

Human Neuronal Cells: Possibilities in Drug Safety Testing and Beyond

Human derived induced pluripotent stem cells (hiPSCs) have revolutionised research and are increasingly used for toxicology screening and disease modelling. Early detection of neurotoxicity induced by potential new therapies is a major challenge, and hiPSC-neuronal cells may provide a solution. These cells demonstrate considerable promise for uncovering drug-induced perturbations to neuronal function such as seizure, and their use extends further to sedation, anti-epileptic drug discovery and modelling of neurological diseases.

Within the field of drug safety hiPSC-cardiomyocytes are now routinely used for early identification of cardiac liabilities in drug discovery. This shift occurred following implementation of the Comprehensive *in Vitro* Proarrhythmia Assay (CiPA) initiative in 2013 and extensive testing of hiPSC-cardiomyocytes with known proarrhythmic drugs. The CiPA initiative aims to engineer an assay to assess the proarrhythmic potential of new drugs with improved specificity compared to current methods. CiPA focusses on the use of emerging new technologies and incorporates ventricular ion channel screening, *in silico* methods and hiPSC-cardiomyocyte analyses to form an integrated assessment of proarrhythmic risk⁽¹⁻⁴⁾. This approach saves time, money, and resources, and ultimately leads to safer medicines going forward to the clinic.

Another area in need of attention is central nervous system (CNS)-safety testing. CNS-related issues account for nearly a quarter of failures during clinical development, a phase where consequences are high in terms of resources and patient impact^(5,6). The range of CNS-related toxicities encountered throughout drug development and post-market approval is vast, and includes abuse liability, suicidal ideation, emesis, sleep disorder and cognitive dysfunction to name a few^(7,8). Modelling any of these endpoints *in vitro* would be especially challenging, however other toxicities that involve perturbations in electrical activity such as seizure may be amenable to earlier detection. Seizures and tremors occur frequently in preclinical drug development and were encountered by 67% and 65% respondents of a nervous system focussed industry survey respectively⁽⁷⁾. The same industry survey highlights that the most frequently used methods for seizure liability assessment are observations/EEG in preclinical rodent and non-rodent studies and the rodent hippocampal slice assay. These models all share similar limitations concerning their

low throughput, interspecies comparison, and cost. It is well known that there are differences between rodent and human brains⁽⁹⁾, so a shift towards human-based models seems like a logical step in the right direction. Interestingly, stem cell derived neurons were used by only 15% respondents as part of their CNS safety assessments⁽⁷⁾ – Is it time to act and increase this number whilst decreasing our reliance on animal studies with questionable translation? The cardiovascular safety revamp was a great success story, perhaps we can improve seizure liability testing in the same way?

A seizure is characterised by periods of excessive neuronal firing and uncontrolled hyper-excitability⁽¹⁰⁾. The use of microelectrode arrays (MEA) to monitor spontaneous electrical activity and drug responses in hiPSC-neuronal cultures stands out as a suitable method to identify seizure liability *in vitro*. This approach enables high-throughput non-invasive measurement of electrical activity from a network of heterogenous neuronal cells. This has great potential for predicting seizure liability of drug candidates as changes in neuronal firing can be measured in real-time with millisecond temporal resolution using specialised plates with electrodes. This allows for analysis of multiple parameters that illustrate the activity of neuronal networks⁽¹¹⁾ (figure 1).

Any potential *in vitro* method for seizure liability testing needs to be reproducible and mimic the complexity of the human brain without being too technically demanding. The recent introduction of commercially available hiPSC-neuronal cells of multiple cell types may provide a solution to issues such as batch-to-batch variation and long, complicated differentiation processes. Commercial cells require a relatively short culture time and neurons can be mixed at desired ratios to mimic the human brain. Normal activity in the brain relies on a balance between inhibitory neurotransmission and neuronal excitation, so our hiPSC-cultures need to contain the right neurons to achieve this balance. We found that a mixture of excitatory glutamatergic neurons (68%), inhibitory GABAergic neurons (17%) and supportive astrocytes (15%) performed well at detecting seizurogenicity of 16 known seizurogenic compounds with various mechanisms of action. Most of these compounds behaved as expected and caused seizure-like behaviour, with one or two exceptions. These results are presented in table 1, alongside rodent hippocampal slice data, which are frequently used in projects where issues with seizure are anticipated^(12,13). We found good alignment between the two models, providing further evidence of the benefit of our assay.

It is now appreciated that hiPSC-cardiomyocytes from different sources and vendors display different electrophysiologic phenotypes which may reflect different levels of maturation. These differences can affect pharmacologic responses and influence assay sensitivity and subsequent risk assessments⁽¹⁴⁾. Similarly, we found that the ratio of cell types is a crucial consideration for detection of different mechanisms of seizurogenic aetiology. For example, in co-cultures containing fewer GABAergic neurons we found that picrotoxin and pilocarpine had no effect. This is presumably due to a lack of extra-synaptic GABA for picrotoxin, and a lack of expression of the relevant target, likely the M1 muscarinic receptor, for pilocarpine. Interestingly, 4-AP caused a dramatic decrease in activity in this culture. This may be due to differential expression of Kv channels - 4-AP is a highly potent inhibitor of Kv3.1 which is expressed on inhibitory GABAergic neurons, and less potently inhibits other Kv channels present on excitatory neurons^(15,16).

The trickiest group of compounds to nail down in our hiPSC neuronal co-culture were the GABA-A antagonists. We observed clear, expected changes with picrotoxin and bicuculline, but these changes are *subtle* compared to the changes observed in primary rodent cortical cultures⁽¹⁷⁾. This could be due to differential expression and activity of GABA-B receptors across the cell models. GABA-B receptors are GPCRs that mediate slow and prolonged inhibitory action, which would be unaffected by application of the GABA-A antagonists⁽¹⁸⁾. This hypothesis warrants further investigation. Pentylentrazole caused some changes to the pattern of electrical firing, but these changes are less clear compared to picrotoxin and bicuculline. We have also performed ion channel screening of the seizurogenic compounds against 15 seizure-associated ion channels, as a complimentary method to identify seizure liability early. Pentylentrazole inhibited the GABA $\alpha_1\beta_2\gamma_2$ ion channel, so perhaps the lack of clear activity in our hiPSC cell model is due to our administration of the compound. We used a single dose, whereas kindling models *in vivo* frequently use repeated doses to create epilepsy models⁽¹⁹⁾. Enoxacin and amoxicillin did not inhibit the GABA $\alpha_1\beta_2\gamma_2$ ion channel at the clinically relevant doses used, suggesting that the epileptogenic mechanism of these compounds is not GABA-A related. No changes in electrical activity in hiPSC-neurons were observed, however seizurogenic responses have been reported in zebrafish and preclinical rodent models, suggesting a species related difference in enoxacin sensitivity^(12,20,21).

These observations highlight the importance of diligently interrogating cell models, considering the expression profile of the cells, and understanding the pharmacology and uses of test compounds. This allows scientists to gain a full understanding of their cell models, tweak their sensitivity, and understand what the model can detect, and more importantly, what the model is unable to detect, and why. Our approach to seizure liability screening is especially pertinent in the context of the recent FDA modernization act which allows applicants to use methods other than animal testing to establish drug safety and effectiveness. Our proposal for a human ion channel assay coupled with assessment of electrical activity in hiPSC neuronal cells fits these criteria^(22,23), and has been highlighted by the FDA/CDER as a useful novel alternative method (NAM) that should be considered as a component of the overall safety assessment for seizure liability⁽²⁴⁾. In addition, the development of our seizure liability assays has been presented at many conferences, and has been recognised by several awards including the Bionow Rising Star award and honours relating to *in vitro* toxicology and drug-oriented toxicological research⁽²⁵⁾.

The possibilities of this model don't end at seizure detection. In addition to further refinement of this assay with respect to GABA-A/GABA-B, we also plan to further characterise the model for a liability that is the direct opposite of seizure – sedation. We have demonstrated that the hiPSC-neuronal cells show decreased activity with GABA agonists such as muscimol, and the benzodiazepine site agonist indiplon. Indiplon is a marketed sleeping aid whose decreased activity was immediately reversed with application of the benzodiazepine site antagonist, flumazenil, a compound that is used clinically to reverse benzodiazepine overdose⁽²⁶⁾. Outside of drug safety, we also plan to adapt our model to anti-epileptic drug discovery. This will be achieved by inducing a seizurogenic phenotype using a known seizurogenic compound and then determining if these changes are reduced by application epilepsy treatments. Looking further afield to disease modelling, studies have been conducted using patient derived iPSC-neuronal cells to identify novel pathological mechanisms in several neurological conditions including Parkinson's Disease, Alzheimer's Disease and Fragile X Syndrome^(27,28).

Since the advent of hiPSC technology almost two decades ago enormous progress has been made in many areas of research⁽²⁹⁾. Within drug safety the development of hiPSC technology represents a paradigm shift as they allow us to assess the potential toxicities of new drugs on a readily available source of human cells – a situation that

wasn't possible before. With further characterisation and refinement of these models we will be able to streamline our drug safety testing, reduce reliance on animal models, and ultimately develop safer medicines.

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