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CONTEMPORARY REVIEW

Can We Panelize Seizure?

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ABSTRACT

Seizure liability remains a significant cause of attrition in drug discovery and development, leading to loss of competitiveness, delays, and increased costs. Current detection methods rely on observations made in *in vivo* studies intended to support clinical trials, such as tremors or other abnormal movements. These signs could be missed or misinterpreted; thus, definitive confirmation of drug-induced seizure requires a follow-up electroencephalogram study. There has been progress in *in vivo* detection of seizure using automated video systems that record and analyze animal movements. Nonetheless, it would be preferable to have earlier prediction of seizurogenic risk that could be used to eliminate liabilities early in discovery while there are options for medicinal chemists making potential new drugs. Attrition due to cardiac adverse events has benefited from routine early screening; could we reduce attrition due to seizure using a similar approach? Specifically, microelectrode arrays could be used to detect potential seizurogenic signals in stem-cell-derived neurons. In addition, there is clear evidence implicating neuronal voltage-gated and ligand-gated ion channels, GPCRs and transporters in seizure. Interactions with surrounding glial cells during states of stress or inflammation can also modulate ion channel function in neurons, adding to the challenge of seizure prediction. It is timely to evaluate the opportunity to develop an *in vitro* assessment of seizure linked to a panel of ion channel assays that predict seizure, with the aim of influencing structure-activity relationship at the design stage and eliminating compounds predicted to be associated with pro-seizurogenic state.

Key words: CNS; ion channels; MEA; seizure; neurotoxicology; drug development; in vitro models; induced pluripotent stem cells; neuron; regulatory sciences.

The average cost of developing and gaining marketing approval for a new drug is around ~$1.2 billion and is steadily increasing at an annual rate of 8.5% (DiMasi *et al.*, 2016). Despite this increasing investment, safety-related attrition remains a major

issue accounting for >50% of failures (Cook *et al.*, 2014, Morgan *et al.*, 2018). Central nervous system (CNS)-related issues account for a small number of failures in preclinical development but nearly a quarter of failures during clinical development (Cook *et al.*, 2014), are associated with serious adverse drug reactions (ADR), and withdrawal from sales (Valentin and Redfern, 2017; Weaver and Valentin, 2019). As well as the issue of attrition due to neurotoxicity, many registered medicines also carry neurotoxicity issues through the label with implications for use and patient quality of life. Of around 46 000 human prescription drugs included in Food and Drug Administration (FDA) Label (Fang *et al.*, 2016), around 400 carry boxed warnings for functional neurotoxicity endpoints such as suicidal ideation, sedation, abuse liability, seizure/convulsion, and headache (Walker *et al.*, 2018). Although abuse liability, suicidal ideation, and numerous generic symptoms (eg headache) are especially challenging, other toxicities such as sedation and seizure may be more amenable to earlier detection (Walker *et al.*, 2018).

Currently, the detection of seizures (see Box 1) is reliant upon observations in preclinical rodent and non-rodent studies (Easter *et al.*, 2009; Nagayama, 2015). These could be CNS-related signs such as tremors or other abnormal movements, but these signs can be misdiagnosed or misinterpreted by inexperienced operators. Additionally, safety pharmacology studies often test relatively low doses so these studies rarely identify adverse events suggestive of potential seizure liability. As such, the primary source of data for seizure liability generally comes from acute and repeat-dose toxicity studies (which include groups at or near the maximum tolerated dose) when either overt convulsions or premonitory clinical signs of convulsions are noted. Screening methods such as the hippocampal brain slice (Easter *et al.*, 2007) or zebrafish larval locomotor assays (Khan *et al.*, 2017) are available. However, there are questions regarding interspecies comparison for both models (Grainger *et al.*, 2018; see Table 1) and hippocampal slice cultures can be of limited throughput. It would be far preferable to have an earlier, higher throughput, human-based model for the prediction of seizure risk that could be used to identify and eliminate liabilities early in discovery while there are still options for the medicinal chemists making potential new drugs.

Attrition due to cardiac adverse events has benefited from the routine screening against the cardiac potassium, sodium and calcium channels, and optimization of medicinal chemistry away from these liabilities (Gintant *et al.*, 2016). These assays are automated and high throughput, translate well to humans and are underpinned by predictive *in silico* models (Park *et al.*, 2018; Pollard *et al.*, 2017). Significantly, this screening strategy has abolished drug withdrawals due to an unacceptable risk of Torsades de Pointes, a fatal ventricular arrhythmia linked to QT prolongation. So, could we reduce attrition due to seizure by using a similar approach to that used to reduce attrition due to cardiac adverse events? Here we assess the current science and take a multidisciplinary approach to evaluating the opportunities and challenges of innovative new methods for seizure detection.

Box 1

"Seizure" refers to a period of rhythmic, synchronized abnormal neuronal activity that may result in a tonic-clonic convulsion and/or more subtle effects such as visual disturbances, tingling, or mood changes. A "convulsant drug" is one that induces overt motor effects of this type; a "proconvulsant drug" increases the likelihood or severity of a convulsion. A proconvulsant drug may have no detectable effect in the absence of "seizure-precipitating factors" (epilepsy, stress, presence of other proconvulsant compounds). Convulsant compounds are typically proconvulsant at lower doses than the dose that produces convulsion. Seizure activity is not always followed by the behavioral changes that define a convulsion.

Table 1. A Comparison of Different Seizure Testing Models, Modified from Grainger *et al.* (2018) and Rockley *et al.* (2019)

Model	Benefits	Limitations
Rodent *ex-vivo* hippocampal slice assay	• Representative of *in vivo* adult rodent brain • Same day experimentation • Validated • Defined architecture • Forms functional network • Current gold standard	• Interspecies comparison – relevance to humans? • Cellular damage • Low throughput • Uses animals • Short lifespan in culture
Larval zebrafish locomotor assay	• Whole organism • Intact CNS • Readily available • Inexpensive	• Interspecies comparison – relevance to humans? • Specificity of locomotor assays to seizure activity
iPCS neuronal culture	• Human based • Retained genotype from donors • Can be higher throughout • Fewer ethical considerations	• Currently expensive • Time consuming • Validation ongoing • Variable cellular composition • Standard protocol evolving • Genetic variability
Ion channel panel	• High throughput • Human relevance • Amenable to mechanistic research	• Research still in infancy • Data interpretation requires bioinformatic modelling

Table 2. Examples of Drug Classes Associated with Seizure (Easter *et al.*, 2009)

Pharmacological Class	Pharmacological Target	Target Type
γ-aminobutyric acid (GABA)ergic antagonists	GABA binding site	Ligand-gated ion channels
CNS stimulants	Dopamine transporter	G-protein-coupled receptors
Nicotinic agonists	Nicotinic acetylcholine receptors (nAChRs)	Ligand-gated ion channels
Sodium channel blockers (anti-arrythmia drugs)	Sodium channel site 2	Voltage-gated ion channels
Cannabinoid agonists	Cannabinoid CB1	G-protein-coupled receptors
Selective serotonin reuptake inhibitor (SSRI) antidepressants (Bupropion)	Serotonin monoamine transporter	Transporter
Phenothiazine antipsychotics (clozapine; chlorpromazine)	GABA B	Ligand-gated ion channels
Antihistamines	Histamine H1 receptor	G-protein-coupled receptors

SEIZURE INCIDENCE

CNS toxicity is a prominent occurrence and accounts for nearly one-quarter of failures across the spectrum of drug discovery and development (Cook *et al.*, 2014). Combining this observation with another industry survey where seizures and tremors represent 67% and 65% respectively, of the CNS issues encountered preclinically (Authier *et al.*, 2016), seizure is likely to be responsible for around 10%–20% of failures. Another important issue raised by these figures is that around 75% of the reported failures occurred in clinical development, a phase where consequences are higher in terms of resources and patient impact (Cook *et al.*, 2014). Confirming this, the seizures/convulsions that were reported in patients for drugs approved in Japan were not identified during preclinical studies (Nagayama, 2015).

Compounds associated with seizure liability span a wide variety of pharmacological classes (Table 2) (Easter *et al.*, 2009) and a wide variety of therapy areas including cardiovascular, gastrointestinal, respiratory, and inflammation as well as the CNS (Cook *et al.*, 2014; Walker *et al.*, 2018). A comparison between therapy areas suggests that the risk of seizure liability is particularly high in neuroscience (Aagaard and Hansen, 2013), although a review of drugs either marketed or in clinical development showed that around two-third of those associated with seizure fall outside the neurological indications (Easter *et al.*, 2009). CNS adverse events may be particularly prevalent in projects where molecules are designed to penetrate the blood-brain barrier to act on their primary target (Aagaard and Hansen, 2013).

CURRENT APPROACHES TO SEIZURE DETECTION

Owing to the potentially serious consequences of drug-induced seizures, significant effort is expended in preclinical safety assessment to identify and mitigate this effect (Authier *et al.*, 2016; Easter *et al.*, 2009; Redfern *et al.*, 2008). Before the year 2000, seizure risk was typically addressed preclinically as part of a larger systematic investigation of CNS risk (Bass *et al.*, 2004). This generally includes observational assessment during *in vivo* (typically rat or dog) general toxicity or other safety pharmacology studies (Haggerty, 1991; Moser, 1991). With the release of ICH S7A guidelines, the comprehensive testing changed to a streamlined package geared to meet the minimal requirements of regulatory agencies primarily aimed at supporting first time in human (FTIH) clinical trials (ICH, 2001). However, these lean packages may fail to detect seizure liability until later in drug development when it is typically revealed in repeat-dose toxicity studies. This motivated the pharma industry to develop strategic approaches to identify seizure liability in early discovery for projects or chemistry of highest risk. Early hazard identification allows time for a project to mitigate or eliminate risk through improved chemistry and/or pharmacokinetic approaches.

To determine if clinical signs of convulsions or premonitory signs noted in animal studies are the result of seizures or abnormal epileptiform electroencephalogram (EEG) activity, EEG studies in the most sensitive or the most relevant nonclinical species can be conducted. EEG has become an increasingly characterized tool to investigate seizure liability in nonclinical drug development but also to assess other non-seizurogenic pharmacological effects such as sedation, stimulation, or sleep disturbances (Authier *et al.*, 2009; 2016; Bassett *et al.*, 2014; Easter *et al.*, 2009; Fonck *et al.*, 2015; Rachalski *et al.*, 2014). As well as "standalone" EEG studies, EEG monitoring is often added to dose range finding studies to give an early indication for programs considered to be at greater risk. Novel telemetry technologies enable the use of more complex EEG protocols to evaluate subtle differences in pharmacological response. Tachyphylaxis, defined as a rapidly diminishing response to successive doses of a drug, could be a concern (ie, apparent false negative) with neurologically active agents in the context of seizure liability testing, especially when testing multiple dose levels in the same animals. Approaches to mitigate this include establishing the minimum washout duration between treatments to prevent tachyphylaxis and the use of a group of drug naïve animals to confirm the established EEG no adverse effect level (NOAEL), noting that a NOAEL is not typically defined in safety pharmacology studies (Baird *et al.*, 2019). However, despite some concerns, a review of historical data suggest that tachyphylaxis is relatively rare in nonclinical seizure liability testing (Authier *et al.*, 2019). The additional value of EEG monitoring in repeat dose toxicity testing includes but is not limited to detection of exacerbation of seizurogenic effect (via drug accumulation, cumulative neurotoxicological effects, or upregulation of receptor expression) or effects mediated by metabolites. EEG can also be used to differentiate seizure profiles including partial or generalized seizures which can manifest with a wide range of clinical signs. Key aspects of study design to detect seizure include species selection, dosing duration, and detailed, extended clinical observations that cover the C_{max} of both the parent drug and major metabolites. C_{max} is generally considered to be the more important parameter compared with area under the curve when establishing a safety margin; this is because seizure is likely to be pharmacologically mediated and thus associated with peak exposure. The use of free versus total drug (unbound vs bound) is still a matter of debate (DaSilva *et al.*, 2020). Studies should include time-synchronized video monitoring to increase EEG analysis sensitivity and improve interpretation accuracy,

specifically related to the exclusion of artifacts. Where possible, toxicokinetic (TK) data should be obtained soon after any epileptiform activity and/or seizures in individual animals because these data can be used to more clearly define the TK thresholds at which adverse effects were observed and inform dose escalation in humans. Time to seizure onset also represents a critical factor to weigh when establishing the testing strategy. Dosing for twice the duration that was required to observe seizure-related clinical signs in prior studies is often viewed as a reasonable strategy when planning seizure liability testing.

The use of quantitative EEG biomarkers in repeat dose toxicology studies involving non-rodent species is a potential approach to assess target engagement or neuromodulation and can serve to support clinical exposure estimation (Authier *et al.*, 2014). Status epilepticus, a potentially fatal condition during which continuous seizure activity is present for more than 5 min, is a major clinical concern. Establishment of the "seizure profile" for a given compound along with treatability using common rescue drugs such as diazepam, propofol, or phenytoin is of paramount importance to clinicians undertaking clinical trials. Many approved drugs are associated with seizure liability at higher exposures and safety testing strategies remain a central success factor in the successful development of neuroactive agents.

Current methods for seizure detection raise two main issues: seizure may be missed in animal studies (hence the tendency toward clinical rather than preclinical failure) and, even if seizure is detected, significant resource has already been invested in the project. Although some progress has been made using *in vivo* detection of seizure using automated video systems that record and analyze animal movements (Yip *et al.*, 2019), there is a need to develop improved screening methods that can be used earlier in drug discovery to identify and predict seizure liability. Advances in stem cell biology coupled with an increased understanding of the role of cellular signaling proteins in seizure offer an opportunity for a new paradigm in screening. Using stem cell-based approaches also offer the ability to incorporate cell-cell interactions with glia that could alter ion channel function to promote seizure during states of neuronal stress that could better inform the drug develop process to avoid seizurogenic activity in new leads. Such mechanism-based approaches could support optimal drug design by influencing structure-activity relationship (SAR) early in development before resources, animals, and time have been wasted.

NEW APPROACHES TO SEIZURE DETECTION

Attrition due to cardiac adverse events has benefited from the assessment of both the proarrhythmic and non-proarrhythmic cardiotoxicity of new drug candidates in human-induced pluripotent stem-cell-derived (hiPSC) cardiomyocytes coupled with routine screening against cardiac ion channels, allowing for optimization of medicinal chemistry away from these liabilities (Gintant *et al.*, 2016). So, can we take a similar approach for seizure liability where we screen compounds in early drug discovery against a panel of seizure-related pharmacological targets (such as voltage- and ligand-gated ion channels, G-protein-coupled receptors, and transporters), coupled with assessment of seizure-like activity in hiPSC-derived neurons?

There is clear evidence for the involvement of ion channels such as the voltage-gated sodium and potassium channels and the ligand-gated ion channels in seizure (Armijo *et al.*, 2005, Lerche *et al.*, 2013). In addition, it is possible to measure the electrical activity of hiPSC-derived neurons in culture where seizure-causing drugs increase the frequency of network bursts (Bradley *et al.*, 2018; Kreir *et al.*, 2018; Odawara *et al.*,2018; Tukker *et al.*, 2020). At the molecular level, these hiPSC-derived neurons express relevant ion channel transcripts and functional ion channels, providing an opportunity for correlation of ion channels with the observed response. Functional ion channel activity assays in hiPSC-derived neurons are also amenable to screening channel activity in co-cultures with glial cells to better identify critical cell-cell interactions that could occur *in vivo*. Glial cells, particularly astrocytes, directly modulate the tone of glutamatergic and GABAergic synapses, activity that is diminished or altered during stress and innate immune responses to seizurogenic agents (Terrone *et al.*, 2020).

Identifying Relevant Ion Channels

There is clear evidence for the involvement of ion channels in seizure (Easter *et al.*, 2009). Genetic or pharmacological studies have pointed to a role for voltage-gated ion channels (NaV1.1, NaV1.2, Kv7.2/7.3), ligand-gated ion channels ($GABA_A$, NMDA1/2A) GPCRs (adrenergic $\alpha 1$ receptors, muscarinic acetylcholine M_1 receptors) and transporters (noradrenaline transporters, 5-HT transporters). Pharmacologically, a number of ion channel modulators are known to be seizurogenic such as chlorpromazine (Table 2).

In determining which ion channels may be informative in predicting seizure liability, one of the major challenges is the complexity of the CNS system. A comprehensive list of ion channels involved in seizure equates to over 100 targets (Kullman and Waxman, 2010; Lerche *et al.*, 2013; Oyrer *et al.*, 2018). Weight of evidence from genetic and pharmacological studies (Armijo *et al.*, 2005; Lerche *et al.*, 2013) suggests an initial panel of ion channels that may be predictive of seizure (Figure 1) as a starting point for testing (Rockley *et al.*, 2019). Building on this, ion channels may be added or removed from the panel, based on expression profiles noted in hiPSC-derived neurons, especially those displaying a seizurogenic phenotype *in vitro*.

There is clearly work to be done on determining which ion channels might constitute a panel. A second key task is to determine the best approach to use for ion channel screening; would it be better to work with recombinant cell lines expressing an individual target, or a more physiologically relevant substrate such as stem-cell-derived neurons? Other considerations when using *in vitro* assays include the involvement of potential metabolites, protein binding/free fractions, tissue accumulation, and delayed neurotoxicity which may account for part of the *in vitro* to clinical translation challenges. These challenges remain to be resolved and are the topic of ongoing work.

Moving forward, whether in transfected cells or in stem-cell-derived neurons, a full ion channel panel would have great utility for *in vitro* detection of seizure. Such a model would allow assessment early in the drug discovery process of whether compounds are likely to cause seizure, allowing for chemical modification in the make-test cycle. Additionally, an ion channel panel that predicts seizure has utility in the development of novel anti-epileptic medicines.

Microelectrode array *in vitro* methods. Over the last decade, there have been many technological advances in microelectrode array (MEA) technology. MEAs can be used both *in vitro* as well as *in vivo* for cardiac, CNS and even peripheral nervous system recordings (Obien *et al.*, 2015). In this instance, MEAs are described for use *in vitro* with multi-well plates containing multiple electrodes that detect neuronal signals from a network

Summary ion channel panel

Sodium	Potassium	Calcium	GABA	TRP	Nicotinic	Misc
SCN1A	KCNQ2	CACNA1A	GABRA1	TRPC1	CHRNA2	HCN1
SCN2A	KCNQ3	CACNA1H	GABRB2	TRPC5	CHRNA4	HCN2
SCN1B	KCNQ4	CACNA1D	GABRB3	TRPM5	CHRNB2	CLC2
SCN8A	KCNA1		GABRG2	TRPM7		
	KCNA2					
	KCNT1					
	KCNB1					
	KCNJ6					
	KCNJ11					
	KCNMA2					

CiPA panel:

KCNH2
SCN5A
CACNA1C
KCNJ12
KCND3
KCNQ1

Figure 1. Summary of a potential ion channel panel. The ion channels proposed are based on weight of evidence from studies of the genetic basis of epilepsy and from pharmacology studies (Armijo *et al.*, 2005; Lerche *et al.*, 2013).

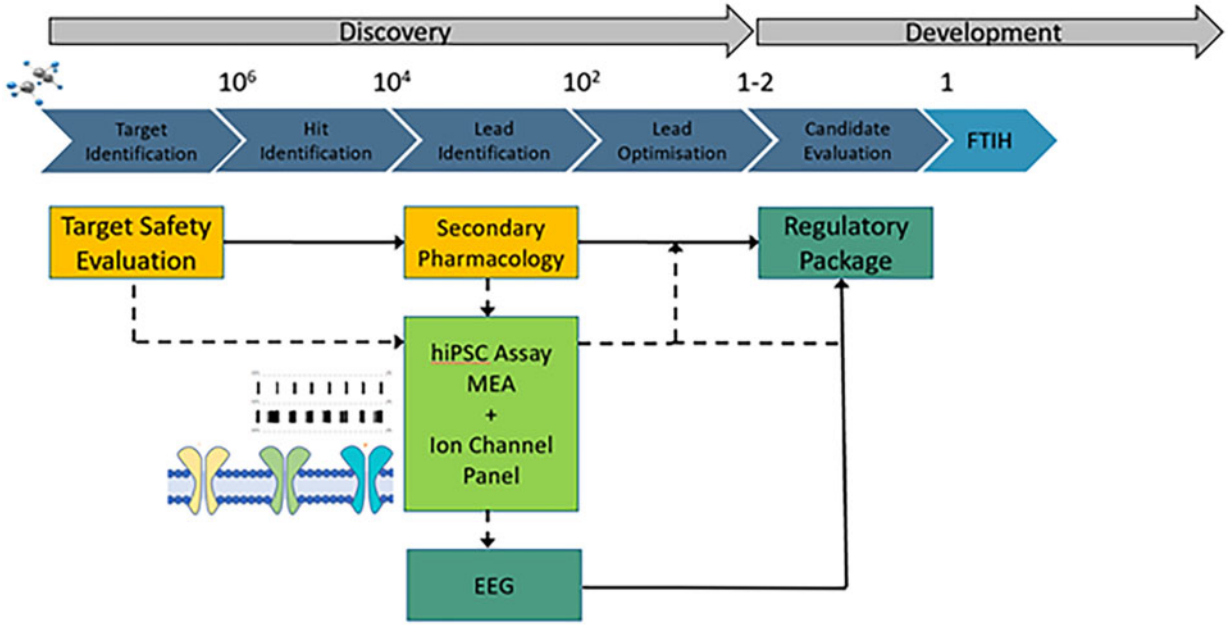

Figure 2. Development of a microelectrode array (MEA) and ion channel focused approach to seizure detection. Conventionally, a target safety evaluation is used to predict target-related risks coupled with secondary pharmacology studies to predict likely chemical-related risks. These earlier assessments are then followed by assessment of CNS-related effects in the safety pharmacology regulatory package required as part of the FTIH submission. We propose the use of an MEA assay coupled with an ion channel panel to provide an earlier assessment of seizure liability with follow up EEG evaluation. Derived and adapted from Easter *et al.* (2009). hiPCS: human-induced pluripotent stem cells; FTIH: first time in humans; EEG: electroencephalogram.

of heterogenous CNS cells (Figure 2). These MEA systems enable high-throughput noninvasive measurement of electrical activity in cells *in vitro* and have great potential for predicting seizure liability of drug candidates. Convulsants have been assessed using MEAs and complex cultures of rodent CNS tissue that contain excitatory and inhibitory neurons as well as glia, cultured neurons, and hippocampus slices (Accardi *et al.*, 2018; Bradley *et al.*, 2018; Bradley and Strock, 2019; Fan *et al.*, 2019; Frega *et al.*, 2012; Koerling *et al.*, 2019; Kreir *et al.*, 2018). The Health and Environmental Science Institute (HESI) Neurotoxicity MEA Subteam (HESI, 2020) has developed and conducted pilot studies using MEAs for predicting the seizure liability of drugs using both rodent- and hiPSC-derived neurons. These human-derived cells are expected to provide a better translation to humans than the rodent cells (Grainger *et al.*, 2018; Ishii *et al.*, 2017; Matsuda *et al.*, 2018; Odawara *et al.*, 2014, 2016, 2018; Ojima and Miyamoto, 2018; Tukker *et al.*, 2018). This remains to be demonstrated, however, as the human neural networks are newer and relatively less well characterized compared with that of rodent models. Rodent models may be an important bridge in understanding this new methodology with the ultimate goal to use human cells to predict human outcomes.

The detection of seizure-like activities has been reported following the administration of typical convulsants such as 4-

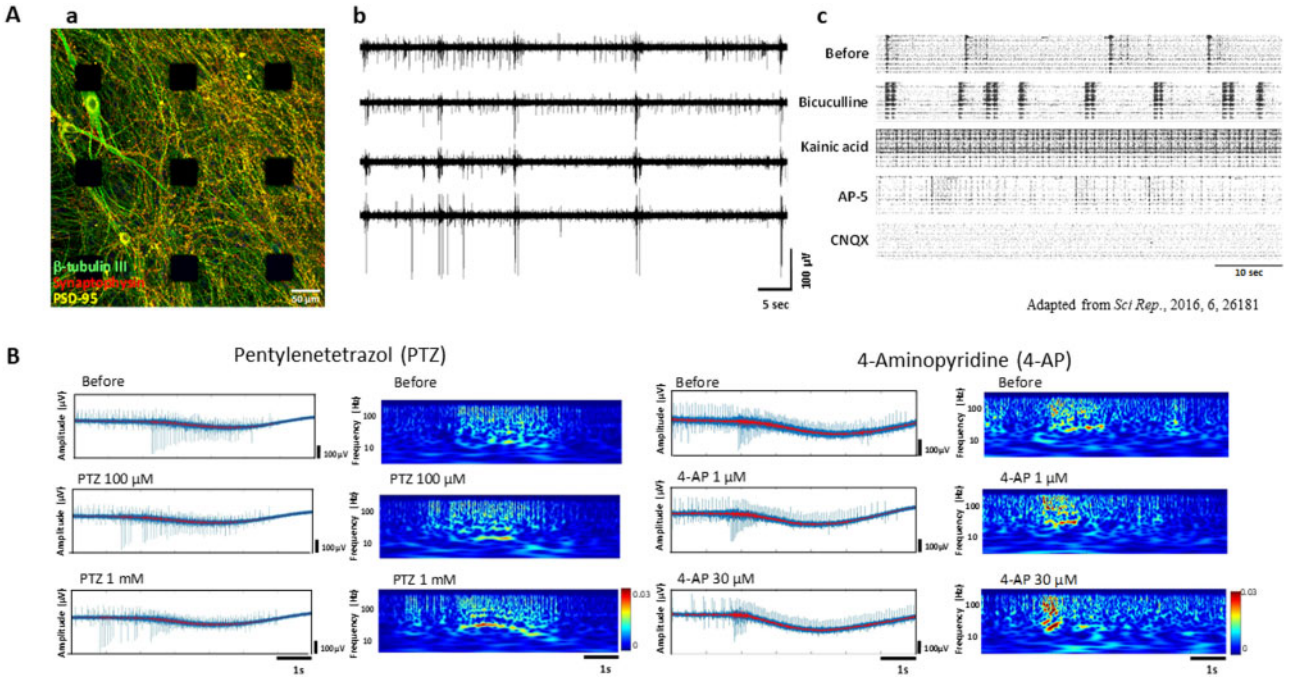

Figure 3. MEA assay in cultured hiPSC-derived neurons. A, HiPSC-derived cortical neurons cultured on an MEA chip (a). (b) Typical waveform of spontaneous firings. (c) Raster plots of spontaneous firings in drug administration. B, Frequency analysis of burst firings with pentylentetrazole (PTZ) and 4-aminopryridine (4-AP) administration. Left: The local field potential (LFP). Right: Corresponding scalograms of temporal scales during the application of PTZ or 4-AP are shown as left traces. Figures (A) and (B) adapted from Odawara *et al.* (2016) and (2018), respectively.

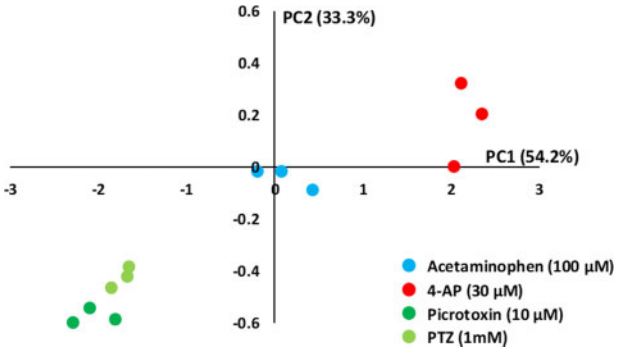

Figure 4. Separation of convulsants and non-convulsant by principal component (PC) analysis using 4 parameters: Number of spikes, Duration in a network burst, maximum frequency in a network burst, and periodicity that quantifies the regularity of network burst cycles (Ishibashi *et al.*, 2018).

aminopyridine (a K^+ channel blocker) and pentylenetetrazol (a γ-aminobutyric acid A ($GABA_A$) receptor blocker) (Figure 3). However, there are challenges involved in analyzing and interpreting the data because the response parameters vary depending on the mechanism of action (MoA) of the drug. Derivation of new analysis parameters and the use of multivariate analysis is expected to improve the effectiveness of the approach. For example, frequency analysis focusing on low-frequency components (250 Hz or less, excluding spike components) is reported to detect concentration-dependent changes. As shown in Figure 3, MEA analysis incorporates local field potentials (LFP) a measurement common to electrocorticography and intracranial electroencephalography (Odawara *et al.*, 2018). Additionally, principal component (PCA)and cluster analyses (both multivariate analyses) can be used to estimate the proconvulsant risks and can also be used to evaluate different mechanisms of action (Figure 4) (Ishibashi *et al.*, 2018). The PCA plot shown in Figure 4 clearly shows that different MOAs are separated, but same MOAs are clustered. These results indicate that MEA holds much promise for *in vitro* seizure prediction. As well as MEA, other approaches are under development such as the optopatch techniques that look at neuronal firing to detect seizurogenic activities (Dempsey *et al.*, 2016; Nguyen *et al.*, 2019). This approach has been used for cardiotoxicity screening with simultaneous optogenic pacing, voltage imaging, and calcium signaling (Dempsey *et al.*, 2016).

NEURO-GLIAL INTERACTIONS IN MODULATION OF SEIZURE

One of the challenges in predicting proconvulsant activity in drug discovery is modeling the complex intercellular signaling mechanisms between neurons and glial cells that regulate neuronal excitability, as well as how such regulation is altered during states of stress and inflammation induced by potential new drugs. An understanding of how glial cells regulate neuronal ion channel activity could aid in designing assays that take account of cell-cell interactions to better detect adverse effects that could lead to seizure. One of the primary receptors involved in seizurogenic hyperexcitability in neurons is the ionotropic class of glutamate receptors, which include the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate (KA) receptors. Excess stimulation of these receptors is an important activator of seizure, which is why the concentration of extracellular glutamate is kept tightly regulated via high-affinity glutamate transporters in astrocytes. The expression of two of the most important of these excitatory amino acid transporters (the Na+-dependent glutamate transporter and the glutamate aspartate transporter [Peterson and Binder, 2020]) is decreased during the activation

of astrocytes during inflammatory stress, associated with diminished uptake of extracellular glutamate (Haroon *et al.*, 2017). Such changes are associated with increases in duration and intensity of seizure, as well as neuronal injury (Peterson and Binder, 2020). Likewise, overstimulation of glutamate receptors such as mGluR3/5 in astrocytes results in release of gliotransmitters, including glutamate that can further enhance neuronal excitability (Bazargani and Attwell, 2016). Thus, approaches that incorporate astrocytes and/or microglia into co-culture systems are more likely to detect alterations in ion channel activity in neurons relevant to seizure and driven by altered astrocytic regulation of extracellular glutamate.

Release of inflammatory cytokines such as interleukin 1 beta (IL-1β) by activated glia can also modulate seizure activity by enhancing excitability of AMPA and NMDA receptors (Sharma *et al.*, 2019), as well as by downregulating GABA-mediated neurotransmission and by decreasing uptake of glutamate by astrocytes (Wang *et al.*, 2000). Supporting a pathogenic role for excessive IL-1β in promoting seizure, antagonizing the IL-1β receptor protects against seizure following traumatic brain injury (Semple *et al.*, 2017). Tumor necrosis factor (TNF) is another inflammatory cytokine produced by activated glial cells that modulates ion channel activity in seizure. TNF increases Ca^{2+}-dependent glutamate release from astrocytes, potentially through the transient receptor potential vanilloid 4 (TRPV4), which could explain its capacity to amplify excitatory currents in adjacent neurons (Wang *et al.*, 2019). Acute changes in inflammatory signaling in astrocytes can occur as a result of damage-associated molecular patterns, such as those occurring from mitochondrial stress, that result in inflammasome activation, release of IL-1β and rapid activation of NF-κB signaling (Haroon *et al.*, 2017). In addition, these alterations can lead to decreased clearance of GABA, decreased special buffering of extracellular potassium and excessive release of ATP, all of which can increase seizureogenic activity in neurons (Nikolic *et al.*, 2020). Detection of such early stress signaling events in neurons and glial cells represents another avenue for predicting potential seizurogenic activity of new drug candidates in conjunction with electrophysiological assays.

Expression of immediate-early stress responsive genes such as c-*fos* occurs early in seizure and has long been used to detect neuronal activation and to map specific brain regions involved in neurological responses to numerous pathophysiological stressors (Curran and Morgan, 1995; Le Gal La Salle, 1988). Newer approaches use fluorescent reporters linked to c-*fos* transcriptional activation sites to detect pathway activation in near real time. Transcriptional activation of c-*fos* is an immediate-early response to stress in neurons and integrates stress signals through multiple pathways including CAM kinase (CAMK) and MAP kinase (MAPK), with rapid stimulation of fluorescent protein transcription within 15–30 min of stimulation through these pathways (Hudson, 2018). Activation of the NF-κB pathway in neurons and glial cells is another potential reporter to help identify possible seizure activity when used in conjunction with MEAs and hiPSC approaches. NF-κB signaling in glia cells modulates latency and severity of seizure, as well as neuronal injury, demonstrating the importance of this pathway in glial-dependent enhancement of seizure activity (Huang *et al.*, 2017; Liu *et al.*, 2017). In neurons, NF-κB activation can parallel activation of c-*fos*, representing an additional measure of stress activation through either Ca^{2+}-dependent stress kinase activation or mitochondrial dysfunction. This has been previously modeled in transgenic mice and in hippocampal slice cultures, where exposure to low levels of kainic acid resulted in regionally selective expression of an NF-κB-EGFP reporter in limbic structures consistent with the regionally selective effects of the compound (Figure 5) that also correlated with seizurogenic activity as determined by EEG (Miller *et al.*, 2014). Finally, the use of real-time, genetically encoded calcium indicators, such as GCaMP6 (Hudson, 2018; Zarowny *et al.*, 2020) and NCaMP7 (Subach *et al.*, 2020) offers the capability of detecting aberrant Ca^{2+} transients induced in neurons coinciding with overstimulation of NMDA or AMPA glutamate receptors preceding seizurogenic event. Collectively, incorporating neuronal and/or glial cells with transgenic reporters for stress-responsive signaling factors such as c-*fos*, NF-κB, GCaMP6, or related signaling factors could be useful in augmenting seizure assessment in high throughput systems using MEAs and hiPSCs.

REGULATORY CONSIDERATIONS

Because seizures can be life threatening and can also increase the risk for future seizures, they represent a significant concern in drug development programs, resulting in delays due to the need for additional *in vivo* studies aimed at addressing the concern. The potential for such delays and the extra resources needed can pose too big of a challenge leading to the discontinuation of potentially useful therapeutic drugs. In general, adverse effects that cannot be monitored in a clinical setting and/or can result in permanent toxicity (such as lowering of the seizure threshold), represent a significant safety concern and result in the need for "large" safety margins (ie, 10-fold based on pharmacokinetic data) in clinical trials. This safety margin is intended to account for human to animal variability and potential intra-animal and intra-human variability in exposures. For some drug classes, such as sodium channel blockers, the application of a 10-fold safety margin can be prohibitive for dose escalation to the required therapeutic exposure.

Standard risk minimization strategies for clinical studies where seizure liability exists include exclusion of patients with a history or family history of seizures, history of head trauma, or indicators of a concomitant risk for seizures such as alcohol use disorder or history of infection with encephalitic or neurotropic viruses. In these instances, inflammation can be a confounding modulator of seizure. Studies should include increased clinical monitoring and have adequate informed consent, assuming the study is allowed to proceed. However, the ability to monitor for abnormal EEG activity in clinical studies is challenging because of confounding normal background EEG signals and the need for prolonged clinical EEG monitoring (usually 24 h/day). Additionally, drugs may have a very narrow margin between the exposure that causes abnormal EEG activity and that which causes seizures, as such, EEG monitoring in humans is not considered adequate to avoid drug-induced seizures. Thus, only patients with an acceptable benefit: risk profile should be included such as those who have failed standard therapies and/or have an unmet medical need.

To date, the U.S. FDA Center for Drug Evaluation and Research has limited experience with alternative assays to characterize seizure risk and the science is not yet sufficiently mature to support amending regulatory guidelines. However, the Agency does critically evaluate the primary and secondary pharmacology binding and functional screens to evaluate biological plausibility of premonitory signs of seizures in nonclinical studies (Papoian *et al.*, 2015; Valentin *et al.*, 2018). Such data may be useful to compare the risk associated with novel compared with currently approved drug products for similar indications. Novel *in vitro* studies or screens with the ability to

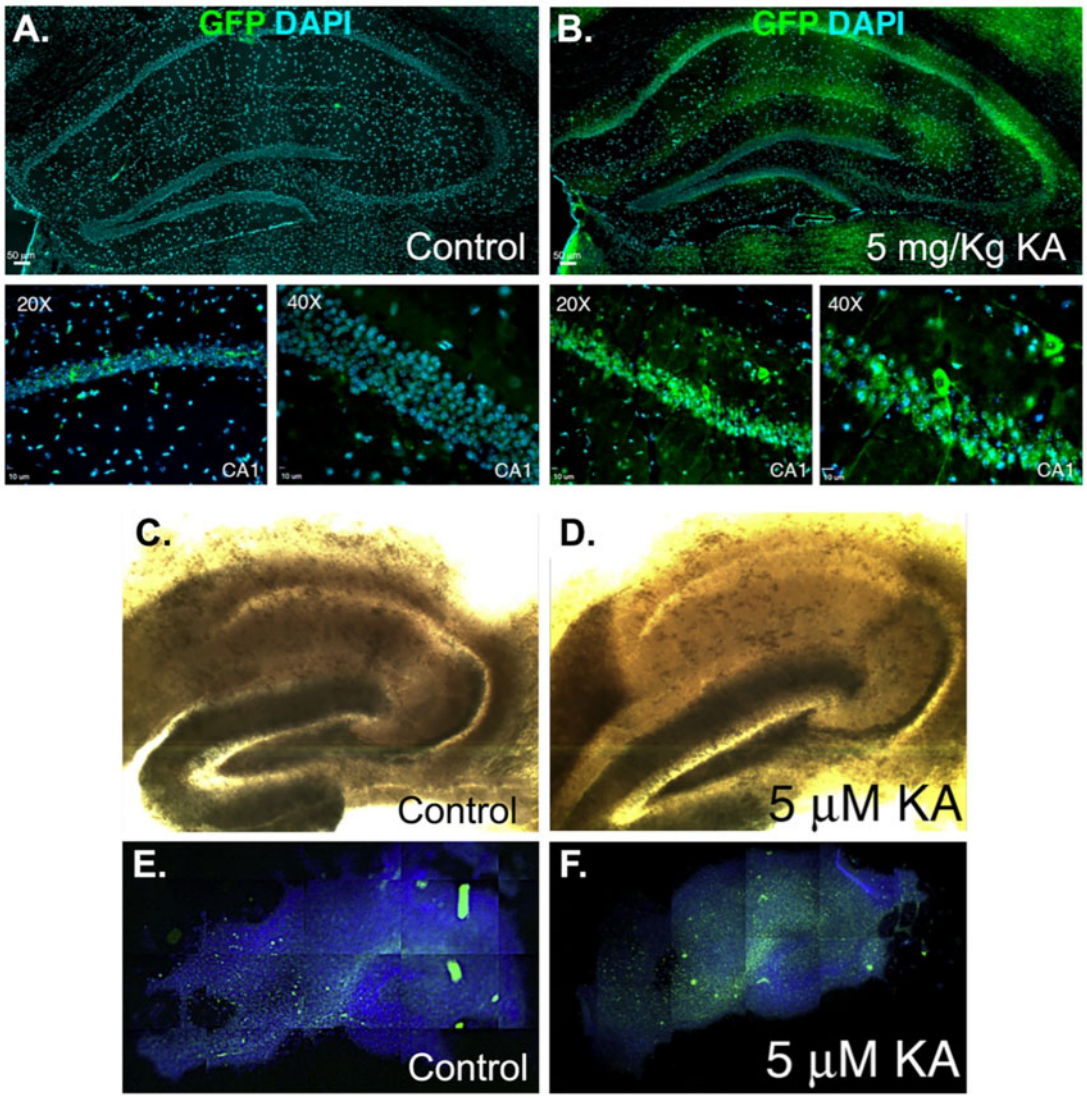

Figure 5. Activation of the NF-kB pathway in the hippocampus of transgenic reporter mice by a single dose of kainic acid *in vivo* and *in vitro* (Miller *et al.*, 2014). (A, B) Representative 10× montage images of intrinsic GFP fluorescence in control (A) and kainic acid-treated (B) NF-κB/EGFP reporter mice. Subset images are high magnification images (20× and 40×) of the CA1 region of the hippocampus demonstrating EGFP expression in the pyramidal cell layer. (C-F) Representative 10× montage brightfield (C, D) and fluorescence (E, F) images of cultured hippocampal slices from NF-κB/EGFP reporter mice treated with saline (C, E) and 5 M KA (D, F). Images in (E) and depict intrinsic EGFP fluorescence (green) and nuclear staining with DAPI (blue).

compare the relative risk of novel compared with approved drugs and can also characterize the potential risk contribution of drug metabolites may provide useful tools that could support proposals to dose beyond the common 10-fold safety margins for these drug development programs. The FDA fully supports the principles of the 3Rs (replace/reduce/refine; NC3Rs 2020; MacArthur, 2018) for animal use testing when feasible and encourages sponsors to consult with review divisions when considering nonanimal testing methods, including methods described in this manuscript that attempt to characterize seizure risk liability.

CONCLUSIONS AND FUTURE PERSPECTIVES

Seizure liability remains a significant cause of attrition in drug discovery and development, leading to loss of competitiveness, delays, reduced risk: benefit and increased costs. Here, we describe a strategy based on an early assessment of target-related risk followed by secondary pharmacology profiling and hiPSC-derived neuronal/MEA assay studies to evaluate whether compounds cause seizure like activity *in vitro*. An ion channel panel can then be deployed for either mechanistic investigations or for guiding medicinal chemistry via influencing SAR away from probable seizure liabilities. There are multiple applications for such a panel including informing discovery chemistry, comparing the relative risk of novel compared with approved drugs and use in characterizing the potential risk of drug metabolites.

There are several issues to address in support of this plan. Currently, the ion channel panel is assembled from literature reports, knowledge from the anti-epileptic drug development field and as such the association between each channel and seizure remains to be validated and tested. In addition, MEA cellular models are working but there is still much to be done. Another key assumption is that this reductionist approach to seizure (where a complex biological response such as seizure is reduced to a panel of ion channels; see CiPA, 2020) will work as

it has for cardiac safety screening. The brain is complex and contains astrocytes and other cells that are required to maintain homeostasis *in vivo*; these can be accounted for in the MEA cultures but not in the ion channel panel. Finally, as described earlier, inflammation plays a key role in seizure; this is not accounted for in the ion channel panel, nor has inflammation been demonstrated to impact *in vitro* CNS cell activity using MEAs. To address this, measurements of stress-related genes could be included in the analysis of the response of hiPSC-derived neurons to seizurogenic compounds.

Regarding MEA, future work needs to consider the types and properties of the different sources and samples of hiPCS neurons with a view to standardization. Specifically, intrinsic properties such as the balance of excitatory and inhibitory neurons, glial cell ratio, and ion channel expression will all alter drug response as well as the more practical considerations such as culture media and conditions. Additionally, drug response detection limitations of *in vitro* hiPSC neurons will also need to be clarified. Although there are challenges, MEA measurements using hiPSC neurons facilitate extrapolation to the human brain and enable testing using neurons derived from diverse sources such as those from neurological disease states. Additionally, a bridging step of using rodent-derived cells for comparison to the current animal EEG models may prove useful. MEA thus holds much promise for *in vitro* seizure prediction.

Regarding the ion channel panels, each assay should be a functional measure of ion channel activity that can measure both channel blockers and activators. With multiple ion channels and the possibility of agonism and antagonism as is seen for cardiac ion channel screening (Crumb *et al.*, 2016), the data generated will be complex to interpret and may require a tailored bioinformatic program as has been developed for the CiPA initiative (CiPA, 2020). Nonetheless, the approach has great potential for moving away from animal testing toward improved human prediction. As *in vitro* screening strategies evolve, strategies that reduce animal use such as including EEG monitoring in early toxicology or dose range finding studies may be considered in reducing animal use while addressing seizure liability risk assessments. Although there are scientific and technical issues to overcome, the ideas and data presented here highlight great potential promise for a more integrated, biologically based and cost-effective system that can be implemented at an early stage in drug discovery for the detection of seizure liability.

DECLARATION OF CONFLICTING INTERESTS

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