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Human iPSC-derived neuronal models for *in vitro* neurotoxicity assessment

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ABSTRACT

Neurotoxicity testing still relies on ethically debated, expensive and time consuming *in vivo* experiments, which are unsuitable for high-throughput toxicity screening. There is thus a clear need for a rapid *in vitro* screening strategy that is preferably based on human-derived neurons to circumvent interspecies translation. Recent availability of commercially obtainable human induced pluripotent stem cell (hiPSC)-derived neurons and astrocytes holds great promise in assisting the transition from the current standard of rat primary cortical cultures to an animal-free alternative.

We therefore composed several hiPSC-derived neuronal models with different ratios of excitatory and inhibitory neurons in the presence or absence of astrocytes. Using immunofluorescent stainings and multi-well micro-electrode array (mwMEA) recordings we demonstrate that these models form functional neuronal networks that become spontaneously active. The differences in development of spontaneous neuronal activity and bursting behavior as well as spiking patterns between our models confirm the importance of the presence of astrocytes. Preliminary neurotoxicity assessment demonstrates that these cultures can be modulated with known seizurogenic compounds, such as picrotoxin (PTX) and endosulfan, and the neurotoxicant methylmercury (MeHg). However, the chemical-induced effects on different parameters for neuronal activity, such as mean spike rate (MSR) and mean burst rate (MBR), may depend on the ratio of inhibitory and excitatory neurons. Our results thus indicate that hiPSC-derived neuronal models must be carefully designed and characterized prior to large-scale use in neurotoxicity screening.

1. Introduction

Human induced pluripotent stem cell (hiPSC)-derived neuronal cultures are becoming increasingly important for *in vitro* neurotoxicity testing. These neuronal cultures could provide an alternative for costly, time consuming and ethically debated animal experiments or *in vitro* work with primary cultures. Moreover, there is a clear need for alternatives of human origin since animal-based models do not always mimic the human physiology and can therefore in some cases be poor predictors for human adverse outcomes (Hartung, 2008).

The use of hiPSC-derived neurons for neurotoxicity testing would circumvent the need for interspecies translation. As such, the recent commercial availability of these neuronal models holds great promise in assisting the transition from the current gold standard of rat primary cortical cultures (Alloisio et al., 2015; Dingemans et al., 2016; Hogberg et al., 2011; Hondebrink et al., 2016; McConnell et al., 2012; Nicolas et al., 2014; Valdivia et al., 2014; Vassallo et al., 2017) to hiPSC-derived models (Tukker et al., 2016). We have recently shown that these commercially available models have the potential to develop spontaneously active neuronal networks that can be used for screening and prioritization of chemically induced effects on neuronal activity (Tukker et al., 2016), whereas others have already shown that hiPSC-derived neurons exhibit the behavior and function of mature neurons (Hyysalo et al., 2017; Odawara et al., 2016; Paavilainen et al., 2018). While costly in comparison to (rodent) primary cultures and (human) neural progenitor cells, an important benefit of hiPSC-derived neurons is the rapid development of a functional neuronal network, in contrast to the time consuming differentiation of hiPSCs into neural progenitor cells that subsequently need to be cultured into functional neurons, a process that can take several weeks (Görtz et al., 2004; Hyysalo et al., 2017; Kuijlaars et al., 2016) till months (Odawara et al., 2016; Paavilainen et al., 2018). Opting for these commercially obtainable cells comes with the additional benefit that they are available in high quantity, allowing for efficient screening (Anson et al., 2011). On the other hand, besides being costly, hiPSC-derived neurons have the disadvantage of being less well characterized for electrophysiological studies and have so far been little used for neurotoxicity screening.

In vitro screening models should represent the *in vivo* situation as

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Table 1
Composition and density of the different cell models.

Type of culture	Cell types and ratio	Seeding density/ well or chamber
iCell® Glutaneuron monoculture	100% Glutaneurons	80,000
iCell® Glutaneuron-iCell® neuron co-culture	20% iCell neurons 80% Glutaneurons	80,000
iCell® Glutaneuron-iCell®-Astrocyte co-culture	50% Glutaneurons 50% Astrocytes	150,000
iCell® Glutaneuron-iCell® neuron-iCell® Astrocyte co-culture	40% Glutaneurons 10% iCell neurons 50% Astrocytes	140,000

closely as possible and must contain sufficient complexity to answer the research question (Pamies and Hartung, 2017; Westerink, 2013). With regard to neurotoxicity testing, this means that the model must capture the complexity and diversity of cell types of the human brain. It thus must form functional networks with a controlled balance of excitatory and inhibitory neurons as well as supportive cells. However, until now, most studies focused on cell cultures containing only hiPSC-derived neurons, whereas other neuronal cell types and/or astrocytes should also be included. Astrocytes, for example, play an important role in the regulation of the development of neurons (Tang et al., 2013) and also have been shown to enhance the development of neuronal networks and action potentials in human iPSC-derived co-cultures (Ishii et al., 2017). The addition of astrocytes to the neuronal model has also been reported to increase synchrony of the networks (Amiri et al., 2013). Moreover, inclusion of astrocytes also adds specific, physiologically relevant targets for toxic insults to the culture that would not be present in a pure neuronal model. Notably in this respect, there is growing evidence that astrocytes can play a protective role against chemical-induced neurotoxicity (Takemoto et al., 2015; Wu et al., 2017).

Currently, however, there is still a knowledge gap that has to be filled before co-cultured hiPSC-derived models can replace the primary rat cortical cultures and become the new gold standard for mwMEA experiments. For example, the role of different cell types in hiPSC-derived neuronal cultures is largely unexplored and concerns regarding the immature phenotype, including limited bursting, have been raised (Meneghello et al., 2015). We therefore composed different (co-)cultures of iCell Glutaneurons® (~90% excitatory glutamatergic neurons/ 10% inhibitory GABAergic neurons) and iCell neurons® (~30% excitatory glutamatergic neurons/70% inhibitory GABAergic neurons) in the absence or presence of astrocytes to explore the role of different cell types in development of neuronal activity and suitability for *in vitro* neurotoxicity testing, including seizure liability testing.

2. Materials and methods

2.1. Chemicals

N2 supplement, penicillin-streptomycin (10.000 U/mL–10.000 µg/mL), Geltrex, phosphate-buffered saline (PBS), fetal bovine serum (FBS), DMEM, trypsin-EDTA, 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). FluorSave was obtained from Calbiochem (San Diego, California, USA). iCell® Neurons Maintenance medium, iCell® Neurons Medium Supplement and Nervous System Supplement were obtained from Cellular Dynamics International (Madison, WI, USA). Rabbit anti-β(III)-tubulin (Ab18207), mouse anti-S100β (Ab11178) and rabbit anti-vGluT1 (Ab104898) were obtained from Abcam (Cambridge, United Kingdom). Mouse anti-vGAT (131001) was obtained from Synaptic Systems (Göttingen, Germany). BrainPhys neuronal medium was obtained from StemCell Technologies (Cologne, Germany). Paraformaldehyde (PFA) was obtained from Electron Microscopy Sciences (Hatfield, Pennsylvania, USA). Picrotoxin (PTX), endosulfan (α:β 2:1 99.9%), methylmercury (MeHg), 50% polyethyleneimine (PEI) solution, laminin, sodium borate, boric acid, bovine serum albumin and all other chemicals (unless described otherwise) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Stock solutions of endosulfan and MeHg were prepared in dimethyl sulfoxide (DMSO). For PTX, stock solutions were freshly prepared in ethanol (EtOH) directly before every experiment.

Table 2
Overview and description of used metric parameters.

Metric parameter	Description
Mean spike rate (MSR)	Total number of spikes divided by recording time (Hz)
ISI coefficient of variation	Standard deviation ISI (time between spikes) divided by the mean ISI. Measure for spike regularity: 0 indicates perfect spike distribution, > 1 signals bursting
Burst duration	Average time from the first spike in a burst till the last spike (s)
Number of spikes per burst	Average number of spikes occurring in a burst
Mean ISI within burst	Mean inter-spike interval within a burst (s)
Median ISI within burst	Median inter-spike interval within a burst (s)
Inter-burst interval (IBI)	Time between the last spike of a burst and the first spike of a subsequent burst (s)
Burst frequency	Total number of bursts divided by recording time (Hz)
IBI coefficient of variation	Standard deviation of IBI divided by the mean IBI. Measure for burst regularity
Burst percentage	Percentage of total number of spikes occurring in a burst
Network burst frequency	Total number of network bursts divided by recording time (Hz)
Network burst duration	Average time from the first spike till the last spike in a network burst (s)
Number of spikes per network burst	Average number of spikes occurring in a network burst
Mean ISI within network burst	Average of the mean ISIs within a network burst (s)
Median ISI within network burst	Average of the median ISIs within a network burst (s)
Number of spikes per network burst/channel	Average number of spikes in a network burst divided by the electrodes participating in that burst
Network burst percentage	Percentage of total spikes occurring in a network burst
Network IBI coefficient of variation	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
Network normalized duration IQR	Interquartile range of network bursts durations. Measure for network burst duration regularity: larger values indicate wide variation in duration.
Area under normalized cross-correlation	Area under inter-electrode cross-correlation normalized to the auto-correlations. The higher the value, the greater the synchronicity of the network.
Full width at half height of normalized cross-correlation	Width at half height of the normalized cross-correlogram. Measure for network synchrony: the higher the value, the less synchronised the network is.