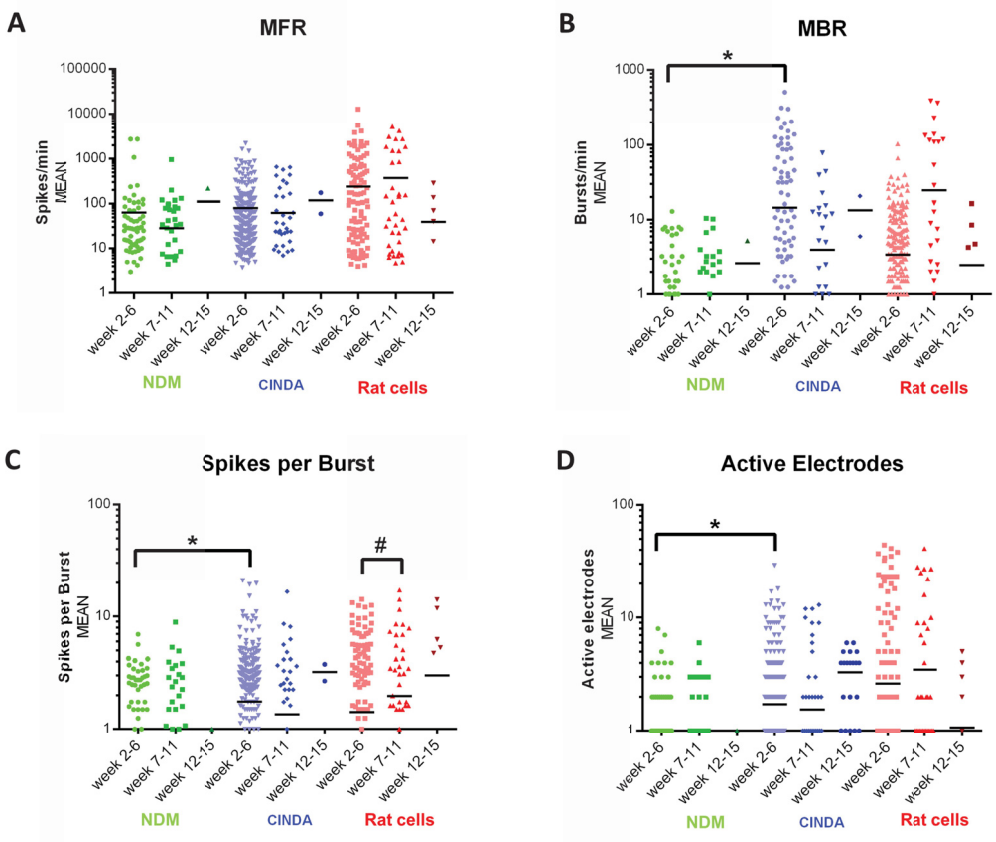
Supplementary Figure 02:



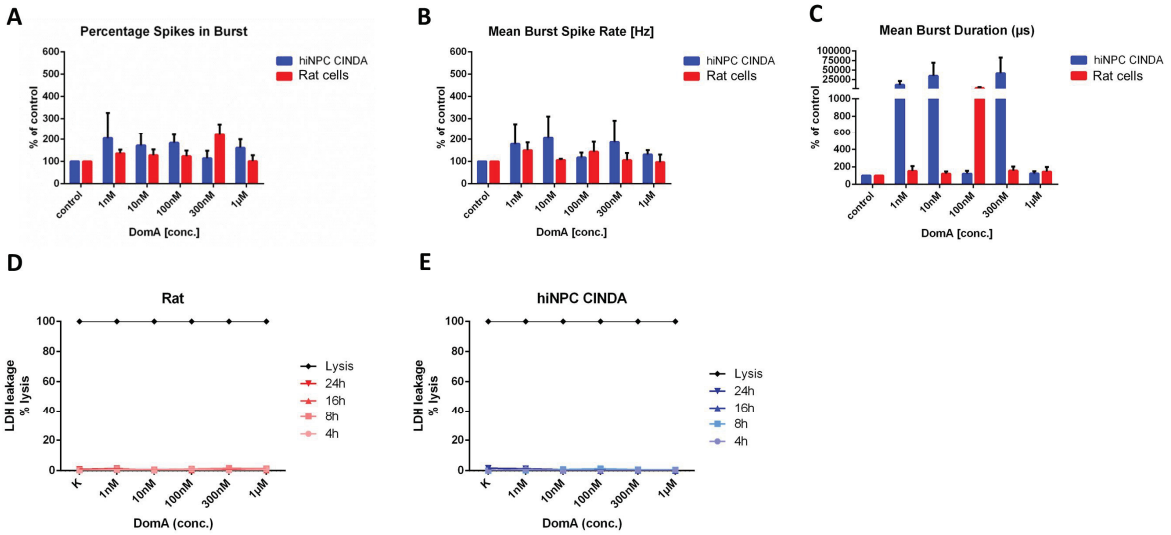
Supplementary Figure 03:



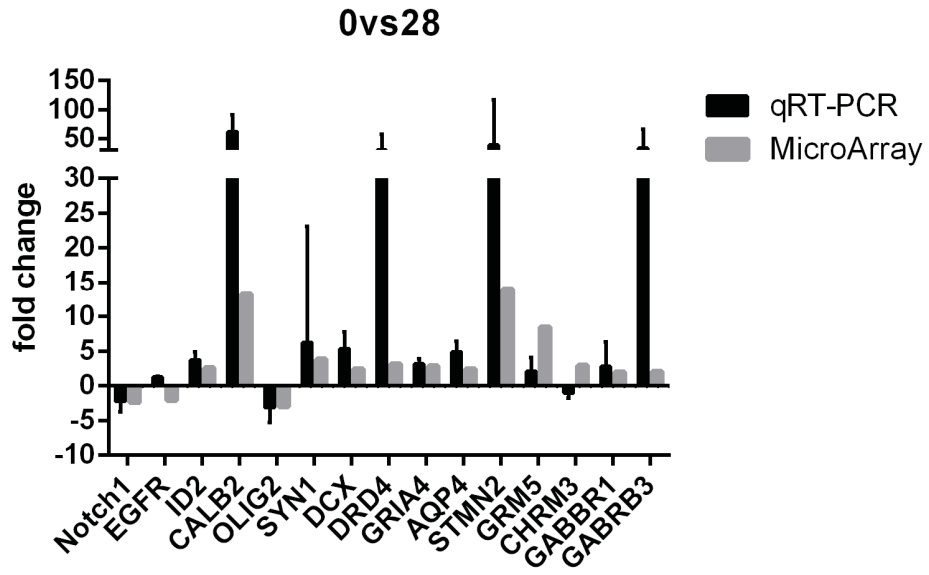
Supplementary Figure 04:



Supplementary Figure 05:



Supplementary Figure 06:



SUPPLEMENTARY MATERIAL

1. MATERIAL AND METHODS:

1.1. Compounds used

γ-Aminobutyric acid (GABA,1mM, A5835), Glutamate (100µM, 49621) and Domoic acid (DA, D6152) were obtained from Sigma Aldrich (Saint Louis, USA). NBQX disodium salt (10µM, sc-222048), DL-AP5 sodium salt (20µM, sc-361139) and Bicuculline (10µM, sc-202498) were obtained from Santa Cruz Biotechnologies (Texas, USA). CytoToxOne Cytotoxicity Assay Kit (G7891) was obtained from Promega Corporation (Madison, USA). Stock solutions of each compound were prepared in the following solvents: deionized water (NBQX, AP5, Glutamic acid, GABA, DA), DMSO (Bicuculline). Stock solutions were diluted 1:1000 to gain a final concentration of the respective solvents of 0,1% vol/vol.

1.2. Cell culture and neural induction

1.2.1. Neural induction medium (NIM):

- DMEM and Ham's F12 (3:1 ratio, Gibco, Invitrogen)
- 20 ng/mL human recombinant epidermal growth factor (EGF, Gibco, Invitrogen)
- 1:50 B27 supplement (50x Stock, Gibco, Invitrogen)
- 1:100 Penicillin/Streptomycin (100x Stock, PAN-Biotech)
- 20% knockout serum replacement (KSR, Thermo Fisher Scientific)
- 10-20µM SB431542 (Merck, Germany)
- 0,5-1µM LDN-193189 (Sigma Aldrich, Germany).

1.2.2. Neural proliferation medium (NPM):

- DMEM and Ham's F12 (3:1 ratio, Gibco, Invitrogen)
- 20 ng/mL human recombinant epidermal growth factor (EGF, Gibco, Invitrogen)
- 1:50 B27 supplement (50x Stock, Gibco, Invitrogen)
- 1:100 Penicillin/Streptomycin (100x Stock, PAN-Biotech)
- for hiNPC: human recombinant FGF (20 ng/mL, R&D systems)
- for rat NPC: rat recombinant FGF (10 ng/ml, R&D systems)

1.3. Cultivation of primary human NPC

Human neural progenitor cells [hNPC, male, gestational week (GW) 16–18] isolated from whole brain were purchased from Lonza Verviers SPRL (CAT. #PT-2599; LOT NO.:0000277385; Verviers, Belgium). HNPC were cultured as described previously [Baumann, 2014]. Briefly, neurospheres were cultivated in neural proliferation medium (NPM, see 3.2.2) at 37°C with 5% CO_2. 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 tissue chopper (McIlwain Tissue Chopper, Vibratome).

1.4. Neuronal differentiation and Immunocytochemistry

1.4.1. Differentiation-medium (NDM):

- DMEM and Ham's F12 (3:1 ratio, Gibco, Invitrogen)

- 1:50 B27 supplement (50x Stock, Gibco, Invitrogen)
- 1:100 N2 Supplement (100x Stock, Gibco, Invitrogen)
- 1:100 Penicillin/Streptomycin (100x Stock, PAN-Biotech)

1.4.2. Differentiation-medium CINDA:

- DMEM and Ham's F12 (3:1 ratio, Gibco, Invitrogen)
- 1:50 B27 supplement (50x Stock, Gibco, Invitrogen)
- 1:100 N2 Supplement (100x Stock, Gibco, Invitrogen)
- 1:100 Penicillin/Streptomycin (100x Stock, PAN-Biotech)
- 5mM creatine monohydrate (Sigma Aldrich, Germany)
- 100 U/ml Interferon-γ (PeproTech, Germany)
- 20 ng/ml neurotrophin-3 (PeproTech, Germany)
- 300µM dibutyryl-cAMP (Sigma Aldrich, Germany)
- 20µM ascorbic acid (Sigma Aldrich, Germany)

Five neurospheres with a diameter of 300 µm were plated on PDL/laminin-coated slides with neural differentiation medium (NDM, see 3.4.1) or maturation-supporting CINDA medium (see 3.4.2). Plated spheres were cultured for 28 days and medium was changed once a week, respectively. Afterwards, cells were fixed with 4% PFA (Sigma Aldrich, Germany) for 30 min at 37 °C and washed with PBS. Cells were stained with primary antibodies for 1 h at 37 °C. After washing with PBS, cells were incubated with the secondary antibodies, respectively, for 30 min at 37°C. Nuclei were stained with Hoechst33258 (Sigma Aldrich, Germany). Samples were analyzed using a fluorescent microscope (Carl Zeiss, Germany) and the ZEN 2 Rel.4.8 software (Carl Zeiss, Germany). Each experiment was performed at least three times with three independent neural inductions.

Primary antibodies:

Antibody	Cat. No., Company
mouse-anti-β(III)-Tubulin	T8660, Sigma Aldrich, Germany
rabbit-anti-β(III)-Tubulin	T2200, Sigma Aldrich, Germany
rabbit-anti-GFAP	G9269, Sigma Aldrich, Germany
rabbit-anti-PSD-95	ab76115, Abcam
mouse-anti-Synapsin1	106001, Synaptic Systems
rabbit-anti-glutamate-receptor 1	AB1504, Millipore
anti-mouse-GABA-A-receptor β	MAB341, Millipore
anti-mouse-NMDA-receptor 1	MAB363, Merck, Germany

Secondary antibodies:

Antibody	Cat. No., Company
anti-mouse-Alexa-546	Invitrogen, USA
anti-rabbit-Alexa488	Invitrogen, USA

1.5. Quantification

Quantification analyses of synapses and receptors were performed by analyzing the neurite mass in μm^2 and number of synapses (synapses/neurite area [μm^2]) using the Omnisphero

software as previously described [Hofrichter, 2017]. In detail, to account for a brighter β(III)-tubulin staining, structuring elements to eliminate uneven background were adjusted as well as thresholding of the resulting images with the Otsu method. Here, algorithms for the segmentation of digital images generated binary images and allowed the recognition of pixels that either represent objects (e.g. neurites and synapses) or their environment. The Otsu method defines a threshold that determines classes in which the dispersion within the classes is as small as possible, but between the classes is as large as possible. The quotient between the two variances is determined and a threshold value is calculated in which the quotient is as large as possible [Otsu, 1979].

For quantification analyses of synapses and receptors of hiNN, three independent experiments consisting of three neural inductions were performed. For each individual n a minimum of 6 images were analyzed for quantifications. Here, both the total number of synapses and the number and area of β(III)-tubulin positive neurites were calculated. Synapses that colocalize with neurites were analyzed using a script written in the Omnisphero software [Schmuck, 2017]. Therefore, the images of β(III)-tubulin stainings were first preprocessed using two different analyzing methods:

i. foreground -pixels were removed using a non-flat structuring element (sphere) (https://www.mathworks.com/help/images/ ref / imopen.html), which allows estimation of the uneven background. This background was then subtracted from the image.
ii. an adaptive threshold method was used on the same images (https://www.mathworks.com/matlabcentral/fileexchange/8647-local-adaptive-thresholding).

The resulting binary image was used as a mask and compared to the image from (i) to extract background regions. After this, the image was provided with a fixed threshold calculated by the Isodata-(Iterative Self-Organizing Data Analysis) technique-algorithm [Ball, 1965]. In a second approach, the same image (background-corrected), was processed with different edge-detection algorithms [Canny, 1986; Lim, 1990; Parker, 2011]. This image was then added to the images resulting from the two-step adaptive thresholding and small holes were filled up using the fill function. Images of immunofluorescent staining of synapses (postsynaptic marker PSD95 and presynaptic marker SYN1) were also processed by removing the foreground pixels to estimate the background. Subsequently, an isodata threshold was applied to filter out large particles that were not considered to be synapses. In a final step, all synapses that were not on a binary component of the neurite image were removed. Finally, the area of the binary neurite components, as well as the number of synapse particles and the relevant density of these particles are calculated as particles per area (synapses / neurite area [μm^2]). The method described above can be applied not only to synapse staining, but also e.g. on the staining of various neuronal receptors (NMDAR1 as a marker for the NMDA receptor, GluR1 as a marker for the AMPA receptor, GABAARβ1 as a marker for the GABA receptor) for the identification and quantification of the neuronal subtypes present in the culture.

1.6. Quantitative reverse-transcription PCR

Gene:	Human Primer:	Rat Primer:
βACTIN	FW:5'-CAGGAAGTCCCTTGCCATCC-3' RV:5'-ACCAAAAGCCTTCATACATCTCA-3'	FW:5'-CAGGAAGTCCCTTGCCATCC-3' RV:5'-ACCAAAAGCCTTCATACATCTCA-3'
NESTIN	FW:5'-CCCCGTCGGTCTCTTTTCTC-3' RV:5'-TCGTCTGACCCACTGAGGAT-3'	FW:5'-GGATGGGGACGAGGATCAAG-3' RV:5'-TGAGGACCAGGAATCCCCAT-3'
MAP2	FW:5'-TGCCTCAGAACAGACTGTCAC-3' RV:5'-AAGGCTCAGCTGTAGAGGGA-3'	FW:5'-CTGGAAGAAGCCTCGAAGATGG-3' RV:5'-TACTTGTGTCCGGCTGAGGA-3'

S100β	FW:5'-CACAAGCTGAAGAAATCCGAAC-3' RV:5'-CACATTCGCCGTCTCCATC-3'	FW:5'-GCTTCTCTGTCTACCCTCCTA-3' RV:5'-CCCTCTCTCCCTGAATACTGAT-3'
AQP4	FW:5'-GGTGCCAGCATGAATCCC-3' RV:5'-TGGACAGAAGACATACTCATAAAGG-3'	FW:5'-CAATTATACCGGAGCCAGCAT-3' RV:5'-TCAGGACAGAAGACATACTCGT-3'
GFAP	FW:5'-GATCAACTCACCGCCAACAGC-3' RV:5'-CTCCTCCTCCAGCGACTCAATCT-3'	FW:5'-ACAGCAAAACAAGGCGCTGGC-3' RV:5'-TCTTGGAGCTTCTGCCTCAGGG-3'
SYN1	FW:5'-GTGCTGCTGGTCATCGAC-3' RV:5'-CCACAAGGTTGAGATCAGAGAA-3'	FW:5'-CAAACCACAGCTGGCTCAGAA-3' RV:5'-GGTCAGAGACTGGGATTTGTTGAG-3'
DLG4	FW:5'-CCAGAAGAGTACAGCCGATTC-3' RV:5'-CTTGGTCTTGTCGTAATCAAACAG-3'	FW:5'-ACCAAGAAATACCGCTACCAAG-3' RV:5'-TCACCTGCAACTCATATCCTG-3'
SLC17A7	FW:5'-CAGAAAGCCCAGTTCAGC-3' RV:5'-ACAATAGCAAAGCCGAAAACTC-3'	FW:5'-CACTGCCTCACCTTGTCAT-3' RV:5'-TTCATCAGCTTTCGCACATTG-3'
GRIA1	FW:5'-CTTAATCGAGTTCTGCTACAAATCC-3' RV:5'-GTATGGCTTCGTTGATGGATTG-3'	FW:5'-CCACTACATCCTCGCCAATC-3' RV:5'-GTCACTTGTCCTCCATTGCT-3'
GRIN1	FW:5'-CTCCTGGAAGATTCAGCTCAA-3' RV:5'-GTGGATGGCTAACTAGGATGG-3'	FW:5'-TCATCTCTAGCCAGGTCTACG-3' RV:5'-GATTCTGTAGAAGCCAGCTGT-3'
GAD1	FW:5'-GACCCCAATACCACTAACCTG-3' RV:5'-GCTGTTGGTCCTTTGCAAG-3'	FW:5'-CTCCAAGAACCTGCTTTCCT-3' RV:5'-TCAACCACCTCCAGTAAGAAC-3'
TH	FW:5'-TTCTCGCAGGACATTGGC-3' RV:5'-CTTACACAGCCCGAACTCC-3'	FW:5'-AGTACAAGCACGGTGAACCA-3' RV:5'-GATGCTGTCCTCTCGGTAGC-3'
ACHE	FW:5'-ATGCGATACTGGGCCAAC-3' RV:5'-GTCCAGACTAACGTACTGCT-3'	FW:5'-TCAGGTTCAGGCTCACGTAT-3' RV:5'-TCAGGTTCAGGCTCACGTAT-3'
SLC6A4	FW:5'-GCTGTGTCTTGGTTCTATGGC-3' RV:5'-GGCTCATCAGAAAACTGCAAA-3'	FW:5'-TGCTCATCTTCACCGTAATCTAC-3' RV:5'-GGACAGAGAGGACAATGTATGG-3'
NOTCH1	FW:5'-GAAGTTCCGGTTCGAGGAGC-3' RV:5'-ATGAAGTCGGAGATGACGGC-3'	
EGFR	FW:5'-ATCACCTGCACAGGACGGGGA-3' RV:5'-GGACGGGATCTTAGGCCCATTCG-3'	
ID2	FW:5'-ACCCGATGAGCCTGCTATAC-3' RV:5'-GCTGACAATAGTGGGAT-3'	
CALB2	FW:5'-GGAAATATGGAAGCACTTTGACG-3' RV:5'-TCACTCTTTGACATCATGCCA-3'	
OLIG2	FW:5'-CCGATGACCTTTTTCTGCCG-3' RV:5'-CCACTGCCTCCTAGCTTGTC-3'	
DCX	FW:5'-GGATCCAGGAAGATCGGAAG-3' RV:5'-TTACGTTGACAGACCAGTTGG-3'	
DRD4	FW:5'-TGCTGCCGCTCTTCGTC-3' RV:5'-GAACCTGTCCACGCTGATG-3'	
GRIA4	FW:5'-CAGGGAATTGACATGGAGAGG-3' RV:5'-TTGTGTAATTGACTCTACGTCCA-3'	
STMN2	FW:5'-CGTCTGCACATCCCTACAATG-3' RV:5'-TGCTTCACTTCCATATCATCGT-3'	
GRM5	FW:5'-TGTGAGAAAGGCCAGATCAAG-3' RV:5'-TGCCTTGCATGTGTACTCATC-3'	
CHRM3	FW:5'-TCACGCTGGGAGCTGTA-3' RV:5'-AGCAAAACCTTATTGTACCTTAGC-3'	
GABBR1	FW:5'-CTGAGGTCTTCACTTCGACTC-3' RV:5'-AAAGTCCCACGATGATTCGG-3'	
GABRB3	FW:5'-AACCGCATGATCCGTCTTC-3' RV:5'-GCCATAGCTTTCAATTTCCAGA-3'	

1.7. NN differentiated on microelectrode arrays (MEA)

Statistical analyses of baseline activity parameters (MFR, MBR, spikes/burst and AE) were done in R version 3.6.0 [R-CoreTeam, 2019]. In case of the hiNPC-derived neural networks (NDM and CINDA), we modelled the activity of the networks (measured by spikes/min (MFR), bursts/min (MBR), spikes/burst and number of active electrodes (AE)) using a mixed model with random intercepts [Laird, 1982] to account for the repeated measurements of each network. The fixed terms of the model include a common intercept, the week of measurement (weeks 2 to 6), the condition and the interaction of week and medium. The model was fitted using the R function package nlme [Pinheiro, 2019].To test for differences between the media for each week and between consecutive weeks for each medium, we used simultaneous confidence intervals for general linear hypotheses controlling the family-wise error rate as implemented in package multcomp [Hothorn, 2008]. In case of the rNN, we used a separate mixed model with random intercepts for each network, where the fixed term only includes the common intercept and the week of measurement. We tested for differences between consecutive weeks using the methodology described above. To analyze long-term effects, we applied the same procedure as described above but replaced the week of measurement in the fixed term by a factor variable indicating short (weeks 1-6), medium (weeks 7- 11) and long term (more than 11 weeks).

For the statistical analyses of all experiments also values from 0-1 are included (not shown in log scale).

1.8. NN characterization with pharmacological treatments

Data acquisition was managed with MC-Rack (Multichannelsystems, Germany). We performed 5min of consecutive 1-min recordings (first minute was deleted subsequently). Raw data were generated using a sampling rate of 20-25kHz on all electrodes using a band pass filter (100-3500Hz). For spike detection we used the SpaNnEr software (Results Medical GmbH, Duesseldorf, Germany) with a variable threshold spike detector of 6x standard deviation of the internal ground noise on each electrode. To be regarded as active each electrode must record ≥5 Spikes/min. A well was defined as active, if ≥3 active electrodes were calculated.

1.9. Cytotoxicity of acute treatment of NN in mwMEA with domoic acid (DA)

Data acquisition was managed with the Multiwell-Screen (Multichannelsystems, Germany). We performed 15min baseline- and treatment-recordings. Raw data were generated using a sampling rate of 20kHz on all electrodes using a band pass filter (300-3500Hz). Spike detection of the MEA-multiwell-plates were performed with the Multiwell-Analyzer (Multichannelsystems, Germany) using an automatic threshold estimation of 500ms baseline duration and rising/falling edge of 5x standard deviation of the noise level. For burst detection the Multiwell-Analyzer Burst Detector (Multichannelsystems, Germany) was used using a max. 100ms interval to start/end a burst, a 20ms interval between bursts with 10ms duration of each burst. A burst was defined as a minimum of 3 spikes. Cytotoxicity was assessed using the Lactate-Dehydrogenase (LDH) Cytotoxicity Assay (Promega, Germany) according to manufacturer's instructions. Briefly, medium from control and treated hiNN and rNN was collected and incubated with the LDH solution for 2 h before fluorescence (540Ex/590Em) was measured using a multimode microplate reader (Tecan, Switzerland) and normalized to lysis control.

1.10. Affymetrics Microarray analysis

Data analyses on Affymetrix CEL files were conducted with GeneSpring GX software (Vers.12.5; Agilent Technologies) and Transcriptome Analysis Console (TAC; Vers. 4.0.1.36 Thermo Fisher Scientific). In GeneSpring, probes within each probe set were summarized by GeneSprings' ExonRMA16 algorithm after quantile normalization of probe-level signal intensities across all samples to reduce inter-array variability [Bolstad, 2003]. Input data pre-processing was concluded by baseline transformation to the median of all samples. After grouping of samples (for biological replicates each) according to their respective experimental condition, a given probe set had to be expressed above background (i.e., fluorescence signal of that probe set was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in all four replicates in at least one of five conditions to be further analyzed in pairwise comparisons. Differential gene expression was statistically determined by Moderated T-Test. The Resulting values were corrected for multiple testing by Bonferroni-correction. A value of ≤0.05 was considered significant. Hierarchical cluster analysis was performed with Euclidian similarity measures and Ward's linkage. The expression profile of single genes in the heatmap (Fig.7A) was prepared with the Multiple Experiment Viewer (MeV 4.9.0; http://mev. tm4.org/). In TAC the Probes within each probe set were summarized by Robust Multichip Average (RMA) after quantile normalization of probe level signal intensities across all samples to reduce inter-array variability. For analysis, samples were grouped according to their respective experimental conditions (four replicates for each condition). For the generation of Venn diagrams (Fig. 7C,D) we compared all genes that were differentially expressed with a false discovery rate (FDR) of ≤ 0.05 and a two-fold change in expression. Duplicate probe sets were deleted so that each differentially expressed gene is represented as 1 probe set. We compared our *in vitro* transcription profiles to *in vivo* samples that originate from homogenates of the prefrontal cortex postconceptional week (pcw) 6 (embryonic) and pcw 12, (fetal; Kang, 2011). These brain regions have been chosen, because they consist both of proliferating progenitor cells as well as differentiating and migrating neurons and glia cells [Silbereis, 2016]. *In vitro* and *in vivo* samples for the generation of Venn diagrams were evaluated with TAC as described above. Data evaluation of all other figures was performed in GeneSpring GX software.

1.11. Flow cytometry analysis

For flow cytometry analysis 50 hiNPC and primary hNPC neurospheres each with a diameter of 300µm were collected. Cells were singularized by incubation with Accutase (Invitrogen, USA) for 10 min at 37°C and 5% CO_2. Singularized cells were stained with Fixable viability dye eFluor® 506 for 30min in the dark at 4°C. Afterwards, cells were fixed with 4% paraformaldehyde (PFA, Sigma Aldrich, Germany) for 30min at 37°C and washed with PBS (Life Technologies, USA). Subsequently, cells were permeabilized with 0.1% Triton-X and blocked with 10% mouse serum (Sigma Aldrich, Germany) in PBS (Life Technologies, USA, 0.1% PBS-T) at room temperature for 15min. Finally, cells were stained with anti-Nestin-Alexa647 (BD Bioscience, Germany) and either anti-PAX6-Alexa488 or anti-SOX2-Alexa488 (BD Bioscience, Germany) antibodies in 0.1% PBS-T supplemented with 10% mouse serum in the dark at 4°C for 60min. Samples were analyzed using a BD FACS-Canto-II flow cytometer and the software FlowJo Version 10.5.3 (BD Biosciences, Germany).

Flow cytometry antibodies:

Antibody	Cat. No., Company
Mouse-anti-nestin-Alexa647	560393, BD Biosciences, Germany
Mouse-anti PAX6-Alexa488	561664, BD Biosciences, Germany
Mouse-anti-SOX2-Alexa488	560301, BD Biosciences, Germany
Fixable Viability Dye eFluor® 506	65-0866, eBioscience, Germany

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Supplementary Figures:

Fig.S1: Representative Flow cytometry analysis of hiNPC and primary human NPC (pNPC). (**A**) Gating strategy for hiNPC (IMR90) and pNPC. (**B**) Flow cytometry analysis of unstained and stained samples for the neural markers PAX6 and Nestin as well as (**C**) SOX2 and Nestin Samples were analyzed using a BD FACSCanto II and FlowJo software 10.5.3 (BD Biosciences, Germany). Number of analyzed cells =20 000.

Fig.S2: Immunocytochemical stainings of rNN. Representative pictures of rNN differentiated in NDM for 28 days into $TUBB3^+$ neurons (red) and $GFAP^+$ astrocytes (green). Nuclei were stained with Hoechst. Scale bars = 100 µm.

Fig.S3: mRNA expression profiles of hiPSC, hiNPC and hNN compared to rNPC and rNN. qRT-PCR analysis of the serotonergic transporter gene *SLC6A4* after 28 DIV in NDM or CINDA medium. Rat samples serve as a positive control. Data are presented as mean+SEM (n = 3).

Fig.S4: Spontaneous electrical activity of hNN- and rNN. Shown are scatter plots of hNN (NDM/CINDA) and rNN differentiated for 15 weeks *in vitro* (WIV) on MEAs. Plotted are the **A**) mean firing rate (MFR), **B**) mean bursting rate (MBR), **C**) spikes per burst and **D**) active electrodes. One dot represents one chip (hiNPC: n=40, Rat: n=43 at start of experiment). Black lines represent the mean of all data points. *****=significant to NDM, **#**=significant within condition ($p < 0.05$).

Fig.S5: Acute treatment of NN in mwMEA with the shellfish toxin domoic acid (DA). Acute treatment of hiNPC and rNN with DA. Mean+SEM of **A**) percentage of spikes per burst, **B**) mean Burst spike rate [Hz], **C**) mean burst duration are plotted in percent of solvent control for the acute exposure time of 15 min. Cytotoxicity of **D**) rNN and **E**) CINDA-NN was assessed using the Lactate-Dehydrogenase (LDH) Cytotoxicity Assay (Promega, Germany) and normalized to lysis control (hNN: n=3, rNN: n=3).

Fig.S6: Validation of microarray experiments by qRT-PCR. qRT-PCR analysis of mRNA samples from hiNPC (four replicates) that were used for transcriptional microarray analysis were compared to results of microarray analysis. Data is presented as mean+SEM fold change between 0 and 28 days (black: qRT-PCR, grey: microarray analysis).

Appendix I

Category
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Appendix II

in vitro:				
GO:2000179 ~positiveregulationofneuralprecursorcellproliferation	GO:2000648 ~positiveregulationofstemcellproliferation	GO:0002052 ~positiveregulationofneuroblastproliferation	GO:0021846 ~cellproliferationinforebrain	GO:1902692 ~regulationofneuroblastproliferation
DCT	DCT	DCT	DCT	DCT
SMO	SMO	SMO	KIF14	SMO
NOTCH1	NOTCH1	NOTCH1	RRM1	NOTCH1
CDON	CDON	VEGFA	IGF2BP1	VEGFA
VEGFA	VEGFA	ASPM	HMGA2	ASPM
GLI2	GLI2			
NR2E1	NR2E1			
ASPM	ASPM			

***Conflict of Interest form**

CONFLICT OF INTEREST AND FINANCIAL DISCLOSURE FORM

Manuscript Titel:

Characterization and application of electrically active neuronal networks established from human induced pluripotent stem cell-derived neural progenitor cells for neurotoxicity evaluation.

I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of us.

I confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing I confirm that we have followed the regulations of our institutions concerning intellectual property.

I further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

By signing below, I represent that the foregoing information is complete and truthful. I will notify the editorial office if there are any changes in my financial relationships prior to the publication of this article.

25.06.2019,

Date, Signature of Corresponding Author

Manuscripts

Characterization and application of electrically active neuronal networks established from human induced pluripotent stem cell-derived neural progenitor cells for neurotoxicity evaluation.

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Journal:	Stem Cell Research (Stem Cell Res)
Impact factor:	3,902 (2017)
Contribution to the publication:	85% Planning and evaluation of all experiments (except for the microarray analyses), performance of almost all experiments, writing of the manuscript
Type of authorship:	first authorship
Status of publication:	Submitted on 25 June 2019

3. Discussion

3.1 Different methods for neural induction of hiPSC in the context of neurotoxicity testing

The field of *in vitro* neurotoxicity is challenged with the task to develop *in vitro* cell culture models that adequately reflect human physiology. A paradigm switch in neurotoxicity assessment from apical endpoint evaluation in rodents towards mode-of-action (MoA)-based testing in human-relevant *in vitro* methods was proposed (Crofton et al., 2011, Dorman et al., 2001, NRC, 2007, NRC, 2017). This switch was inspired by the high attrition rates in new drug development when moving from the preclinical phase of animal experimentation into clinical trials with humans. Therefore *in vitro* methods based on human cells might increase predictivity of compound testing due to the elimination of species differences. Ethically disputed primary human cell cultures closely resemble the *in vivo* situation but exhibit donor-to-donor variability as well as limitations in their ability to proliferate *in vitro* (Eglen and Reisine, 2011). Thus human-relevant alternative methods are preferred that are indefinitely available and can predict adverse effects on the central nervous system. Currently human *in vitro* stem cell-derived biology using hESC or hiPSC holds great potential to predict neurotoxic effects of various reagents (Scott et al., 2013, Pei et al., 2016, Hofrichter, Nimtz et al., 2017). The discovery of techniques for reprogramming adult somatic cells, e.g. fibroblasts, into hiPSC with the potential of self-renewal allows the possibility to produce a large number of cells *in vitro*, resembling human physiology without ethical concerns (Gerecht-Nir and Itskovitz-Eldor, 2004, Takahashi et al., 2007, Zhang et al., 2012, Toivonen et al., 2013). Moreover, this approach has opened the door to produce patient-specific pluripotent stem cells to study either common but also rare types of existing neurodevelopmental and -degenerative diseases, such as Parkinson's and Alzheimer's disease, autism and schizophrenia (Nguyen et al., 2011, Israel et al., 2012). Disease models derived from hiPSC are being used to explore therapeutic potentials of pharmacological chemicals or transplantations. Nevertheless a critical part of hiPSC is that their epigenome is related to the tissue of origin and might persist throughout reprogramming (Ghosh et al., 2010, Kim et al., 2010,

Lister et al., 2011). To what extent this “epigenetic memory” influences the potential to differentiate into specific cells and tissues is currently an issue of special interest. Moreover, a cell line-to-cell line variability exists that impede the development of hiPSC-based models for *in vitro* use (Carcamo-Orive et al., 2017, Schwartzentruber et al., 2018). Thus it needs to be demonstrated that hiPSC-lines can be differentiated reproducibly into relevant cell types that exhibit characteristics of the *in vivo* equivalent (Trosko and Chang, 2010, Csobonyeiova et al., 2016, Tukker et al., 2018).

Until now numerous different protocols are published to neurally induce hiPSC into a co-culture of neurons and glial cells to recapitulate neural processes. The differentiation of embryonic tissue into the neuroectodermal lineage, resulting in NPC, is triggered by several key signaling pathways and transcriptional programs (Masserdotti et al., 2016). Extrinsic and intrinsic factors can set specific regions (cortex, ventral midbrain, etc.) and morphogenic gradients instruct NPC to express cell type-specific transcription factors. A commonly used method is the inhibition of the TGFβ/BMP-pathway by specific SMAD-inhibiting agents, e.g. a combination of CHIR99021, noggin, dorsomorphin, SB431542 or LDN193189 (Lamb et al., 1993, Moreau and Leclerc, 2004, Denham and Dottori, 2011, Ladewig et al., 2012, Qiang et al., 2013, Zhang et al., 2015, Li et al., 2015, Hu et al., 2015). These compounds are added to a defined neural induction medium, often containing a mixture of neurobasal B27 and/or N2 supplements (see Manuscript 2.1 for more details) that is known to induce neural differentiation (Gerrard et al., 2005, Hibaoui et al., 2014). Classic neural induction protocols utilize the spontaneous self-organization of stem cells into embryoid bodies followed by a blossom-like neural rosette formation when cells are plated on an adhesive matrix (Chambers et al., 2009, Neely et al., 2012, Pistollato et al., 2014). The differentiation into rosette structures is a primitive architecture common in the neural lineage which exists *in vivo* (Elkabetz et al., 2008). Within the *in vivo* embryonic neurodevelopment neural stem cells (NSC) aggregate to epithelial-like layers and form the neural tube which subsequently gives rise to neurons and glia cells. *In vitro* cultured NSC are called neural rosettes due to their radial aggregation comparable to early differentiated NSC *in vivo* (Conti and Cattaneo, 2010). These cells are adherent and also form multilayered structures, similar to those of the neural tube, and were also recognized after transplantation of neural stem cells into the

rodent brain (Brustle et al., 1997). Moreover, neural rosettes exhibit characteristics similar to embryonic primary neural populations, for example the expression of the transcription factors SOX1 (SRY-box containing gene 1), Nestin and PAX6 (paired box gene 6) (Zhou et al., 2010, Neely et al., 2012, Pistollato et al., 2014). There are protocols published, however that neurally induced hiPSC under 3D-cultuing conditions – free-floating 3D aggregates termed neurospheres (Toivonen et al., 2013, Walsh et al., 2017, Hofrichter et al., 2017) without going through monolayer rosette formation. For such protocols hiPSC colonies are cut into colony-fragments and transferred into low-attachment plates containing neural induction media often supplemented with knock-out serum replacement (KSR), with or without SMAD inhibitors (Manuscript 2.1. and 2.4). Within a few hours the cell aggregates round up and form free-floating spheroids. Comparison of 2D versus 3D induction approaches suggested that the early self-organization of stem cells into a 3D neural culture can promote the expression of early neural markers, such as PAX6 and Nestin, as well as differentiation of young neurons with extended neurites and glial progenitor formation (Chandrasekaran et al., 2017). Nevertheless long term differentiations of both, 2D and 3D neural induction methods, revealed that NPC can be further differentiated into a heterogeneous culture of post-mitotic neuronal subtypes and GFAP-positive astrocytes (Manuscript 2.1, 2.4, Zagoura et al., 2017). We further demonstrated that neuronal networks generated with a 3D spheroid-forming induction protocol exhibited the presence of synaptic proteins and spontaneous electrophysiological activities, suggesting that this method is suitable for generating electrically active neurons (Manuscript 2.1 and 2.4). However, hiPSC-derived neuronal networks lack synchronized bursting events that are known to be associated with matured network activity (Heikkila et al., 2009). Synchronization is a highly precise process wherein multiple neurons adjust incoming electrical signals and simultaneously transmit those impulses to postsynaptic membranes and therefore configure synchronous firing (Kamioka et al., 1996, Selinger et al., 2004, Uhlhaas and Singer, 2006, Kapucu et al., 2016). Simultaneous transmission requires a fine tuning of interacting neurotransmitters and their respective receptors (Harsch and Robinson, 2000, Voigt et al., 2001, Reimbayev et al., 2017, Iida et al., 2018). We showed that spontaneously differentiated neuronal networks from hiNPC spheres exhibit electrical activities but lack several classes of neurotransmitter systems as well as synchronized

network activities. This is possibly due to a combination of limited maturation time *in vitro* and the combination of neurothrophic factors used. For example, we differentiate mainly functional GABAergic neurons while Izsak et al., 2019 receive mostly glutamatergic neurons with their protocol (see Manuscript 2.4 for more details). The two protocols differ in the generation of NPC (neurospheres vs. neural rosette generation) and the neuronal differentiation medium components and neurotrophic factors which support the differentiation into specific neuronal subtypes. Therefore protocols still need optimization for containing a physiological ratio of excitatory and inhibitory neurons. This can either be achieved by factor optimization or by a defined combination of pre-differentiated neuronal subtypes mixed together in a defined ratio.

3.2 Neuronal networks for functional analyses

Human pluripotent stem cells can be differentiated into the main cell types of the human brain and are therefore a great cell source for producing functional neuronal networks *in vitro* (Lappalainen et al., 2010, Odawara et al., 2016b, Cotterill et al., 2016a, Heikkila et al., 2009, Pistollato et al., 2017b, Izsak et al., 2019). Differentiation of NPC into neuronal networks results in a heterogeneous culture consisting of neurons and glia cells. It is known that in human stem cell-derived culture neurogenesis occurs before astrogenesis (Grandjean and Landrigan, 2006, Liu and Zhang, 2011, Yuan et al., 2011, Kiiski et al., 2013, Nat et al., 2007, Itsykson et al., 2005). The hiNPC-derived neural cultures differentiated during my thesis show that the maturation-supporting medium causes astrocyte, yet not so much neuronal maturation as the medium does not influence MAP2 but strongly aquaporin4 (AQP4) upregulation during 28 days of differentiation (Manuscript 2.4). Interestingly the astrocytic marker glial fibrillary acidic protein (GFAP) is hardly expressed during these time points indicating that the type of astrocytes is possibly protoplasmic instead of fibrillary (Molofsky and Deneen, 2015, Molofsky et al., 2012). Protoplasmic astrocytes are found in the grey matter of the brain and have many short branches that predominantly envelop synapses and possess synaptic tasks (Molofsky and Deneen, 2015). Beside the development of supporting astrocytes a

healthy and functional neural cell culture consists of neurons which spontaneously transmit electrical signals from one neuron to multiple others thereby forming electrically active neuronal connections (Carpenter et al., 2001, Itsykson et al., 2005, Johnson et al., 2007, Erceg et al., 2008). In manuscript 2.4 we demonstrated that the detailed constitution of a functional neuronal network is extremely variable regarding the development of pre- and postsynapses within a variety of neuronal subtypes. Across the mammalian species synapse development of newborn neurons undergoes a period of overproduction to increase synaptic density (Andersen, 2003). This boost of arborization is followed by a successive elimination and synaptic pruning of network refinement. For example, the density of synaptic connections in the mammalian cortex is reduced by about 50% during neuronal maturation due to competitive, activity-dependent synapse pruning and elimination (Huttenlocher, 1990, LaMantia and Rakic, 1990, Colman et al., 1997). We showed that in differentiating hiNPC-derived neuronal networks synaptic genes are expressed within a differentiation time of 4 weeks (Manuscript 2.4). These cells resemble human prenatal neurodevelopmental stages where elimination of synapse contacts and synaptic pruning begins but continues until adolescence (Huttenlocher, 1979, Uylings and van Eden, 1990, Bourgeois, 1997, Petanjek et al., 2011). Once a neuronal connection is strengthened by a consistent signal transmission it survives and increases the neurotransmitter release, known as neuronal maturation, leading to long term potentiation (LTP) of specific receptors (Malenka and Nicoll, 1993, Colman et al., 1997, Turrigiano and Nelson, 2000, Volianskis et al., 2015). For example, the glutamate-dependent NMDA receptor is a major part involved in the LTP of the mammalian brain (Malenka, 1993, Turrigiano and Nelson, 2000, Volianskis et al., 2015). When a stimulus activates the release of glutamate into the synaptic cleft the fast glutamate-dependent AMPA-receptor depolarizes the postsynaptic membrane and the signal is transmitted further along the dendrite. If this happens consistently the NMDA-receptor pushes off an Mg^{2+} ion which leads to a Ca^{2+} influx and enzyme activation. As a result more AMPA-receptors are developed and become more sensitive towards the agonist glutamate (Moosmang et al., 2005). Because *in vitro* neuronal networks lack network input due to their very reduced nature LTP cannot be studied in such a set-up. The postnatal brain requires a fine-tuning of a balanced proportion between excitatory and inhibitory neurons to correctly form neuronal circuits. As a counterpart to the

excitatory neurotransmitter glutamate GABA is the prevalent inhibitory neurotransmitter in the brain. Within the immature brain GABA first has excitatory function by inducing postsynaptic depolarization and works in synergy with glutamate on neuronal differentiation and maturation (Ben-Ari et al., 1989, Cherubini et al., 1991, Ben-Ari, 2002). Soon after birth the polarity of the GABA-receptor switches permanently and GABA becomes inhibitory leading to hyperpolarization of the postsynaptic membrane (Rivera et al., 1999). Interestingly GABA is produced in the presynaptic membrane from glutamate via the glutamic acid decarboxylase (Petroff, 2002). Thus both most important counteracting neurotransmitters are developed from one precursor molecule. Supporting astrocytes play a major role in the Glutamate/GABA metabolism (Figure 3-1). The astrocytic membrane contains specific transporters that re-uptake both neurotransmitters from the synaptic cleft. Astrocytes exclusively express the glutamine synthetase which converts recycled glutamate into glutamine and can subsequently be transferred back into both, glutamatergic as well as GABAergic neurons (Reubi et al., 1978, Norenberg and Martinez-Hernandez, 1979, Sonnewald et al., 1993). In manuscript 2.4 immunocytochemistry stainings and gene expression analyses of spontaneously differentiated neuronal networks derived from hiNPC confirmed the development of GABA-receptors as well as glutamate-dependent AMPA and NMDA receptors. In contrast to the GABAergic system, however which is functional as an inhibitory transmitter system the electrophysiological measurements on MEAs suggest that the glutamatergic system is immature and thus non-functional. A possible reason could be that within the neural induction protocol and the subsequent neuronal differentiation we inhibited the bone morphogenic protein (BMP)/WNT-pathway by using dualSMAD components and therefore induced the differentiation into the ventral forebrain known for GABAergic neuronal development (Tao and Zhang, 2016, Li et al., 2009). Moreover, the CINDA medium contains neuronal supporting compounds but not the brain-/glial-derived neurotrophic factors (BDNF/GDNF) that promote maturation of glutamatergic neurons (Reyes et al., 2008). Therefore the glutamatergic receptors in our hiNPC-derived neuronal networks are present but not functional due to a lack of pre-synaptic glutamatergic neurons and might need different supporting supplements to mature. In addition astrocytes also absorb GABA from the synaptic cleft to recycle it via α-ketoglutarate to glutamine which then can be transferred to glutamatergic or GABAergic

neurons to synthesize glutamate or GABA (Schousboe et al., 2013). The enzyme pyruvate carboxylase involved in this catalysis is entirely located in cells of the astrocytic lineage (Yu et al., 1983, Shank et al., 1985, Cesar and Hamprecht, 1995). This demonstrates how important a co-culture of neurons and supporting astrocytes is to uphold neurotransmitter homeostasis within the synaptic cleft.

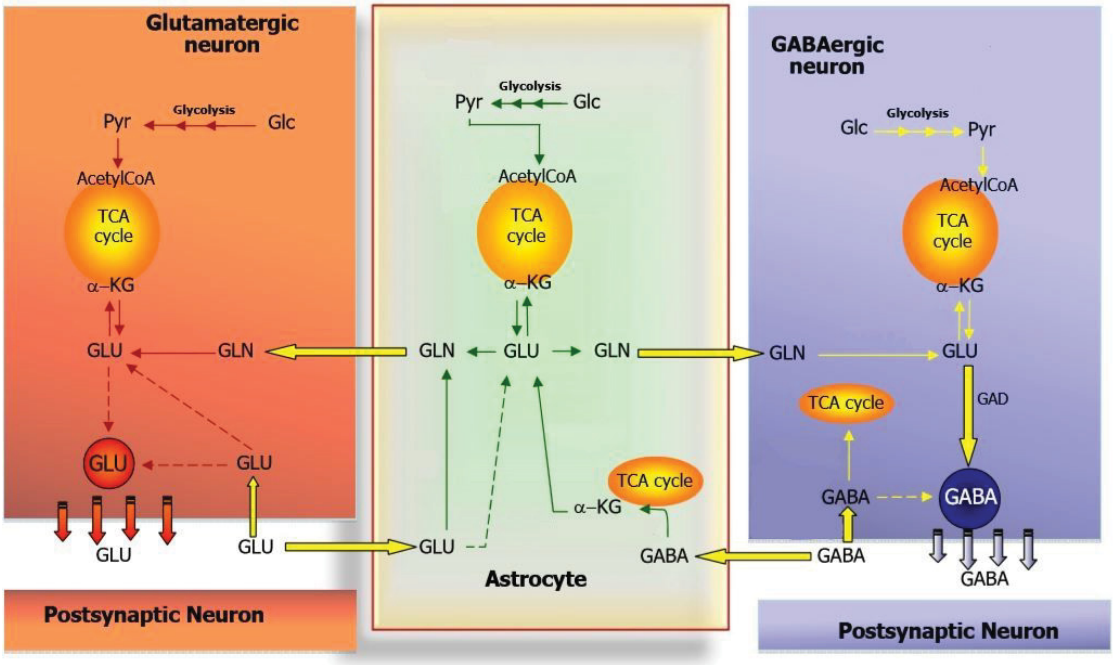

Figure 3-1: Schematic illustration of the metabolic interaction of astrocytes with glutamatergic and GABAergic neurons (Adapted from Schousboe et al., 2013).

A fully developed neuronal network not only consists of glutamatergic and GABAergic neurons. There are several endogenous compounds functioning as neurotransmitters, for example dopamine (DA), serotonin, and acetylcholine (ACh). Excitatory dopaminergic neurons synthesize DOPA from tyrosine and further metabolize DOPA to dopamine which acts as an agonist for five different dopamine receptors (D_1-D_5). 5-hydroxytryptamine (5-HT), known as serotonin, is a mainly excitatory CNS neurotransmitter molecule which is hydroxylated and decarboxylated from the amino acid tryptophan (Ruddick et al., 2006). Serotonin is released from the presynaptic membrane and activates 5-HT receptors of postsynaptic dendrites. ACh is a

neurotransmitter prominently used at neuromuscular junctions and lead to an arbitrary muscle contraction (Sanes and Lichtman, 1999, Wu et al., 2010). In the CNS it also serves as an agonist for excitatory muscarinic and nicotinic acetylcholine receptors (Cortes-Altamirano et al., 2018), and has a variety of effects on plasticity, excitation and reward. A multitude of additional neurotransmitters exist in the CNS which can be generally classified into monoamine, opioid, neuropeptide and amino acid transmitters (Lodish et al., 2000), all having specific functions and effects. In this thesis, the presence of a few neuronal subtype-specific genes and proteins, including glutamatergic, GABAergic, cholinergic and dopaminergic neurons was demonstrated (for more details see manuscript 2.4). Amongst the neuronal subtypes studied, we were not able to detect the expression of the serotonergic gene SLC6A4. Serotonin increases in humans within the first and second year of infancy (Hedner et al., 1986) which might be the reason why serotonergic neurons are not present in these cultures. To resemble equilibrium of neuronal circuits in a neuronal network *in vitro*, a physiological representation of neuronal subtypes is crucial. Yet neuronal composition differs between brain regions and should be considered in *in vitro* network differentiation (Tao and Zhang, 2016, Zeng et al., 2010). Synaptic modification and communication between neurons are an extremely sensitive and equilibrated concert of molecular interactions. The connectivity of a neuronal network can be measured by the electrical potential of a neuronal network combined with molecular characterization of neuronal constitutions. Microelectrode arrays are a non-invasive method which besides measuring a single stimulus transmission, can analyze network activity with multiple recording electrodes embedded into a surface (Gross et al., 1977, Pine, 1980). Hence it allows measuring network responses to specific agonists or antagonists for aforementioned neuronal subtypes that help analyzing network constitutions in real time (Xiang et al., 2007, Odawara et al., 2014, Alloisio et al., 2015, Fukushima et al., 2016, Odawara et al., 2016b). Moreover, this technology is seen as a promising tool to rapidly and sensitively screen potentially neurotoxic chemicals (Johnstone et al., 2010, McConnell et al., 2012, Mack et al., 2014, Wallace et al., 2015, Brown et al., 2016, Vassallo et al., 2017). Nevertheless two-dimensional (2D) *in vitro* grown hiNPC-derived neuronal networks exhibit low and often not synchronized activity levels compared to rodent networks (manuscript 2.4). The *in vivo* brain is grown in three dimensions (3D) and much more complex and branched

than neurons in a 2D *in vitro* culture (Baker and Chen, 2012, Bourke et al., 2018). It underlies spatiotemporal exposure to growth factors, cell-to-cell contacts and physiological properties from the surrounding extracellular matrix (Discher et al., 2009). Neurons in a 2D network are grown in a simplified manner and lack neuronal interconnections and cell-matrix adhesions (Hopkins et al., 2015). One way to tackle this issue and bring more complexity into *in vitro* neuronal networks is to differentiate them in a 3D-matrix, for example using hydrogels (Aurand et al., 2012, Ahmed, 2015). Nonetheless hydrogels and other biomaterials need to be cytocompatible and facilitate cell attachment, proliferation and differentiation into the intended tissue (Jhala and Vasita, 2015, Mawad et al., 2016). This field of research is relatively young and thus no standardized protocol for 3D differentiation of neural cells on MEAs has been published yet.

3.3 Microelectrode arrays and their analyses

In 1972, Thomas and colleagues published a first experiment with the use of an array with planar electrodes to monitor electric activity of cultured cardiomyocytes (Thomas et al., 1972). 10 years later, as a first *in vitro* experimental use of neural tissue, mouse spinal cord neurons were cultured on MEAs which allowed recordings from simultaneously emitted electrical signals (Gross et al., 1982). From that point substrate-integrated MEA research became a more flexible and complex application through commercial vendors and custom adjusted productions. The more complex the technology became, the more variable opportunities arose to analyze tissue-specific data. Recorded electrical activities by MEAs are extracellular action potentials but a multitude of additional information can be gained from such recordings. Beside single action potentials (“spikes”) and trains of action potentials (“bursts”) that can be displayed by each electrode, spatio-temporal parameters including the interspike and interburst intervals, the number of spikes in a burst, the burst duration and the overall network synchronicity are transmitted. From such readouts many other complex activity analyses can be performed, such as changes in individual spike waveforms, e.g. amplitude and duration, and network functions as cross-correlations and network connectivity (Baruchi

and Ben-Jacob, 2004, Gold et al., 2006, Nam and Wheeler, 2011, Buzsaki et al., 2012). This suggests that MEAs are a useful high-content *in vitro* approach that provide a multitude of detailed information on network activity and can detect changes in network function due to compound exposure (Manuscript 2.4).

So far, mainly rodent-derived neuronal networks as primary cortical cultures have been used for MEA recordings because they are easily cultured, widely accepted and well-characterized regarding their electrophysiological behavior (Xiang et al., 2007, Hogberg et al., 2011, Robinette et al., 2011, Mack et al., 2014, Alloisio et al., 2015). The unlimited availability of material and its rapid development make primary rat cortical cells the current standard culture for neurotoxicity *in vitro* studies (Hogberg et al., 2011, Novellino et al., 2011, Harrill et al., 2011, Wallace et al., 2015, Brown et al., 2016, Vassallo et al., 2017). A variety of potentially neurotoxic chemicals like pesticides, impair important neuronal activity pathways by a direct inhibition or activation of neuronal receptors or ion channels. Therefore acute neurotoxicity evaluations on spontaneous electrical activity are of high interest. For example, a screening study on effects of 30 compounds on spontaneous activity in rat cortical networks demonstrated characteristic changes in the recorded electrical activities (McConnell et al., 2012). A relatively high concentration of 50µM of each compound was added to the cultures between 12 and 22 days *in vitro* (DIV) and measured for 33 min. Here a control baseline activity measurement was performed before the treatment and therefore each well served as its own control. Compound-specific changes in network activity were directly compared to the activity level and patterns before the treatment and thus made it relatively simple to analyze respective specific effects. The data were shown as the percentage of changes in Spikes/min (Mean-Firing-Rate (MFR)) in relation to the number of active electrodes. Important threshold levels for activity validity were set according to previously published values (Shafer et al., 2008, Meyer et al., 2008, Novellino et al., 2011).

Beside studies on acute neurotoxicity, few *in vitro* approaches to assess chemical effects on the developing CNS have been performed (Brown et al., 2016, Frank et al., 2017, Dingemans et al., 2016). Yet cultures are still missing higher level functions of living neurons. Neuronal coordination and connectivity are the fundamental key processes for cognitive function (Korte and Schmitz, 2016). The main challenging fact is that spontaneously differentiating neuronal networks always show high variability

regarding network constitutions (Novellino et al., 2011, Mack et al., 2014, Vassallo et al., 2017). This is driven by the differentiation of a variable number of neurons, flexible accumulation of different cell types (e.g. neurons and glia cells) and/or neuronal subtypes with variable densities of activity-driving receptors formed by chance. Positioning of cells across electrodes is something that we cannot promote, posing another variability to the system. High variability leads to low reproducibility which prevents a direct comparison of activity levels from one network to the other, i.e. a treated one with an untreated control network. Variability might be decreased by including more replicates and having the knowledge to avoid variability-inducing factors. Those factors could be chip-to-chip differences by manual cell seeding, day-to-day, and culture-to-culture differences. Nevertheless by increasing the number of replicates and/or experiments combined with an automatic and reproducible cell plating strategy variability might be reduced. A recently published study on effects of 86 compounds demonstrated characteristics and concentration-dependent activity modifications in developing primary cortical rat networks over 12 days of differentiation (Frank et al., 2017). Here the experimental setup was composed of 48-well-MEA multiwell-plates which are a useful tool for increasing replicates without being too laborious. A pilot work for this study characterized the cell cultures and validated the production of highly similar results within a screening of 6 different compounds (Brown et al., 2016, Cotterill et al., 2016b). The low inter-experimental variability within a well-established network formation assay laid the foundation for the screening of additional 80 potential developmental neurotoxic compounds (Frank et al., 2017). However, these cells were cortical neurons and glia and therefore exhibit less variability than cells differentiating from stem cells. Rat networks that were derived from NPC within my thesis showed more variability than the published rat networks from cortical cultures (Brown et al., 2016, Frank et al., 2017) due to the stem cell differentiation taking place *in vitro.*

It is known that neurite outgrowth in rat primary cortical cultures exhibit less variability compared to human neural cultures (Harrill et al., 2011). Therefore analyses of human neuronal network data need even more attention regarding their network variability. In addition, the network activity level of human stem cell-derived neuronal networks is not very high compared to rodent networks which in general differentiate faster and exhibit higher and more synchronized electrical activities (manuscript 2.4; (Alloisio et al., 2015,

Cotterill et al., 2016b). In manuscript 2.4 the comparison of a conventional medium and a self-made neuronal maturation medium (CINDA) suggests that adding additional neural supporting compounds increases electrical network activity levels as well as neuronal and astrocyte maturation within the first 4 weeks of differentiation but with high chip-to-chip variabilities. This makes analyses of adverse effects of potentially toxic chemicals more complicated. Due to this limitation, there are only few reports on successfully developed neuronal networks from human-based material (Heikkila et al., 2009, Yla-Outinen et al., 2010, Weick et al., 2011, Cotterill et al., 2016a, Fukushima et al., 2016, Pistollato et al., 2017a, Tukker et al., 2018). Available research provides fundamental knowledge of electrical signal transmission and synapse function (Eccles, 1990, Huttenlocher, 1990, Connors and Long, 2004, Curti and O'Brien, 2016) but does not cover the precise processes of neuronal and astrocytic interactions within a spontaneously differentiated neuronal network *in vitro*. Moreover, the definition of both species- and cell type-specific thresholds for network activities bears challenges. For example, the overall spike detection threshold varies from a background noise standard deviation of ±4,5 up to ±7 (Tukker et al., 2018, Mayer et al., 2018). In contrast, others set no thresholds that must be fulfilled by networks to be regarded as active (Heikkila et al., 2009, Kiiski et al., 2013, Odawara et al., 2014, Fukushima et al., 2016, Odawara et al., 2016a). Some research groups defined their own spike detection and analyzing algorithms (Mayer et al., 2018) or set a threshold for the numbers of spikes but without counting the number of electrodes that record these activities which would verify the spatial connection of neurons and therefore network function (Yla-Outinen et al., 2010). In addition the question if data from single electrodes versus the mean of all electrodes from one chip is presented is handled very differently (Brown et al., 2016, McConnell et al., 2012, Novellino et al., 2011).

In this dissertation neuronal network activity must reach defined activity thresholds to be regarded as active. A minimum of 5 spikes/min determine an active electrode and a minimum of 3 out of 59 active electrodes/chip is the inclusion criteria for an active chip (Manuscript 2.4). These thresholds are commonly used for MEA analyses (Kasteel and Westerink, 2017, Paavilainen et al., 2018, Tukker et al., 2018). However, due to the high variabilities of our individual networks, we wanted to enhance a reliable baseline activity

for treatment applications which for evaluating data of compound testing should be the basis.

Nevertheless electrophysiological assessment of neuronal function with the MEA technology might be a useful tool to investigate chemically induced changes of potential neurotoxicants on network functionality and neuronal transmission but requires training and expertise as well as more defined thresholds and establishment to be more reproducible.

3.4 Suitability of hiPSC-derived network activity for *in vitro* neurotoxicity testing

Current regulatory neurotoxicity testing strongly relies on *in vivo* animal experiments. These are mandatory for the registration of new chemicals and drugs but are often too laborious and expensive and might also not well reflect the human situation because of inter-species variation (Leist et al., 2013, Perel et al., 2007, Matthews, 2008, Leist and Hartung, 2013). Moreover, those *in vivo* experiments are not suitable for screening large numbers of chemicals (Bal-Price et al., 2008). Therefore innovative medium- to high-throughput *in vitro* test strategies are clearly needed that not only reduce the number of animals but also give insights on compounds' MoA (Bal-Price et al., 2008, Llorens et al., 2012, van Thriel et al., 2012). In the current form, human *in vitro* models are not suitable for regulatory neurotoxicity testing because they do not reflect the complexity of the *in vivo* brain and do not cover the necessary applicability domains covering the neurotoxic chemical space (Masjosthusmann et al., 2018a). For neurotoxicity evaluation *in vitro*, neuronal networks should consist of a mixed population of excitatory and inhibitory neurons, contain the broad variety of neuronal subtypes with different neurotransmitters, their receptors and ion channels, as well as different glia populations, i.e. astrocytes, oligodendrocytes and microglia. To assess composition and neuronal morphology of a neuronal network specific protein can be visualized by stainings with immunofluorescent antibodies. Thus the presence and location of neuronal subtypes and glia cells can be identified. The MEA technology then allows evaluation of *in vitro* neuronal network activity by real-time recording of extracellular local field potentials from multiple

electrodes (Johnstone et al., 2010). Such networks possess spontaneous electrical activity and can respond in a MoA-specific manner to pharmacological compounds which represent characteristics of *in vivo* functional neurons (Heikkila et al., 2009, Odawara et al., 2014, Tukker et al., 2016, Kasteel and Westerink, 2017). Here network responses reflect the cellular composition, maturation and complexity of the networks. In this work, hiPSC-derived networks only represent part of the brain's diversity, as the differentiated neurons are mainly inhibitory GABAergic neurons. This needs to be accounted for when assessing chemical toxicity. In this case, additional networks are needed that close the gap to the MoA that cannot be studied here like excitotoxicity. To analyze adverse effects of chemicals there is a need of identifying specific molecular mechanisms and constitutions of the respective testing model. Human stem cell-derived cultures used on MEAs to date are less well characterized compared to rodent cortical neurons. This makes data interpretation difficult. In addition, the spontaneous and self-organized differentiation of cells in a neuronal network elicits variations between single network constitutions. This MEA-to-MEA variability hampers direct comparisons of exposed neuronal networks to untreated control ones. Due to that it will need more research work on creating a refined and directed neuronal differentiation. The differentiation into brain organoids might be a solution to differentiate a directed brain region-specific cell culture (Sloan et al., 2018). HiPSC-derived 3D neural organoids are self-organized and recapitulate developmental processes and organization of the human brain (Lancaster et al., 2017). The so called 'mini-brains' can represent several different brain regions (Lancaster and Knoblich, 2014a, Qian et al., 2016) and therefore offer the possibility for modeling specific neurological disorders like autism or schizophrenia (Lancaster and Knoblich, 2014b, Mariani et al., 2015). Besides brain organoids, precise positioning of distinct differentiated cell types onto the electrode surface by using the bioprinting technology might also help to increase network reproducibility. Recent studies of 3D printed brain-like structure showed successful encapsulation, survival and networking of rodent primary neurons and glia cells in 3D printed hydrogels (Lozano et al., 2015, Zhuang et al., 2018). Still, this cannot exactly resemble the *in vivo* brain because this technology is restricted to create layers with horizontal configurations (Lozano et al., 2015). But electrophysiological analyses of 3D-grown neuronal networks exhibited recordings of extended burst durations which were more synchronous

compared to a 2D network (Bourke et al., 2018, Tedesco et al., 2018). This demonstrates higher oscillatory activity through multiple network pathways indicating an extensive network formation throughout the 3D matrix. Until today neuronal networks combining both, bioprinting and 3D-configurations, are rare and hardly analyzed for their electrophysiological behavior on MEAs.

In general hiPSC-derived neural cultures offer other medical applications besides toxicological testing. These cultures provide an enormous potential in the field of regenerative medicine. Here progenitor cells of specific tissue can be differentiated from stem cells *in vitro* and transplanted into damaged tissue (Riazi et al., 2009). A unique feature of hiPSC is the generation of patient-specific pluripotent stem cells that can be further differentiated into desired target tissues. Such can be used for the identification of individual cell therapies, e.g. for brain disorders and diseases (Durnaoglu et al., 2011, Young et al., 2012, Brennand et al., 2015). Alternative strategies to test for potential neurotoxic compounds will require proving their predictive capacity. This will involve responsible development, validation and acceptance of data interpretation. HiPSC-derived neuronal networks on MEAs may lay the foundation for animal-free neurotoxicity testing in the future but at present can be used for electrophysiological investigations and help to reduce resource-consuming *in vivo* studies.

4. Abstract

Today developmental toxicity testing is mostly performed on animals according to guidelines from the OECD or U.S. EPA. These methods are time and resource-intensive and bear the problem of inter-species extrapolation. Due to the anticipated resources, it is impossible to test the large number of chemicals needed in such animal studies. Therefore a paradigm shift in toxicological testing from apical endpoint evaluation in rodents towards mode-of-action (MoA)-based, human-relevant *in vitro* testing is strived for. One organ with high failure rates for effect prediction for safety as well as efficacy is the central nervous system (CNS). Thus alternative methods are needed that predict compounds' effects on human brain cells with high accuracy.

The two reviews included in this thesis summarize the *in vitro* development and availability of alternative human stem cell-based scientific concepts for endpoint evaluation. Here for assay establishment neurodevelopmental endpoints should be chosen that (i) can be used to set up a stem cell-based DNT testing battery covering spatiotemporal neurodevelopmental processes, (ii) increase predictivity for humans and (iii) accomplish regulatory requirements.

Furthermore, this thesis contains two research manuscripts revealing the cell biological characterization of human induced pluripotent stem cell-derived neural progenitor cells (hiNPC). These hiNPC are organized as 3D-floating spheroids, so called neurospheres, and differentiate spontaneously into a heterogeneous co-culture of neurons and astrocytes, forming electrophysiologically active neuronal networks. Analyses of immunocytochemical stainings and mRNA expression profiles demonstrate that neuronal networks express pre- and postsynaptic molecules as well as certain neuronal subtype-specific receptors or enzymes. Upon plating on extracellular matrix-coated single- or multi-well microelectrode arrays (MEAs), neurons exhibit electrical activity (spikes and bursts) and network synchronicity over time. Treating networks with pharmacological compounds indicates presence and functionality of inhibitory GABAergic synapses but lack of glutamatergic transmission. These data suggest that hiNPC-derived neuronal networks are suited to contribute to a battery of test methods for neurotoxicity evaluation *in vitro*.

5. Zusammenfassung

Die aktuellen Analysen der entwicklungstoxikologischen Tests gemäß den Richtlinien der OECD oder der US-amerikanischen EPA, zeigt, dass diese primär in Tierversuchsstudien durchgeführt werden. Die dabei angewandten Methoden sind zeit- und ressourcenintensiv und hinsichtlich der Übertragung von Ergebnissen zwischen verschiedenen Spezies problematisch. Aufgrund der benötigten hohen Menge an Versuchstieren, ist es unmöglich, die große Anzahl an Chemikalien in wissenschaftlichen Experimenten an oder mit lebenden Tieren zu testen. Daher wird ein Paradigmenwechsel angestrebt, der von einer apikalen Endpunktbewertung bei Nagetieren zu humanrelevanten Wirkmechanismen innerhalb alternativer *In-vitro*-Tests geändert wird. Ein Organ mit hohen Ausschlussraten für die Vorhersage von Risiko und Wirksamkeit potenziell toxischer Substanzen ist das zentrale Nervensystem (ZNS). Daher sind alternative Methoden gefordert, mit denen die Auswirkungen auf humane Gehirnzellen präzise vorhergesagt werden können. Die beiden in dieser Arbeit enthaltenen Reviews fassen die Entwicklung und Verfügbarkeit alternativer *In-vitro*-Konzepte auf der Basis menschlicher Stammzellen für eine Endpunktbewertung zusammen. Für eine Assay-Etablierung sollten solche Endpunkte ausgewählt werden, die (i) zum Aufbau einer auf Stammzellen basierenden DNT-Testbatterie verwendet werden können und räumlich-zeitliche neurologische Entwicklungsprozesse abdecken; (ii) ferner die Vorhersagbarkeit für den Menschen verbessern und (iii) regulatorische Anforderungen erfüllen. Darüber hinaus enthält diese Dissertation zwei Forschungsmanuskripte, welche die biologische Charakterisierung von aus human-induzierten pluripotenten Stammzellen abgeleiteten neuralen Vorläuferzellen (hiNPC) aufzeigen. Diese hiNPC sind in 3D-schwimmenden Sphäroiden, den sogenannten Neurosphären, organisiert und differenzieren spontan zu einer heterogenen Ko-Kultur aus Neuronen und Astrozyten. Diese bilden zusammen ein elektrophysiologisch aktives neuronales Netzwerk. Analysen von immunzytochemischen Färbungen und mRNA-Expressionsprofilen zeigen, dass die neuronalen Netzwerke Prä- und Postsynapsen exprimieren und bestimmte subtypspezifische neuronale Rezeptoren und Enzyme ausbilden. Beim Ausplattieren dieser hiNPC auf Einzelwell- oder Multiwell-Mikroelektrodenarrays (MEAs), die zuvor mit einer extrazellulären Matrix beschichtet wurden, zeigen sich im Laufe der Zeit elektrische Aktivitäten (Spikes und Bursts) und erste vereinzelte Netzwerk-Synchronitäten. Die pharmakologische Behandlung der Netzwerke bestätigt das Vorhandensein und die Funktionalität von inhibitorischen GABAergen Synapsen, allerdings wird auch ein Mangel an einer glutamatergen Reizweiterleitung deutlich. Die Ergebnisse zeigen, dass hiNPC-abgeleitete neuronale Netzwerke durchaus einen Beitrag für eine *In-vitro*-Testbatterie zur Neurotoxizitätsbewertung leisten können.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
5-HT	5-Hydroxytryptamine
ACH	Acetylcholine
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazol-Propion acid
AP	Action potential
AP5	2-Amino-5-Phosphovalerian acid
AQP4	Aquaporin 4
ATP	Adenosine triphosphate
BDNF	Brain-Derived neurotrophic Factor
BMP7	Bone Morphogenetic Protein
BSA	Bovines Serum Albumin
cAMP	Cyclic adenosine monophosphate
CD15	3-Fucosyl-N-Acetyl-Lactosamine
CINDA	Neuronal maturation supporting medium
c-Myc	Myelocytomatosis oncogene
CNS	Central nervous system
CoA	Coenzyme A
DA	Dopamine
dH2O	Deionized water
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

Abbreviations

DNT	Developmental neurotoxicity
dualSMAD	Dual Small Mothers Against Decapentaplegic
EB	Embryoid Bodies
ECM	Extracellular Matrix
ECVAM	European Centre for the Validation of Alternative Methods
eEF1	Eukaryotic translation Elongation Factor 1
EGF	Epidermal Growth Factor
EPA	Environmental Protection Agency
ESC	Embryonic stem cells
FACS	Fluorescence-Activated Cell Sorting
FGF	Fibroblast Growth Factor
FOXA2	Forkhead box protein A2
GABA	γ-Aminobutyric acid
GDNF	Glial-Derived neurotrophic Factor
GDP	Guanosine-5'-diphosphate
GFAP	Glial Fibrillar Acidic Protein
GLU	Glutamate
GTP	Guanosine-5'-triphosphate
h	Human
hESC	Human embryonic stem cells
hiPSC	Human induced pluripotent stem cells
hNPC	Human neural progenitor cells
hUCBSC	Human umbilical cord blood mesenchymal stem cells
IgG	Immunoglobulin G
IgM	Immunoglobulin M
iPSC	Induced pluripotent stem cells

Abbreviations

K^+	Potassium
KE	Key events
Klf4	Kruppel-like factor 4
KSR	Knockout Serum Replacement
Lin-28	Lineage protein 28
LTP	Long-term potentiation
LUHMES	Immortalized Human Dopaminergic Neuronal Precursor Cells
MAP2	Microtubule-Associated Protein 2
MBR	Mean bursting rate
MEA	Microelectrode Array
MeHgCl	Methylmercury chloride
MFR	Mean firing rate
MoA	Mode-of-action
mRNA	Messenger Ribonucleic acid
Na^+	Sodium
Nanog	Nanog homeobox
NBQX	2,3-Dihydroxy-6-Nitro-7-Sulfamoyl-Benzochinonxalin-2,3-dion
NMDA	N-Methyl-D-Aspartate
NPC	Neural progenitor cells
NRC	National Research Council
NSC	Neural stem cells
NT	Neurotoxicity
NT-3	Neurotrophin-3
O4	Oligodendrocyte marker
Oct4	Octamer binding transcription factor 4
OECD	Organization for Economic Co-operation and Development

Abbreviations

OPPTS	Office of Prevention, Pesticides and Toxic Substances
PAX6	Paired box 6
PC	Personal computer
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PND	Postnatal day
PSD95	Postsynaptic Density Protein 95
qRT-PCR	Quantitative real-time Polymerase Chain Reaction
r	Rat
ReNcell CX	Immortalized human neural progenitor cell line
rNPC	Rat neural progenitor cells
ROCK	Rho-associated protein kinase
S100β	Calcium-binding protein beta
SD	Standard deviation
SLC6A4	Serotonin transporter
SOX1/SOX2	SRY (Sex determining region Y)-box containing gene 1/2
SSEA4	Stage-Specific Embryonic Antigen 3
SSEA4	Stage-Specific Embryonic Antigen 4
TGFβ	Transforming Growth Factor beta
Tra-1-60	Tumor rejection antigen-1-60
Tra-1-81	Tumor rejection antigen-1-81
TTX	Tetrodotoxin
vs	Versus
WNT	*wingless* Int-Gen

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Danksagung

An dieser Stelle möchte ich mich bei allen Personen bedanken, die meine Promotion ermöglicht haben:

Frau Prof. Dr. Ellen Fritsche möchte ich für die Bereitstellung des sowohl sehr interessanten als auch zukunftsversierten Themas und die umfassende Betreuung, Unterstützung und Förderung während der gesamten Promotion danken.

Bei Frau Prof. Vlada Urlacher möchte ich mich für die fakultätsübergreifende Betreuung, durch die meine Dissertation erst möglich war, und die Übernahme der Mentorenschaft bedanken.

Ein weiterer Dank gilt Stephan Theiss von der HHU Düsseldorf für die sehr hilfreiche Unterstützung bei der Auswertung der MEA-Daten, der ausgesprochen angenehmen Zusammenarbeit und den herzlichen Treffen auf wissenschaftlichen Kongressen.

Ein ganz großes Dankeschön geht an alle ehemaligen, alten und neuen Kollegen des IUFs und natürlich besonders der AG Fritsche. Hier danke ich besonders meiner Betreuerin Julia Tigges, die mich besonders in den letzten Phasen meiner Arbeit in jeglicher Hinsicht immer kompetent und herzlich unterstützt und mir mit ihrer umfangreichen wissenschaftlichen Erfahrung immer hilfsbereit zur Seite stand.

Außerhalb der wissenschaftlichen Arbeit danke ich den Jungs und Mädels im IUF für die großartige Zeit, die spaßigen Aktivitäten (Rollnacht, LaserTag, Weihnachtsmarkt, Kirmes, etc) und für die exorbitanten Partys und so ziemlich jegliche Abende und Nächte, wo auch immer diese stattfanden. Besonders sind hier auch die feucht-fröhlichen Weihnachtsfeiern zu erwähnen. Vielen Dank an unser Büro, an Farina, Saskia, Gaby, Jördis, Ulrike und allen neuen und alten „Mitbewohnern“, wo es täglich unglaublich viel zu lachen gab und ich mich während meiner Zeit in der AG unfassbar wohl gefühlt habe.

Danke, Farina und Jördis für unsere kulinarischen Ausflüge und Ines für das sportliche Engagement, die Kalorien wieder mit Laufen im Volksgarten abzutrainieren. Denen, die mit in Brüssel (Stefan, Ines, Jördis) und Hannover (Kristina, Saskia, Jördis, Ines) waren, für die schönen chaotischen Tage fern ab von Gut und Böse. Und natürlich alle, die ich jetzt nicht erwähnt habe für die immer unterhaltsamen Pausen und die kontinuierliche Hilfe bei allen aufkommenden Fragen.

Danke auch an Marius, für die arbeitsgruppen-übergreifenden, tiefgründigen Gespräche, den fulminanten Abenden am Rhein oder sonst wo, und vor allem der körperlichen Beeinträchtigung am nächsten Tag.

Last but not least, geht ein besonderer Dank an Marcel, der mich während stressiger Phasen unaufhörlich unterstützt, und auch die stressbedingten miesen Launen, besonders in der letzten Zeit, nicht immer aber häufig kommentarlos ertragen hat. Abschließend möchte ich mich bei meinen Eltern, meinem Bruder und meinen Freunden bedanken, die mich während der

Danksagung

gesamten Promotionszeit unermüdlich unterstützt haben und mir zu jeder Zeit den notwendigen Rückhalt gaben.

Vielen Dank für alles!

Eidesstattliche Erklärung/Declaration

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit „Entwicklung und molekulare Charakterisierung von elektrisch aktiven neuronalen Netzwerken auf Mikroelektroden Arrays für *in vitro* Neurotoxizitätstestung“ 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 “Development and molecular characterization of electrically active neuronal networks on microelectrode arrays for neurotoxicity testing *in vitro*” 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.

Laura Nimtz

Düsseldorf, Juni 2019