Research Article

Towards Animal-Free Neurotoxicity Screening: Applicability of hiPSC-Derived Neuronal Models for In Vitro Seizure Liability Assessment

Anke M. Tukker, Regina G. D. M. van Kleef, Fiona M. J. Wijnolts, Aart de Groot, Remco H. S. Westerink
Neurotoxicology Research Group, Toxicology Division, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Abstract

A sizeable proportion of drug attrition is due to drug-induced seizures. Current available animal models frequently fail to predict human seizure liability. Therefore, there is a need for in vitro alternatives, preferably based on human-derived neurons to circumvent interspecies translation. The increasing number of commercially available human induced pluripotent stem cell (hiPSC)-derived neuronal models holds great promise for replacing rodent primary cultures. We therefore tested three different hiPSC-derived neuronal models for their applicability for in vitro seizure liability assessment.

Using immunofluorescent staining and multi-well micro-electrode arrays we show that all models develop functional neuronal networks that exhibit spontaneous activity and (network) bursting behavior. Developmental patterns differ between the models, probably due to differences in model composition and seeding density. Nevertheless, neuronal activity and (network) bursting can be reproducibly modulated with the seizurogenic compounds strychnine, picrotoxin (PTX) and 4-aminopyridine (4-AP). However, the sensitivity and degree of chemical-induced effects differ between the models, which can likely be explained by differences in seeding density, maturation and different ratios of inhibitory and excitatory cell types. Importantly, compared to rat primary cortical neurons, the hiPSC-derived neuronal models were equally, or even better in the case of 4-AP, suited to detect seizurogenicity. Overall, our data indicate that hiPSC-derived neuronal models may in the future be used as a first screening tool for in vitro seizure liability assessment. However, before hiPSC-derived neuronal models can fully replace animal experiments, more compounds should be tested and the available models must be further characterized to fully understand their applicability.

1 Introduction

Drug development and safety testing are often done using in vivo and ex vivo experiments. These experiments are however not fully predictive for adverse effects in humans. While human drug approval rates have been rising in recent years, only 10.4% of drugs in phase I of drug development will ultimately gain final approval (Hay et al., 2014). Most drugs thus fail the trajectory, often due to safety concerns related to the central nervous system (CNS). Even when compounds reach the market, drug attrition as a result of neurotoxicity and CNS problems remains a challenging concern (Onakpoya et al., 2016). An increased risk of seizures, i.e., periods of abnormal, rhythmic and uncontrolled hyper-excitability of neurons characterized by hyper-synchronicity of electrical activity (Easter et al., 2009; Jiriuska et al., 2013), is amongst the most encountered CNS related problems during pre-clinical drug development (Authier et al., 2016). Drug-induced seizures can be life threatening and are classified as severe adverse drug reactions. It is therefore of utmost importance that (increased) seizure liability can be detected early during drug development.

Since animal models frequently fail in human seizure liability predictions, their use is debated (Little et al., 2019). Consequently, in vitro alternatives are required to reduce the number of animal tests and to improve safety screening. However, in vitro seizure liability testing is challenging because the CNS contains many different cell types with a diverse range of drug targets that can all have effects on brain function and excitability (Easter et al., 2009). In vitro models for seizure liability screening should ideally thus model the in vivo brain as closely as possible (Westerink, 2013).

The most commonly used ex vivo model for seizure liability assessment in drug research is the brain slice assay (Authier et al., 2016). While the brain slice assay accurately mimics the in vivo organization of the brain (Grainger et al., 2018), including active and intact neuronal networks, these slices have a relatively short life-span (Buskila et al., 2015). More importantly, brain slices are still of animal origin and recordings require specific expertise and equipment, and are labor
intensive, thereby limiting high-throughput screening (Grainger et al., 2018). The recent introduction of multiwell microelectrode array (mwMEA) recordings eliminates some of these concerns. For this technique, cells are grown on a culture surface area with an integrated array of micro-electrodes allowing for non-invasive recordings of extracellular local field potentials simultaneously at different locations in the in vitro network (for review see Johnstone et al., 2010). MEA recording yield a broad range of data on parameters that describe the state of the network. Parameters related to activity and synchronicity can be used to detect a hyper-active and/or hyper-synchronized state of the network, linked to seizure like events in the in vivo situation (Ishii et al., 2017; Bradley et al., 2018). Many cell types can be grown on MEAs, but rat primary cortical cultures are the current gold standard (Authier et al., 2016). Primary cortical cultures grown on MEAs possess many characteristics of in vivo neurons, such as development of spontaneous network activity including (network) bursting (Cotterill et al., 2016) and responsiveness to neurotransmitters, pharmacological agents and toxicological modulation (Hogberg et al., 2011; McConnell et al., 2012; Nicolas et al., 2014; Valdivia et al., 2014; Hordebrink et al., 2016). Rodent cortical cultures grown on mwMEAs have been used for seizure liability assessment with positive results as known seizurogenic compounds increased spiking, (network) bursting activity and/or synchronicity (Bradley et al., 2018; Kreir et al., 2018; Fan et al., 2019). Although the combination of mwMEA and rodent primary cultures is promising, it is still based on animal cells.

As also outlined in NC3R’s Crack-It Neuratect Challenge2, there is a clear need for new in vitro drug screening models that are able to reliably detect seizures and can be used in a time and cost-efficient manner. These models should preferably be of human origin to circumvent interspecies translation and reduce the number of animal experiments. Human induced pluripotent stem cell (hiPSC)-derived neurons lack the ethical concerns of embryonic stem cells and animal experiments. It has been shown that hiPSC-derived neurons can be cultured on MEAs and exhibit spontaneous neuronal activity with (network) bursting behavior of mature neurons (Odawara et al., 2016; Paavilainen et al., 2018; Sasaki et al., 2019). On top of that, these hiPSC-derived neurons can be modulated with known neurotoxicants and drugs (Tukker et al., 2016; Hordebrink et al., 2017; Odawara et al., 2018). However, one of the challenges in using hiPSC-derived neurons is the long time it takes to generate the neurons, which can vary from weeks (Kuijlaars et al., 2016) till even months (Odawara et al., 2016), and the potential variability between batches of differentiated cells (Little et al., 2019). The introduction of commercially available hiPSC-derived neurons can help to overcome these concerns as they can be purchased in large, quality-controlled quantities (Anson et al., 2011; Little et al., 2019) and can be used for neurotoxicity screening following a relatively short culture duration (Tukker et al., 2018).

With the increasing availability of hiPSC-derived neurons, the differences between these models also increase. Human neuronal models can differ for example in the ratio of excitatory and inhibitory neurons as well as in the presence or absence of astrocytes. These factors greatly affect the (bursting) behavior of the hiPSC-derived model (Tukker et al., 2018). Also, variations in the differentiation protocol as well as the level of maturation before freezing may affect the culture. Therefore, this research aims to explore whether these models, despite their differences, are suitable for animal-free seizure liability testing. To that aim, we cultured three different commercially available hiPSC-derived neuronal models to assess the development of spontaneous neuronal network activity. Subsequently, these models were challenged with known seizurogenic compounds and the results were compared to data from rat primary cortical cultures that were exposed in parallel.

2 Materials and methods

2.1 Animals

All experiments were conducted in accordance with the Dutch law and approved by the Ethical Committee for Animal Experiments of Utrecht University. Animals were treated humanely, and all efforts were made to alleviate suffering. Primary cultures of rat cortical neurons were prepared from postnatal day (PND) 0-1 pups of timed pregnant Wistar rat dams (Envigo, Horst, The Netherlands) as described in section 2.2.1.

2.2 Chemicals

Neurobasal®-A medium, Dulbecco’s Modified Eagle Medium (DMEM)-F12, Fetal Bovine Serum (FBS), penicillin – streptomycin (5000 U/mL – 500 µg/mL for rat primary cortical culture media and 10,000 U/mL – 10,000 µg/mL for supplemented Brainphys™ medium), B27 supplement, N2 supplement, L-glutamine, 4’,6-diamidino-2-phenylindole (DAPI), donkey anti-rabbit Alexa Fluor® 488 and donkey anti-mouse Alexa Fluor® 594 were obtained from Life Technologies (Bleiswijk, The Netherlands). Goat anti-chicken Alexa Fluor® 647 was obtained from Jackson ImmunoResearch Europe (Ely, UK). Paraformaldehyde (PFA) was obtained from Electron Microscopy Sciences (Hatfield, Pennsylvania, USA). Rabbit anti-

β(III) tubulin (Ab18207), mouse anti-S100β (Ab11178) and chicken anti-β-actin (Ab5392) were obtained from Abcam (Cambridge, United Kingdom). FluorSave was obtained from Calbiochem (San Diego, California, USA). iCell® Neural Supplement B and Nervous System Supplement were provided by Cellular Dynamics International (Madison, WI, USA). BrainPhys™ neuronal medium was obtained from StemCell Technologies (Cologne, Germany). Neuro.4U® basal medium A and CNS.4U® supplement were provided by Ncardia (Leiden, The Netherlands). SynFire® seeding basal medium, short-term basal medium, long-term basal medium and accessory supplements were provided by Neucyte (Sunnyvale, CA, USA). Laminin (L2020), 50% polyethyleneimine (PEI) solution, sodium borate, boric acid and all other chemicals (unless stated otherwise) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Stock solutions of strychnine HCl were made in purity-checked dimethyl sulfoxide (DMSO) and stored at 4°C. 4-Aminopyridine (4-AP) was freshly dissolved in medium at the day of experiments. Stock solutions of picrotoxin (PTX) were made in ethanol (EtOH; VWR, Amsterdam, The Netherlands) on the day of the experiment. Final concentration of solvent in exposure solutions was always kept at or below 0.1% (vol/vol).

2 http://www.NC3Rs.org.uk
2.3 Cell culture

Primary rat cortical cultures and all hiPSC-derived neuronal co-cultures were kept at 37°C in a humidified 5% CO2: incubator. 48-Wells MEA plates (Axion BioSystems Inc., Atlanta, GA, USA) and µ-slide 8-well chambered coverslips (Ibidi GmbH, Planegg, Germany) were pre-coated with 0.1% PEI solution diluted in borate buffer (24 mM sodium borate/50 mM boric acid in Milli-Q, pH adjusted to 8.4). See Table 1 for an overview of the culture details of the four different models, including the day at which their chemical sensitivity was assessed (exposure DIV). Notably, the exposure days used to determine the drug response differs between hiPSC-derived neuronal models as a consequence of manufacturer’s recommendations. Nevertheless, all models did show a mature network phenotype with (network) bursting on the day of exposure.

Tab. 1: Composition, density and exposure DIV of the different neuronal culture models

<table>
<thead>
<tr>
<th>Cell models</th>
<th>Cell types (%)</th>
<th>Excitatory: inhibitory neurons (ratio)</th>
<th>Seeding density</th>
<th>Exposure DIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>iCell® Glutamatergic neurons – iCell® Astrocytes co-culture</td>
<td>Neurons (85%, of which 70% glutamatergic and 30% GABAergic) / Astrocytes (15%)</td>
<td>2.3:1</td>
<td>140k</td>
<td>DIV14</td>
</tr>
<tr>
<td>CNS.4U® co-culture</td>
<td>Glutamatergic neurons (~40%) / GABAergic neurons (~40%) / Dopaminergic neurons (~10%) / Astrocytes (~10%)</td>
<td>1:1</td>
<td>36k</td>
<td>DIV23</td>
</tr>
<tr>
<td>SynFire® iNs co-culture</td>
<td>Glutamatergic neurons (52%) / GABAergic neurons (22%) / Astrocytes (26%)</td>
<td>2.3:1</td>
<td>270k</td>
<td>DIV28</td>
</tr>
<tr>
<td>Rat primary cortical neurons</td>
<td>Neurons (55%, of which 70-80% glutamatergic and 20-30% GABAergic) / Astrocytes (45%)</td>
<td>3:1</td>
<td>100k</td>
<td>DIV9-11</td>
</tr>
</tbody>
</table>

*a* Ncardia https://ncardia.com/product/cns.4u-sup-sup-kit.html#product_specification accessed on June 20, 2019
*b* Markram et al., 2004
*c* Görtz et al., 2004; Tukker et al., 2016

2.3.1 iCell® Glutamatergic neurons – iCell® Astrocytes co-culture

iCell® Glutamatergic neurons (Lot# 103288; Cellular Dynamics International, Madison, WI, USA) and iCell® Astrocytes (Lot# 11493, 1414 and 1444; Cellular Dynamics International, Madison, WI, USA) were thawed and cultured according to manufacturer’s protocol. Briefly, each cell type was thawed separately in supplemented BrainPhys™ medium (BrainPhys™ medium supplemented with 2% iCell® Neural Supplement B, 1% Nervous System Supplement, 1% N2, 1% penicillin – streptomycin and 0.1% laminin). The cell pellet was diluted in dotting medium (supplemented BrainPhys™ medium with 10% laminin) at a density of 15k cells/µL for iCell® Glutamatergic neurons or 6,6k cells/µL for iCell® Astrocytes. Before plating, iCell® Glutamatergic neurons and iCell® Astrocytes were premixed into a co-culture containing 120k iCell® Glutamatergic neurons (85%) and 20k iCell® Astrocytes (15%) and then plated in 11 µL droplets (140k cells/droplet) over the electrode field of pre-coated MEA wells or in a chamber of the µ-slide coverslip. Cells were allowed to adhere for ~1hr before 300 µL (MEA) or 200 µL (coverslip) room temperature (RT) supplemented BrainPhys™ medium was added. 50% Medium changes with RT supplemented BrainPhys™ medium took place at DIV1, 2, 4, 6, 8, 10, 12 and 14.

2.3.2 CNS.4U® culture

CNS.4U® (Lot# CN39CL_V-2M and CN59CL_V-x2; Ncardia, Leiden, The Netherlands) were obtained as a mixture of ~40% glutamatergic neurons, ~40% GABAergic neurons, ~10% dopaminergic neurons and ~10% astrocytes. Cells were thawed and cultured according to manufacturer’s protocol. Briefly, a vial was thawed in Neuro.4U® basal medium A and the cell pellet was dissolved in complete Neuro.4U® medium A (basal medium and supplement) at a density of 12k cells/µL. Cells were plated in 3 µL droplets (36k cells in total) directly over the electrode field of pre-coated MEA wells or in the chamber of the µ-slide coverslip. Cells were allowed to adhere for ~1hr before 300 µL (MEA) or 200 µL (coverslip) RT Neuro.4U® medium was added. At DIV1, 7, 14 and 21, 100% medium changes were performed with complete Neuro.4U® medium. At DIV2, 4, 9, 11, 16, 18, 50% of the medium was replaced with complete Neuro.4U® medium.

2.3.3 SynFire® iNs co-culture

SynFire® glutamatergic neurons (Lot#104, 109 and 116), SynFire® GABAergic neurons (Lot#105, 109, 110 and 116) and Synfire® astrocytes (Lot#12854 and 13029; all from NeuCyte, Sunnyvale, CA, USA) were thawed and cultured according to manufacturer’s protocol. In short, each cell type was thawed separately in DMEM-F12 medium. The cell pellet was dissolved in complete seeding medium (containing seeding supplement) at a density of 10k cells/µL (for all cell types). Next, a mixture was made containing 140k glutamatergic neurons (52% of total cell number), 60k GABAergic neurons (22%) and 70k astrocytes (26%). The mixture was plated in 50 µL droplets (270k cells in total) over the electrode field of pre-coated MEA wells or in a chamber of the µ-slide coverslip. Cells were left overnight to adhere. Next day (DIV1), 250 µL (MEA) or 150 µL (coverslip) RT complete short-term maintenance medium (containing short-term supplement) was added. At DIV3 and 5, 50% medium changes with complete short-term maintenance medium took place. The remaining 50% medium changes at DIV7, 10, 13, 16, 19, 22 and 25 were performed with RT complete long-term maintenance medium (containing long-term supplement A and B).

2.3.4 Rat primary cortical culture

Primary rat cortical cells were isolated from PND0-1 Wistar rat pups as described previously (Dingemans et al., 2016; Tukker et al., 2016). Briefly, PND0-1 pups were decapitated and cortices were rapidly dissected on ice and kept in dissection medium.
(Neurobasal®-A supplemented with 25 g/L sucrose, 450 µM 1-glutamine, 30 µM glutamate, 1% penicillin/streptomycin and 10% FBS, pH 7.4) during the entire procedure. Cortices were dissociated to a single-cell suspension by mincing with scissors, triturating, and filtering through a 100 µm mesh (EASYstrainer, Greiner). The cell suspension was diluted to 2 x 10⁵ cells/mL.

Droplets of 50 µL (100k cells in total) were placed on the electrode fields in wells of pre-coated MEA plates. Cells were left to adhere for ~2 h before adding 450 µL dissection medium. At DIV2, 90% of the dissection medium was replaced with glutamate medium (Neurobasal®-A supplemented with 25 g/L sucrose, 450 µM 1-glutamine, 30 µM glutamate, 1% penicillin/streptomycin and 2% B27 supplement, pH 7.4) to prevent glial overgrowth. At DIV4 90% of the glutamate medium was replaced with FBS medium (Neurobasal®-A supplemented with 25 g/L sucrose, 450 µM 1-glutamine, 1% penicillin/streptomycin and 10% FBS, pH 7.4).

2.4 Immunocytochemistry

The µ-slide coverslips with different hiPSC-derived neuronal co-cultures were fixated on the day of exposure with 4% PFA in 0.1 M PBS (pH 7.4) for 15 min at RT. Following fixation, coverslips were quenched for PFA, permeabilized and incubated for 20 min at RT with 20 mM NH4Cl in blocking buffer (2% bovine serum albumin and 0.1% saponin in PBS) as described previously (Tukker et al., 2019). Hereafter, coverslips were incubated overnight at 4°C with rabbit anti-

2.5 MEA measurements

Each well of a 48-well MEA plate contains 16 nanotextured gold micro-electrodes (~40-50 µm diameter; 350 µm spacing) with 4 integrated ground electrodes yielding a total of 768 channels that can simultaneously be recorded. Spontaneous electrical activity was recorded on the days of medium changes and on the day of exposure (Table 1) as described previously (Nicolas et al., 2014; Tukker et al., 2019). In short, signals were recorded using a Maestro 768-channel amplifier with integrated heating system and temperature controller and a data acquisition interface (Axion BioSystems Inc., Atlanta, GA, USA). Data acquisition was managed with Axion’s Integrated Studio (AxIS 2.4.2.13) and recorded as .RAW files. All channels were sampled at the same time with a gain of 1200x and a sampling frequency of 12.5 kHz/channel with a 200-5000 Hz band-pass filter. Prior to the recording, MEA plates were allowed to equilibrate for ~10 min in the Maestro.

To determine effects of the known seizurogenic compounds PTX, 4-AP and strychnine on spontaneous activity (spiking and (network) bursting behavior) of the different cell cultures, activity was recorded prior to exposure to generate a baseline recording. Immediately following this recording, cells were exposed to the seizurogenic compounds or the appropriate solvent control and activity was recorded for another 30 min plus the time it took to expose all wells. Concentrations tested were determined by the NC3R CRACK-it team based on the list from the HESI NeuTox MEA Subteam, and were in line with earlier studies assessing seizure liability (Kreir et al., 2018). Each well was exposed to only one single concentration of one compound in order to prevent receptor (de)sensitization or other compensatory cellular processes. For each experimental condition, MEA plates from at least two different plating rounds or culture preparations were used.

2.6 Data analysis and statistics

To determine (modulation of) spontaneous activity, .RAW data files were re-recorded to obtain Alpha Map files. In this re-recording, spikes were detected with the AxIS spike detector (Adaptive threshold crossing, Ada BandFit v2) and a variable threshold spike detector set at 7x (rat p

The effects of seizurogenic compounds on spontaneous activity were determined by comparing the baseline activity with activity following exposure. A custom-made MS Excel macro was used to calculate treatment ratios (TR) per well for the different metric parameters (Table 2) by: (parameter_exposure/parameter_baseline) x 100%. Hereafter, TRs were normalized to appropriate vehicle control (medium, DMSO or EtOH). To prevent inclusion of exposure artefacts, effect analysis was performed in the window of 20-30 min post-exposure for all tested compounds.

Wells that showed an effect two times SD above or below average were considered outliers and removed for further data analysis (4% for iCell® Glutaneurons – iCell® Astrocytes co-culture; 3% for CNS.4® cu-culture; 4% for SynFire® iNs co-culture and 3% for rat primary cortical culture). Concentration-dependent effects were determined by a one-way Welch
Tab. 2: Overview and description of different metric parameters
Adapted from Tukker et al., 2018

<table>
<thead>
<tr>
<th>Metric parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean spike rate (MSR)</td>
<td>Total number of spikes divided by recording time (Hz)</td>
</tr>
<tr>
<td>ISI coefficient of variation</td>
<td>Standard deviation ISI (time between spikes) divided by the mean ISI. Measure for spike regularity: 0 indicates perfect spike distribution, &gt;1 signals bursting</td>
</tr>
<tr>
<td>Number of active electrodes</td>
<td>Average number of electrodes with a spike rate of at least 0.1 spike/s</td>
</tr>
<tr>
<td>Number of active wells</td>
<td>Number of wells that exhibits activity that meets spike criteria</td>
</tr>
<tr>
<td>Mean burst rate (MBR)</td>
<td>Total number of bursts divided by recording time (Hz)</td>
</tr>
<tr>
<td>Number of bursting electrodes</td>
<td>Average number of electrodes in the well with number of bursts/second that is higher than the burst criterion of 0.005 bursts/s</td>
</tr>
<tr>
<td>Number of bursting wells</td>
<td>Number of wells that meets the bursting criteria</td>
</tr>
<tr>
<td>Burst duration</td>
<td>Average time from the first spike in a burst till the last spike (s)</td>
</tr>
<tr>
<td>Number of spikes per burst</td>
<td>Average number of spikes occurring in a burst</td>
</tr>
<tr>
<td>Mean inter-spike interval (ISI) within burst</td>
<td>Mean inter-spike interval within a burst (s)</td>
</tr>
<tr>
<td>Inter-burst interval (IBI)</td>
<td>Time between the last spike of a burst and the first spike of a subsequent burst (s)</td>
</tr>
<tr>
<td>IBI coefficient of variation</td>
<td>Standard deviation of IBI divided by the mean IBI. Measure for burst regularity</td>
</tr>
<tr>
<td>Burst percentage</td>
<td>Percentage of total number of spikes occurring in a burst</td>
</tr>
<tr>
<td>Mean network burst rate (MNBR)</td>
<td>Total number of network bursts divided by recording time (IBI)</td>
</tr>
<tr>
<td>Network burst duration</td>
<td>Average time from the first spike till the last spike in a network burst (s)</td>
</tr>
<tr>
<td>Number of spikes per network burst</td>
<td>Average number of spikes occurring in a network burst</td>
</tr>
<tr>
<td>Mean ISI within network burst</td>
<td>Average of the mean ISIs within a network burst (s)</td>
</tr>
<tr>
<td>Number of electrodes participating in network burst</td>
<td>Average number of electrodes with spikes that participate in the network burst</td>
</tr>
<tr>
<td>Network bursting wells</td>
<td>Number of wells that meets the network burst criteria</td>
</tr>
<tr>
<td>Network burst percentage</td>
<td>Percentage of total spikes occurring in a network burst</td>
</tr>
<tr>
<td>Network IBI coefficient of variation</td>
<td>Standard deviation of network IBI divided by the mean network IBI. Measure of network burst rhythmicity: value is small when bursts occur at regular interval and increases when bursts occur more sporadic</td>
</tr>
<tr>
<td>Network normalized duration IQR</td>
<td>Interquartile range of network bursts durations. Measure for network burst duration regularity: larger values indicate wider variation in duration.</td>
</tr>
<tr>
<td>Area under normalized cross-correlation</td>
<td>Area under inter-electrode cross-correlation normalized to the auto-correlations. The higher the value, the greater the synchronicity of the network.</td>
</tr>
<tr>
<td>Full width at half height (FWHH) of normalized cross-correlation</td>
<td>Width at half height of the normalized cross-correlogram. Measure for network synchrony: the higher the value, the less synchronized the network is.</td>
</tr>
</tbody>
</table>

ANOVA followed by a post hoc Dunnett test. P-values < 0.05 were considered statistically significant. All statistical analyses were performed in R version 3.6.0<sup>4</sup> using the DescTools package version 0.99.28<sup>5</sup>. Spider plots and heat maps were also created in R with the packages fmsb version 0.6.3<sup>6</sup> and pheatmap version 1.0.12<sup>6</sup>, respectively. All data are presented as mean ± standard error of the mean (SEM) from the number of wells (n) indicated, derived from at least 2 independent plating rounds (N).

## 3 Results

### 3.1 Immunofluorescent staining of the different hiPSC-derived neuronal models

As a first characterization, the three different hiPSC-derived neuronal co-cultures were labelled with microtubule-associated protein 2 (MAP2, marker for mature neurons), β(III) tubulin (general neuronal marker) and S100 calcium binding protein β (S100β, marker for astrocytes) antibodies to visualize the heterogeneity and complexity of the in vitro networks (Fig. 1). The iCell® Glutaneurons – iCell® Astrocytes co-culture (A), CNS.4U<sup>®</sup> co-culture (B) and SynFire® iNs co-culture (C) all show network formation with a high degree of complexity at their corresponding days of exposure (Table 1). Images also indicate that neurons and astrocytes are spread evenly through the culture.

These results show that all three different hiPSC-derived neuronal co-cultures consist of mature neurons and astrocytes. The data also indicate that neuronal networks are formed. The network complexity and heterogeneity are comparable to the degree of the complexity present in the rat primary cortical culture (Hondebrink et al., 2016). These data thus suggest that all models may exhibit spontaneous network activity.

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<sup>3</sup> fmsb: Functions for Medical Statistics Book with some Demographic Data. https://CRAN.R-project.org/package=fmsb
<sup>4</sup> Pheatmap: Pretty Heatmaps. https://CRAN.R-project.org/package=pheatmap

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Fig. 1: Immunofluorescent images of iCell® Glutaneurons – iCell® Astrocytes co-culture (A), CNS.4U® co-culture (B) and SynFire® iNs co-culture (C) illustrating (neuronal) network structures with MAP2 (mature neurons; grey), β(III) tubulin (general neuronal marker; green) and S100β (astrocytes; red) Nuclei are stained with DAPI (blue). Scale bar depicts 20 µm.

Fig. 2: Development of mean spike rate (MSR; left), mean burst rate (MBR; middle) and mean network burst rate (MNBR: right) in solid lines and percentage of spiking wells (left), bursting wells (middle) and network bursting wells (right) in dashed lines for iCell® Glutaneurons – iCell® Astrocytes co-culture (A), CNS.4U® co-culture (B) and SynFire® iNs co-culture (C)

Data are expressed as mean ± SEM from n = 26-208 wells, N = 5 plates for the iCell® Glutaneurons – iCell® Astrocytes co-culture; n = 4-170 wells, N = 5 plates for the CNS.4U® co-culture and n = 2-202 wells, N = 8 plates for the SynFire® iNs co-culture.

3.2 Development of spontaneous neuronal activity and (network) bursting behavior

To further test the applicability of these different hiPSC-derived neuronal models for seizure liability assessment, cells were cultured according to continuously improving manufacturer’s recommendations up to DIV14 (iCell® Glutaneurons – iCell® Astrocytes co-culture), DIV23 (CNS.4U® co-culture) or DIV 28 (SynFire® iNs co-culture) on mwMEAs to assess the development of spontaneous neuronal network activity and bursting behavior over time. All models develop spontaneous activity (Fig. 2). The mean spike rate (MSR) is relatively stable for the iCell® Glutaneurons – iCell® Astrocytes co-culture, whereas MSR of the SynFire® iNs co-culture increases during the entire culture period (Fig. 2C, left). The percentage of spiking wells is stable and comparable (>75%) for all models.

The mean burst rate (MBR) of the CNS.4U® co-culture is stable during the whole culture period, however the rate is low compared to the other two models, especially at the day of exposure (Fig. 2B, middle). The SynFire® iNs co-culture has...
the highest MBR at the end of the culture period (Fig. 2C, middle). The percentage of bursting wells is >75% for all three models at the day of exposure.

At the beginning of the culture period, the iCell® Glutaneurons – iCell® Astrocytes co-culture has a high mean network burst rate (MNBR; Fig 2A, right), especially compared to the other two models (Fig. 2B/C, right). However, this rate decreases rapidly over time to levels comparable with the other two models. At the day of exposure, all cultures exhibit a comparable MNBR, but the percentage of wells that exhibit network bursts is low in the CNS.4U® co-culture (<50%) compared to the other two models (>75%).

Further analysis of additional (network) burst related parameters reveals additional differences between the models, as is depicted in spider plots (Fig. 3). It becomes clear from the lay-out of these graphs that the models develop in a different manner. Whereas the burst duration becomes shorter during prolonged culture of the iCell® Glutaneurons – iCell® Astrocytes co-culture (Fig. 3A), it slightly increases for the other two models (Fig. 3B-C). For the iCell® Glutaneurons – iCell® Astrocytes co-culture (Fig. 3A) and the CNS.4U® co-culture (Fig. 3B) the number of spikes per network burst and network burst percentage increase over time, in contrast to the SynFire® iNs co-culture (Fig. 3C) where this pattern is relatively stable. The IBI is longest in early culture DIVs for the iCell® Glutaneurons – iCell® Astrocytes co-culture and the SynFire® iNs co-culture, whereas for the CNS.4U® co-culture the IBI is relatively stable during the entire culture period. The number of spikes per burst increases during the culture period resulting in an increasing burst percentage for the iCell® Glutaneurons – iCell® Astrocytes co-culture and the SynFire® iNs, whereas this is relatively stable for the CNS.4U® co-culture.

Fig. 3: Spider plots illustrating the differences in development of neuronal activity between the iCell® Glutaneurons – iCell® Astrocytes co-culture (A), CNS.4U® co-culture (B) and SynFire® iNs co-culture (C). The yellow and lighter green lines depict earlier developmental DIVs, whereas darker shades belong to DIVs later in development. The grey grid represents 0% at the center, whereas the outer ring represents 100%. For each model, the highest value of a parameter during the culture period of that model is set to 100%. The axis represent (counter clockwise): burst duration (BD), number of spikes per burst (NoSpB), burst percentage (BP), inter-burst interval (IBI), network burst duration (NBD), network burst percentage (NBP) and number of spikes per network burst (NoSpNB), from n = 15-208 wells, N = 5 plates for the iCell® Glutaneurons – iCell® Astrocytes co-culture; n = 4-167 wells, N = 5 plates for the CNS.4U® co-culture; n = 2-202 wells, N = 8 plates for the SynFire® iNs co-culture. 
Each row depicts one electrode, each tick mark represents one spike in a 200 s interval illustrating the pattern of activity of the iCell® Glutaneurons – iCell® Astrocytes co-culture (A), the CNS.4U® co-culture (B), the SynFire® iNs co-culture (C) and the primary rat cortical neurons (D) at the exposure DIV. The cumulative trace (green box) above each plot depicts the population spike time histogram, indicating synchronized activity between the different electrodes.

The pattern of spontaneous electrical activity at the exposure DIV, the day in vitro at which the chemical sensitivity of the compound was assessed, is depicted in spike raster plots (Fig. 4) and further described by different metric parameters (Table 3). The raster plots show that all three models exhibit a mature phenotype with synchronized firing with (network) bursts. The raster plots also confirm that the pattern and level of activity differs between the models. The higher MSR for the iCell® Glutaneurons – iCell® Astrocytes co-culture (Fig. 4A) and SynFire® iNs co-culture (Fig. 4C), as described in Table 3 and Fig. 2, is also visible in the raster plots. The spike time histogram (green box, Fig. 4) clearly shows the different levels of synchronicity that are outlined in Table 3, and indicate the high number of spikes within a network burst in the SynFire® iNs co-culture (C) and the primary rat cortical neurons (D) at the exposure DIV. The cumulative trace (green box) above each plot depicts the population spike time histogram, indicating synchronized activity between the different electrodes.

**Fig. 4:** Spike raster plots of the different hiPSC-derived co-cultures

**Tab. 3:** Mean ± SEM of different metric parameters at the exposure DIV

<table>
<thead>
<tr>
<th>Metric parameter (unit)</th>
<th>iCell® Glutaneurons – iCell® Astrocytes (DIV14)</th>
<th>CNS.4U® (DIV23)</th>
<th>SynFire® iNs (DIV28)</th>
<th>Rat cortical neurons (DIV11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSR (Hz)</td>
<td>64.5 ± 2.39</td>
<td>20.5 ± 1.60</td>
<td>81.3 ± 5.86</td>
<td>21.4 ± 0.82</td>
</tr>
<tr>
<td>ISI coefficient of variation</td>
<td>1.86 ± 0.029</td>
<td>1.93 ± 0.046</td>
<td>3.36 ± 0.15</td>
<td>4.74 ± 0.10</td>
</tr>
<tr>
<td>Number of active electrodes</td>
<td>14.4 ± 0.13</td>
<td>8.68 ± 0.29</td>
<td>13.3 ± 0.27</td>
<td>13.4 ± 0.23</td>
</tr>
<tr>
<td>Number of active wells</td>
<td>208</td>
<td>166</td>
<td>201</td>
<td>236</td>
</tr>
<tr>
<td>MBR (Hz)</td>
<td>0.44 ± 0.017</td>
<td>0.16 ± 0.014</td>
<td>0.69 ± 0.057</td>
<td>0.77 ± 0.028</td>
</tr>
<tr>
<td>Number of bursting electrodes</td>
<td>9.78 ± 0.28</td>
<td>3.96 ± 0.28</td>
<td>11.5 ± 0.43</td>
<td>13.2 ± 0.23</td>
</tr>
<tr>
<td>Number of bursting wells</td>
<td>208</td>
<td>141</td>
<td>172</td>
<td>234</td>
</tr>
<tr>
<td>Burst duration (s)</td>
<td>0.98 ± 0.036</td>
<td>1.15 ± 0.06</td>
<td>0.97 ± 0.033</td>
<td>0.32 ± 0.012</td>
</tr>
<tr>
<td>Number of spikes per burst</td>
<td>38.9 ± 1.35</td>
<td>28.7 ± 1.07</td>
<td>73.3 ± 3.50</td>
<td>22.7 ± 0.55</td>
</tr>
<tr>
<td>Mean ISI within burst (s)</td>
<td>0.03 ± 0.0007</td>
<td>0.045 ± 0.002</td>
<td>0.023 ± 0.001</td>
<td>0.02 ± 0.001</td>
</tr>
<tr>
<td>IBI (s)</td>
<td>33.8 ± 0.97</td>
<td>44.0 ± 2.22</td>
<td>37.66 ± 2.47</td>
<td>25.5 ± 0.94</td>
</tr>
<tr>
<td>IBI coefficient of variation</td>
<td>0.69 ± 0.019</td>
<td>0.80 ± 0.031</td>
<td>0.80 ± 0.046</td>
<td>0.83 ± 0.037</td>
</tr>
<tr>
<td>Burst percentage (%)</td>
<td>34.8 ± 0.76</td>
<td>34.7 ± 1.62</td>
<td>52.3 ± 1.84</td>
<td>83.3 ± 0.52</td>
</tr>
<tr>
<td>MNBR (Hz)</td>
<td>0.1 ± 0.006</td>
<td>0.08 ± 0.012</td>
<td>0.074 ± 0.005</td>
<td>0.073 ± 0.002</td>
</tr>
<tr>
<td>Number of spikes per network burst</td>
<td>0.92 ± 0.088</td>
<td>0.88 ± 0.076</td>
<td>2.12 ± 0.18</td>
<td>0.75 ± 0.015</td>
</tr>
<tr>
<td>Mean ISI within network burst (s)</td>
<td>0.004 ± 0.0002</td>
<td>0.007 ± 0.0004</td>
<td>0.003 ± 0.0002</td>
<td>0.004 ± 0.000</td>
</tr>
<tr>
<td>Number of electrodes network burst</td>
<td>13.2 ± 0.14</td>
<td>10.1 ± 0.36</td>
<td>14.4 ± 0.20</td>
<td>13.6 ± 0.15</td>
</tr>
<tr>
<td>Number of network bursting wells</td>
<td>206</td>
<td>54</td>
<td>154</td>
<td>225</td>
</tr>
<tr>
<td>Network burst percentage (%)</td>
<td>37.1 ± 1.32</td>
<td>29.8 ± 2.87</td>
<td>64.7 ± 2.53</td>
<td>93.0 ± 0.62</td>
</tr>
<tr>
<td>Network IBI coefficient of variation</td>
<td>0.73 ± 0.023</td>
<td>0.56 ± 0.039</td>
<td>0.83 ± 0.064</td>
<td>0.78 ± 0.035</td>
</tr>
<tr>
<td>Network normalized duration IQR</td>
<td>0.51 ± 0.043</td>
<td>0.51 ± 0.091</td>
<td>0.56 ± 0.13</td>
<td>0.71 ± 0.030</td>
</tr>
<tr>
<td>Area under normalized cross-correlation</td>
<td>0.11 ± 0.003</td>
<td>0.016 ± 0.002</td>
<td>0.25 ± 0.012</td>
<td>0.56 ± 0.011</td>
</tr>
<tr>
<td>FWHH of normalized cross-correlation</td>
<td>128 ± 1.60</td>
<td>210 ± 18.0</td>
<td>734 ± 21.3</td>
<td>228.2 ± 0.36</td>
</tr>
</tbody>
</table>
co-culture. Further details regarding the other developmental days of the hiPSC-derived co-cultures can be found in the supplemental material (Table S1-S3 and Fig. S1).2

As can be seen in the raster plots, rat primary cortical neurons exhibit a spike frequency comparable to the CNS.4U® co-culture (Fig. 4D, Table 3), whereas the burst frequency is more in line with the SynFire® iNs co-culture. Network burst activity of rat primary cortical neurons occurs in a frequency comparable to the CNS.4U® co-culture and the SynFire® iNs co-culture. The burst duration of the primary rodent culture is much lower than in the hiPSC-derived co-cultures. In rat primary cortical neurons most spikes are incorporated in (network) bursts as is reflected in the (network) burst percentage. This is in sharp contrast with hiPSC-derived neuronal models, where single spikes occur.

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**Fig. 5:** Toxicological modulation of spontaneous network activity with strychnine (left), PTX (middle) and 4-AP (right) of iCell® Glutaneurons – iCell® Astrocytes co-culture (black), CNS.4U® co-culture (blue), SynFire® iNs co-culture (green) and rat cortical neurons (striped)

Effects are depicted on mean spike rate (MSR; A), mean burst rate (MBR; B), mean network burst rate (MNB; C), burst duration (D) and network burst duration (E) as average in % change of control (solvent control set to 100%; dashed line) ± SEM from n = 3-17 wells, N = 2-3 plates for iCell® Glutaneurons – iCell® Astrocytes co-culture; n = 2-16 wells, N = 4 plates for CNS.4U® co-culture; n = 3-14 wells, N = 4-5 plates for SynFire® iNs co-culture and n = 19-29, N = 5-8 for rat primary cortical culture. * p < 0.05.
3.3 Seizure liability assessment

Since all models developed spontaneous neuronal activity and (network) bursting behavior, all hiPSC-derived neuronal models are amenable for neurotoxicity testing. To assess their applicability for seizure liability assessment, the different models were exposed to known seizurogenic compounds with different modes of action: strychnine (0.3-30 µM), 4-AP (1-100 µM) and PTX (0.1-10µM). We also exposed rat primary cortical neurons as the current gold standard for MEA seizure liability assessment.

Exposure to the known neurotoxicant strychnine, a glycine receptor antagonist, results in an increase of the MSR followed by a decrease at higher test concentrations in the SynFire Glutaneurons – iCell Astrocytes co-culture and the CNS.4U iNs co-culture. MBR increases in the CNS.4U iNs co-culture and rat primary cortical culture (Fig. 5A; left). However, compared to the rat primary cortical neurons, the increase starts in the SynFire iNs co-culture at a concentration 3 times lower. Strychnine significantly decreases the MSR of the iCell Glutaneurons – iCell Astrocytes co-culture and the CNS.4U iNs co-culture. MBR increases in the CNS.4U iNs co-culture and rat primary cortical culture (Fig 5B; left). However, this increase is only significant in the latter. At the highest concentration tested, the MBR significantly decreases in the iCell Glutaneurons – iCell Astrocytes co-culture and the CNS.4U iNs co-culture. Strychnine exposure significantly increases the MNBR in the rat primary cortical culture (Fig. 5C; left). However, this cannot be observed in the hiPSC-derived neuronal models. Burst duration increases in the CNS.4U iNs co-culture and the iCell Glutaneurons – iCell Astrocytes co-culture, but only significantly in the latter (Fig. 5D; left). A significant decrease in network burst duration following strychnine exposure can be observed in rat primary cortical neurons (Fig. 5E; left).

PTX, a widely used seizurogenic reference compound and GABAA-receptor antagonist, shows limited effects on the MSR of iCell Glutaneurons – iCell Astrocytes co-culture and CNS.4U iNs co-culture (Fig. 5A; middle). However, PTX significantly increases the MSR of the SynFire iNs co-culture and the rat primary cortical culture at the higher concentrations tested. Higher concentrations of PTX significantly increase the MBR of the SynFire iNs co-culture and the rat primary cortical culture, but have little effect on iCell Glutaneurons – iCell Astrocytes co-culture and the CNS.4U iNs co-culture (Fig. 5B; middle). In all cultures, PTX increases the MNBR (Fig. 5C; middle), but only significantly in the rat primary cortical culture. A significant increase of burst duration can be observed for the iCell Glutaneurons – iCell Astrocytes co-culture, whereas a decrease occurs in the rat primary cortical culture (Fig. 5D; middle). Network burst duration significantly increases for the iCell Glutaneurons – iCell Astrocytes co-culture and the SynFire iNs co-culture (Fig. 5E; middle). However, network burst duration decreases in the rat primary cortical culture.

4-AP, a known CNS stimulant and potassium channel blocker, only increases the MSR of the SynFire iNs co-culture (Fig. 5A; right). MBR increases when the iCell Glutaneurons – iCell Astrocytes co-culture and SynFire iNs co-culture are exposed, although not significantly (Fig. 5B; right). Exposure to 4-AP significantly decreases the MBR of the CNS.4U iNs co-culture. This is in contrast with the increased MBR following 4-AP exposure of the CNS.4U iNs co-culture (Fig. 5C; right). An increase can also be observed in the SynFire iNs co-culture, whereas the iCell Glutaneurons – iCell Astrocytes co-culture and rat primary cortical culture are relatively unaffected. Following exposure to 4-AP, burst duration significantly decreases in all hiPSC-derived co-cultures, but this decrease occurs at different concentrations (Fig. 5D; right). Network burst duration increases in the SynFire iNs co-culture, but decreases in the CNS.4U iNs co-culture and rat primary cortical culture (Fig. 5E; right).

The activity patterns of the different models change following exposure. More details can be found in the supplemental data (Fig. S2 for strychnine, Fig. S3 for PTX and Fig. S4 for 4-AP).

We created a heatmap of the concentration-response curves of the three test compounds on the four different models to further illustrate the effects on different metric parameters (Fig. 6). By the different colour pattern in the heatmap, it becomes clear that strychnine, PTX and 4-AP have different mechanisms of action. For all compounds, but particularly for PTX, it is visible that network burst related parameters are most affected when it comes to excitation. Burst related parameters are more sensitive to inhibition, especially following 4-AP or strychnine exposure.

Several similarities and differences between the models can be observed. Following exposure to strychnine, seizurogenicity is most noticeable in the network burst related parameters in the SynFire iNs co-culture. Inhibition following strychnine exposure is most pronounced in the CNS.4U iNs co-culture.

When the models are exposed to PTX, all models show a clear excitation. The iCell Glutaneurons – iCell Astrocytes co-culture and the CNS.4U iNs co-culture react in a comparable pattern, whereas the SynFire iNs co-culture shows more similarities with the rat primary cortical culture.

The only model that is excitabile with 4-AP on all three traditional parameters (MSR, MBR and MNBR) is the SynFire iNs co-culture. The iCell Glutaneurons – iCell Astrocytes co-culture shows excitability on burst and network burst parameters at concentrations lower than the rat primary cortical culture. Overall, the SynFire iNs co-culture is most easily excited, whereas the CNS.4U iNs co-culture most easily inhibited.

4 Discussion

In this study we investigated the applicability of three different human iPSC-derived neuronal models for more predictive in vitro seizure liability assessment without the use of animals.

Our immunocytochemistry data (Fig. 1) demonstrated the mixed nature of the different hiPSC-derived neuronal models. The images show that all models form highly complex neuronal networks comparable with the ones formed by rat primary cortical cultures (Hondebrink et al., 2016). All three models develop spontaneous neuronal network activity and (network) bursting behavior over time (Fig. 2-4). However, the level of activity and bursting varies between the different models, despite culturing according to manufacturer’s protocol. There are several possible explanations for these differences. One likely explanation relates to cell density as the amount of cells plated in mwMEAs has been shown to greatly influence the spontaneous firing rate, with higher densities resulting in a higher spontaneous activity rate (Jun et al., 2007). In line with this notion, the model with the lowest seeding density (CNS.4U iNs co-culture; Table 1) also has the lowest spike and burst.
Fig. 6: Heatmap of the effects of strychnine (0.3 – 30 μM; top), PTX (0.1 – 10 μM; middle) and 4-AP (1 – 100 μM; bottom) on selected metric parameters on iCell® Glutaneurons – iCell® Astrocytes co-culture (black), CNS.4U® co-culture (blue) SynFire® iNs co-culture (green) and rat primary cortical neurons (grey)

Color scaling is based on the magnitude of the % of change relative to the control based on n = 1-18 wells, N = 2-3 plates for iCell® Glutaneurons – iCell® Astrocytes co-culture; n = 1-16 wells, N = 4 plates for CNS.4U® co-culture; n = 2-15 wells, N = 4-5 plates for SynFire® iNs co-culture and n = 11-30 wells, N = 5-8 plates for rat primary cortical culture. For white compartments no average could be calculated.

activity (Fig. 2). The cell density of the CNS.4U® co-culture is also low compared to densities reported in literature (Odashara et al., 2016; Matsuda et al., 2018; Sasaki et al., 2019). Cell densities of the iCell® Glutaneurons – iCell® Astrocytes co-culture and in particular of the SynFire® iNs co-culture are more in line with literature and this is paralleled by a higher spontaneous activity level. Spontaneous firing rate is also enhanced by the presence of astrocytes (Tang et al., 2013; Ishii et al., 2017; Tukker et al., 2018). The higher ratio of astrocytes in the iCell® Glutaneurons – iCell® Astrocytes co-culture and SynFire® iNs co-culture (Table 1) could contribute to their higher spike and burst rate (Fig 2A-B). The SynFire® iNs co-culture is the culture with the highest ratio of astrocytes, which coincides with the highest degree of synchronization (Fig. 4). Additionally, human iPSC-derived neuronal cultures with a high ratio of glutamatergic neurons exhibit more synchronous bursting events compared to cultures with a low proportion glutamatergic neurons (Sasaki et al., 2019). At the first culture days, the iCell® Glutaneurons – iCell® Astrocytes co-culture, the culture with the highest number of glutamatergic neurons, exhibited the highest MNBR (Fig. 2C). However, at the day of exposure, MNBR was comparable for all cultures. Despite their differences, all the models tested, exhibited a pattern of development comparable to that of the rat primary cortical culture (Brown et al., 2016) with MSR, presence of (network) bursts and synchronized activity increasing over time.
Since all models develop spontaneous activity, a neurotoxicity screen was performed using the known seizurogenic compounds strychnine, PTX and 4-AP. These compounds are used in vivo studies to induce seizures (Peña and Tapia, 2000; Mackenzie et al., 2002; Alachkar et al., 2018). Strychnine and 4-AP have also been reported to cause convulsions (Pickett and Enns, 1996; van Berlo-van de Laar et al., 2015). Overall, our data indicate the potential of the hiPSC-derived neuronal models for in vitro seizure liability assessment as all three compounds increased activity and synchronicity of the networks.

The potential for seizure liability assessment is further confirmed when comparing our data with two seizure prediction patterns described in literature (Bradley et al., 2018). Seizure prediction pattern 1 is indicative for the response induced by GABA_A-receptor antagonists, such as PTX. This pattern is characterized by an overall increase in activity as well as an increased organization and synchronization, reflected in an increased number of spikes per burst, increased ISI coefficient of variation, increased burst duration and a decrease in IBI. All four tested models showed an increase in overall activity following exposure to PTX (Fig. 5). Spiking activity increased in a concentration-dependent manner only in the SynFire® iNs co-culture and primary rat cortical neurons similar to results reported in literature for rat primary cortical cultures (Mack et al., 2014; Kreir et al., 2018) and hiPSC-derived neuronal co-cultures (Kreir et al., 2019). A rise in activity was observed for all models in (network) bursting and comparable to results from Kreir et al. (2019). Also, the organization and synchronization are enhanced according to prediction pattern 1 (Fig. 6).

Seizure prediction pattern 2 is characterized by a decrease in overall activity and a deterioration of network organization, reflected in an increased IBI, a decreased number of spikes per burst and a decreased ISI coefficient of variation and burst duration. This pattern is indicative for exposure to the glycine receptor antagonist strychnine (Bradley et al., 2018). The iCell® Glutaneurons – iCell® Astrocytes co-culture and CNS.41® co-culture exhibited decreased activity (Fig. 5) and network disintegration (Fig. 6) following the described prediction pattern. Changes in spike and burst activity at the lowest test concentration in the iCell® Glutaneurons – iCell® Astrocytes co-culture are statistically significant, indicating a high sensitivity of this model for the tested glycine receptor agonist. However, this does not necessarily indicate biological relevance. In contrast to earlier reported findings (Bradley et al., 2018; Kreir et al., 2018), activity of the rat primary cortical culture increased following strychnine exposure. However, this can be due to the differences in maturation stage between E18-19 and PND1 cortices. During embryonic development, glycine receptors have an excitatory function, whereas they become inhibitory later on (Dutez et al., 2012).

Exposure to the potassium channel blocker 4-AP is reported to follow seizure prediction pattern 1 (Bradley et al., 2018), however our data do not support that notion (Fig. 5-6). An increase in overall activity is only observed in the SynFire® iNs co-culture and an increase in bursting activity in the iCell® Glutaneurons – iCell® Astrocytes co-culture (Fig. 5), but the increased organization and synchronization to match pattern 1 is not present (Fig. 6). Kreir et al. (2019) reported an increase in spike and network burst frequency following exposure of hiPSC-derived neuronal cultures to strychnine. This matches with our SynFire® iNs co-culture data. The other cultures showed a decreased activity pattern, but with a network deterioration that did not match prediction pattern 2. Rat hippocampal neurons cultured on MEA plates exhibit increased activity (Fan et al., 2019). It could thus be that a more hippocampal-like phenotype is required to detect the seizurogenic activity of 4-AP.

When comparing the differences in sensitivity of the models, the aforementioned points in regards to ratio of excitatory neurons to inhibitory neurons, number of astrocytes present and seeding density must be kept in mind, since these can all affect chemical sensitivity. Although cells were exposed at a moment of network maturation and according to continuously improving manufacturer’s protocol, network complexity (e.g. the number of contacts that the different cell types establish) can still be different for the different models. This may influence how the models react to chemical insults. Also, receptor expression may differ between the different hiPSC-derived neuronal models. Spontaneous activity and drug responses are dictated by a complex interplay of a large number of different receptor types, ion channels and transporters all consisting of different subtypes. To better understand the differences in chemical sensitivity between the models, full protein profiles of the expression levels of the different receptors, channels and transporters present must be made. However, not only the number of receptors, channels and transporters are crucial, also the interplay, location and distribution of the receptors, channels and transporters define chemical sensitivity.

We showed that all tested models were able to form spontaneously active networks with (network) bursting behavior. Our data also indicated that these models can be used for animal-free in vitro seizure liability assessment. More importantly, the iPSC-derived models were capable of detecting seizures at the same level or even better than the rat primary cortical neuronal culture. However, in order to detect seizures in vitro with an MEA system, multiple parameters must be assessed. When only spike behavior is investigated, seizure-like activity might be missed. Also, when comparing data from hiPSC-derived neuronal models with rat primary cortical cultures, it must be kept in mind that there are differences between rodents and humans. Hence, rodent experiments are not always predictive for human risk (Olson et al., 2000). It is thus logical that results differ between in vitro assays that are performed using human cells and assays using rodent cells (Hondebrink et al., 2017). This in turn results in different hits and sensitivities (Malik et al., 2014). However, there are also cases where the rodent and human system show comparable results (Kasteel and Westerink, 2017). The discrepancies between the rat primary rodent cortical culture and our hiPSC-derived neuronal models should not be the main concern. Rather, attention must be on further characterization and optimization of hiPSC-derived neuronal models to make them more closely resemble the human brain, specifically those parts that are involved in epileptic activity such as the amygdala, hippocampus and neocortex (Rogawski and Löscher, 2004). Also, it must be kept in mind that this study was done with a limited number of test compounds. To fully understand the applicability of hiPSC-derived neuronal models for in vitro seizure liability assessment, a broader set of compounds should be tested, including besides seizurogens, anti-seizurogenic and negative compounds.

Nevertheless, the current data show that hiPSC-derived neuronal models may already be used as a first screen for epileptic activity before performing follow-up studies. With that we are one step closer to animal-free in vitro seizure liability assessment. Nevertheless, to fully move away from animal testing for in vitro seizure liability assessment, hiPSC-models must be developed in which all targets involved in the onset and duration of seizures are present and validated using a large library of diverse reference compounds. With respect to the comparison of hiPSC-derived neuronal cultures with rodent primary...
neuronal cultures; such a comparison cannot be performed one on one. Actually, our data show some striking differences between the different models, highlighting the need to move to human model systems, thereby eliminating the need for interspecies extrapolation.

References


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

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