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The emerging role of *in vitro* electrophysiological methods in CNS safety pharmacology

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ABSTRACT

Adverse CNS effects account for a sizeable proportion of all drug attrition cases. These adverse CNS effects are mediated predominately by off-target drug activity on neuronal ion-channels, receptors, transporters and enzymes — altering neuronal function and network communication. In response to these concerns, there is growing support within the pharmaceutical industry for the requirement to perform more comprehensive CNS safety testing prior to first-in-human trials. Accordingly, CNS safety pharmacology commonly integrates several *in vitro* assay methods for screening neuronal targets in order to properly assess therapeutic safety. One essential assay method is the *in vitro* electrophysiological technique — the 'gold standard' ion channel assay. The *in vitro* electrophysiological method is a useful technique, amenable to a variety of different tissues and cell configurations, capable of assessing minute changes in ion channel activity from the level of a single receptor to a complex neuronal network. Recent advances in automated technology have further expanded the usefulness of *in vitro* electrophysiological methods into the realm of high-throughput, addressing the bottleneck imposed by the manual conduct of the technique. However, despite a large range of applications, manual and automated *in vitro* electrophysiological techniques have had a slow penetrance into the field of safety pharmacology. Nevertheless, developments in throughput capabilities and *in vivo* applicability have led to a renewed interest in *in vitro* electrophysiological techniques that, when complimented by more traditional safety pharmacology methods, often increase the preclinical predictability of potential CNS liabilities.

1. Introduction

Upon the adoption of the International Conference on Harmonisation (ICH) S7A guidelines (FDA, 2001), the central nervous system (CNS) was recognized as a central pillar within the field of safety pharmacology (Pugsley, Authier, & Curtis, 2008). Testing strategies and practices aimed at addressing adverse CNS effects represent an important aspect to the development of any new chemical entity (NCE) since they attempt to prevent serious adverse drug reactions (ADR) from occurring within the clinical setting. Despite this, drug candidates continue to fail within clinical studies due to adverse CNS effects (Arrowsmith & Miller, 2013) — increasing pressure to minimize drug attrition rates within pharmaceutical development (Hay, Thomas, Craighead, Economides, & Rosenthal, 2014; Kola & Landis, 2004; Palmer & Alavijeh, 2012; Pangalos, Schechter, & Hurko, 2007; Waring et al., 2015). This pressure is further exacerbated by the fact that 10% of all marketed pharmaceuticals withdrawn between 1960 and 1999 were withdrawn due to adverse CNS effects stemming mostly from neurologic, psychiatric and abuse liabilities (Fung et al., 2001; Hamdam et al., 2013). Thus, with an ageing global population and a growing demand for novel pharmaceutical therapies, the need to adequately assess adverse CNS effects earlier in the drug development is paramount.

As defined by the ICH S7A, the core battery of CNS safety pharmacology studies consists of investigations conducted in accordance with good laboratory practice (GLP) standards and involve *in vivo* observations together with follow-up studies, if appropriate (Fonck, Easter, Pietras, & Bialecki, 2015; Hamdam et al., 2013). Traditionally, the most common core battery tests used within CNS safety pharmacology are behavioral assays such as the Irwin assay (Irwin, 1968) and the functional observational battery (FOB) (Moser, Cheek, & MacPhail, 1995). These tests rely heavily upon subjective endpoints and require highly experienced observers to ensure experimental reproducibility (Fonck et al., 2015). Technological advances such as electroencephalography (EEG) (Durmuller, Guillaume, Lacroix, Porsolt, & Moser, 2007), conducted either alone or paired with telemetry (Authier et al., 2014; Kramer & Kinter, 2003) and/or integrated video systems (Authier et al., 2009), have afforded safety pharmacologists more quantitative and objective endpoints with which to assess adverse CNS effects in unstressed animals. When paired with behavioral assays, EEGs represent a robust strategy linking clinical manifestations to altered neuronal activity.

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Unfortunately, these techniques possess limitations such as low signal-to-noise and a lack of regional accuracy (Castagné et al., 2013; Fonck et al., 2015; Hamdam et al., 2013; Porsolt, Lemaire, Durmuller, & Roux, 2002) preventing complete predictability of results to the clinical setting. Thus, follow-up CNS safety studies are required, and if conducted at earlier stages of drug development, should help to improve clinical outcome predictability and ultimately reduce drug attrition rates.

Neuronal-based assays for screening ion-channel targets are rapidly emerging as key assays implemented early during CNS safety pharmacology testing (Bowes et al., 2012; Gonzalez, Oades, Leychkis, Harootunian, & Negulescu, 1999; Mattheakis & Savchenko, 2001; Terstappen, 2005; Terstappen, Roncarati, Dunlop, & Peri, 2010; Xu et al., 2001). Ion-channels are pore-forming, membrane-bound proteins, permeable to specific ions, which play fundamental roles in inter- and intracellular communication and neuronal excitability. As such, ion-channels have been subjected to rigorous research investigations into a very broad range of important therapeutic areas since numerous disease states are associated with their dysfunction (Babcock & Li, 2013; Kaczorowski, McManus, Priest, & Garcia, 2008; Kullmann, 2010). Adding to this point, roughly 15% of the most successfully marketed pharmaceutical drugs target ion-channels (Overington, Al-Lazikani, & Hopkins, 2006) with a market value in excess of $10 billion (USD) (Terstappen et al., 2010). However, ion-channels also serve as highly promiscuous targets for small molecules and thus contribute to ADRs and off-target interactions (Lounkine et al., 2012). Some of the most notorious off-target interactions are associated with ion-channels such as the human *ether-à-go-go*-related gene potassium channel (hERG, also known as KCNH2), voltage-gated sodium channel (SCN5A), γ-aminobutyric acid type-A receptor ($GABA_AR$) and N-methyl-D-aspartate receptor (NMDAR). Accordingly, these ion-channels have been suggested to be screened in the early assessment of the potential hazard of a NCE (Bowes et al., 2012).

Some of the most common neuronal-based assays for screening drug-induced effects on ion-channel functionality rely on exploiting key aspects of ion-channel activity such as ligand binding, ion flux or the resultant modulation of neuronal membrane potential (see Table 1). Of these assays, the 'gold standard' for assessing ion-channel function is the patch-clamp electrophysiological technique (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981; Neher & Sakmann, 1976). Patch-clamp electrophysiology is a technique in which one can measure the biophysical (*e.g.*, activation and decay kinetics) and pharmacological properties of ion-channels on millisecond timescales with up to single channel resolution. Thus, this technique is capable of generating precise, high quality data. The advent of automated robotic patch-clamp systems in recent years has made patch-clamp electrophysiology a high-through technique able to meet the demand within the pharmaceutical industry (Dunlop, Bowlby, Peri, Vasilyev, & Arias, 2008; Terstappen et al., 2010). Together, manual and automated patch-clamp systems represent only a small window into the world of *in vitro* electrophysiology where various other techniques and platforms are also available for use; however each with their own merits and limitations. Due to the robustness and utility of the method, *in vitro* electrophysiological techniques are beginning to emerge as important fixtures in safety pharmacology. This is, in part, due to the growing support for the requirement to perform more comprehensive CNS safety testing prior to clinical studies (Lindgren et al., 2008; Valentin & Hammond, 2008). As such, this review will briefly highlight common electrophysiological methods with the greatest translational potential for safety pharmacology as well as provide a brief look into the emerging role of *in vitro* electrophysiology within CNS safety pharmacology.

2. *In vitro* electrophysiological approaches

Despite the embracement of *in vivo* electrophysiological approaches early in CNS safety investigations (*e.g.*, EEG), *in vitro* electrophysiology methods have often been regulated to follow up CNS studies in late phase testing. This is surprising since EEGs record the ensemble electrical activity of the brain and thus it would be useful to also provide mechanistic information related to drug-induced modulation at the level of the individual neuron, receptor or ion channel early in safety testing. As an example, studies on the hERG channel have shown that subtle blockade in channel activity, which may contribute to long QT interval and Torsade de pointes, may go unnoticed during electrocardiograph evaluation (Moller & Witchel, 2011; Viskin et al., 2005). Accordingly, the ICH S7B guidelines emphasize that *in vitro* electrophysiology studies must play a key role in addressing potential hERG channel interactions (FDA, 2005). A similar approach has not yet been mandated for CNS ion-channels, but a systematic *in vitro* electrophysiological exploration of neuronal ion channels are included by a number of large pharmaceutical companies for the characterization of a NCE.

In vitro electrophysiological techniques in CNS studies exploit ionic conductance of ion-channels and transient modulation of the membrane potential of a neuron. If the membrane potential becomes sufficiently depolarized, an action potential will trigger. Many *in vitro* electrophysiology techniques have been developed to detect and manipulate ion-channel function and/or action potential generation. The applicability of each technique is dependent upon numerous factors including, but not limited to, the biophysiological properties of the recorded cell, the tissue preparation, the use of current- and/or voltage-clamp, manipulation of the intra- and/or extracellular milieu, the detection of a single channel or ensemble responses, the study of responses at the channel, neuron or network level, and intra- *vs.* extracellular recordings.

Table 1
Common ion channel assays.

Method	Assay Type	Throughput	Temporal resolution	Comments
Radioligand binding assay	Biochemical	High	N/A	Does not provide functional data
Radioactive flux assays	Functional	Medium–high	Seconds–minutes	Use of radioactive isotopes
Nonradioactive flux assays				
Based on atomic absorption spectrometry	Functional	High	Seconds–minutes	K^+ and nonselective cation channels
Based on ion-specific fluorescence dyes	Functional	Medium–high	Seconds	Ca^{2+} and nonselective cation channels
Fluorescence assays				
Based on voltage-sensing dyes	Functional	Medium–high	Seconds–minutes	Useful in cell lines that pass little or no Ca^{2+}
Fluorescence resonance energy transfer (FRET)	Functional	High	Sub-second	Indirect measurement of channel activity; ratiometric
Electrophysiology				
Manual patch-clamp	Functional	Low	Sub-millisecond	Sensitive to single channel resolution
Automated robotic patch-clamp systems	Functional	Medium–high	Millisecond	10-fold higher throughput than manual patch-clamp
Planar array based recording interfaces	Functional	High	Millisecond	Up to 1000-fold higher throughput than manual patch-clamp
Multielectrode array	Functional	Medium–high	Millisecond	Neuronal population responses
Oocyte recording techniques (manual and automated)	Functional	Low–medium	Millisecond	High heterologous protein expression within a short period

Thus, due to the diversity of *in vitro* electrophysiology techniques and applications, a comprehensive review of all electrophysiology methods is beyond the scope of this review. This review will instead highlight common *in vitro* electrophysiological techniques that hold the greatest translational potential for CNS safety pharmacology testing and those which are currently employed within the field of safety pharmacology.

2.1. Patch-clamp techniques

The most commonly employed *in vitro* electrophysiological technique is the patch-clamp method. This method requires the initial formation of a giga-ohm (GΩ) seal between the plasma membrane and the blunt tip (0.5–2 μm diameter) of a heat-polished glass or quartz micropipette (electrode) (Hamill et al., 1981; Sigworth & Neher, 1980). This '*cell-attached*' configuration is a stable non-invasive technique from which all other patch-clamp configurations derive (Fig. 1A). This configuration maintains the integrity of the plasma membrane (*i.e.*, the membrane seal is not ruptured) preventing the pipette solution from dialyzing into the cell. However, this lack of seal rupture consequently restricts electrical access to the cell interior resulting in an inability to control the membrane potential of the cell. Consequently, the cell-attached configuration allows only control of the patch membrane potential relative to the cell's resting potential. Therefore, by controlling either the magnitude of the seal resistance (*loose seal vs. tight seal*) and/or whether the recording electrode is current- *vs.* voltage-clamped, the cell-attached configuration can be used to measure single channel currents, spontaneous neuronal cell firing and synaptic potentials as well as evoked action potentials within the cell.

To increase electrical access to the cell interior, two methods can be employed. First, the internal pipette solution can be made to contain antibiotic or antifungal agents (*e.g.*, nystatin, gramicidin, amphotericin-B). These agents form small, monovalent ion-permeable pores that '*perforate*' (Fig. 1A) the membrane allowing electrical assess to the entire cell. Importantly, these pores do not allow passage of proteins thus ensuring that the intracellular milieu remains intact preserving intracellular signaling pathways (Horn & Marty, 1988). However, this *perforated patch* technique suffers from several limitations including higher electrical noise, loss of single channel resolution and patch instability. Additionally, the perforation process is associated with considerable time requirements (Sarantopoulos, 2007). Together, these concerns have dissuaded safety pharmacologists attempting to elucidate pharmacological effects, and hence drug safety profiles of novel NCEs on neuronal ion channels, from widely using this technique. An alternative approach to the perforated patch technique is to apply a strong suction, or brief voltage transient, after giga-seal formation in an attempt to rupture the plasma membrane. Upon rupture, a low-resistance electrical and physical continuity is established between the pipette and the cell interior known as the *whole-cell* configuration (Fig. 1A). Accordingly, this configuration permits direct measurements of the cell's membrane potential (*via* current-clamp) and its manipulation (*via* voltage-clamp). Due to the physical continuity between the cell interior and the pipette solution, the cytosolic contents can be reasonably controlled. Furthermore, unlike the perforated patch, pharmacological or ionic manipulations of both the intracellular and extracellular environment can lead to the isolation of individual ion-currents. However, this physical continuity between the pipette lumen and cytosol may also dialyze out and/or alter the activity of endogenous second messenger systems. Thus, it is critical to assess current 'rundown' of the system and cells within whole-cell CNS studies.

'Cell-free' variations of patch-clamp techniques also exist. For instance, upon giga-seal formation, the electrode can be gently retracted pulling the membrane patch into the bath solution. This arrangement, known as the *inside-out* configuration (Fig. 1A), enables the complete manipulation of the cytoplasmic face of the plasma membrane *via* the

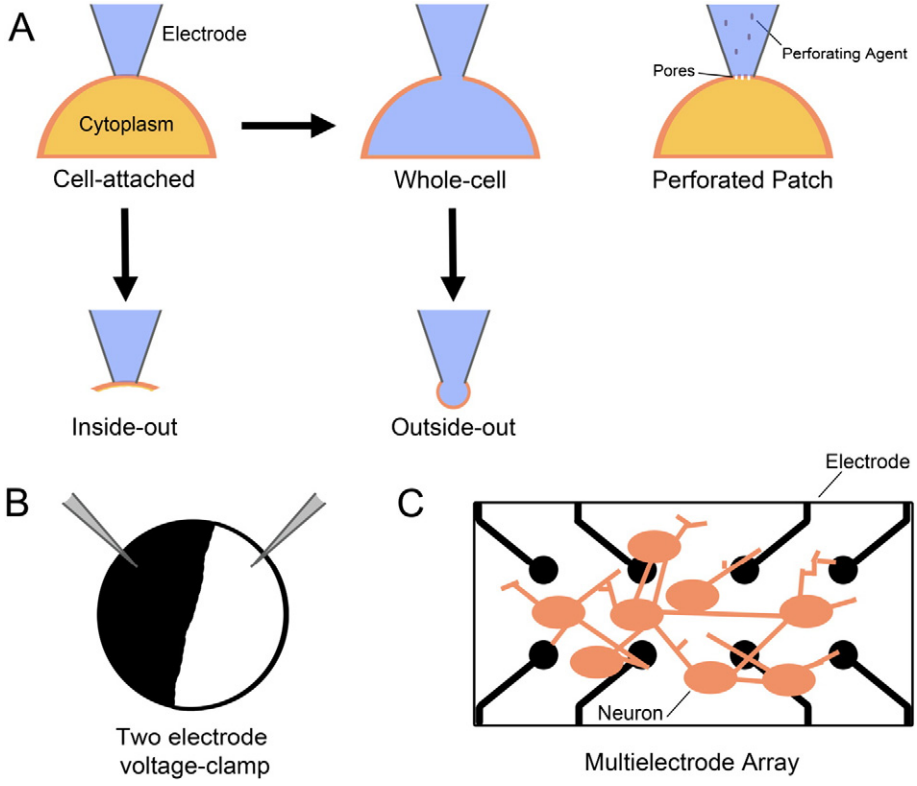

Fig. 1. Common *in vitro* electrophysiological methods. A) The cell-attached patch-clamp method is a stable non-invasive technique from which all other patch-clamp configurations derive. The perforated patch, outside-out and inside-out configurations are not commonly used in CNS safety pharmacology studies. To show potential dialysis, the pipette lumen and cytoplasm are represented by blue and orange, respectively. B) A representation of the impalement technique known as 'Two electrode voltage-clamp' performed on a *Xenopus laevis* oocyte. C) A cartoon illustrating a section of a multielectrode array with dissociated neurons cultured directly over the electrodes.

bath perfusion – a feature not possible in the cell-attached configuration. As a result, inside-out patches extend the utility of single channel recordings through the ability to manipulate the immediate environment of the inner membrane face. Unfortunately, this arrangement suffers from the loss of intracellular signaling pathways acting on the ion-channels upon patch excision; a particularly important consideration due to altered channel activity (Covarrubias & Steinbach, 1990; Trautmann & Siegelbaum, 1983). Conversely, an *outside-out* patch (Fig. 1A) also requires the gentle retraction of the patch electrode from the whole-cell configuration. However, in this situation the pipette retraction forces the plasma membrane surrounding the electrode tip to detach from the cell and reseal forming a cell-independent patch whose extracellular membrane is facing the bathing solution. As a result, an experimenter has complete control over the intracellular environment (albeit without endogenous signaling pathways) and can rapidly exchange different external physiological or pharmacologic drugs over the same patch. This rapid external solution exchange enables the acquisition of concentration-response curves within a single patch up to single ion-channel resolution.

2.1.1. Manual vs. automated patch-clamp techniques

The manual patch-clamp is considered the 'gold standard' for conduct of *in vitro* electrophysiology studies due to its versatility and high-quality data production. Unsurprisingly, it is widely employed throughout the academic arena where highly trained personnel are capable of producing around 10 data points/day (Wood, Williams, & Waldron, 2004). However, when compared to other ion-channel assays (*e.g.*, ion- or voltage-sensitive fluorescence-based assays, binding assays or ion flux measurements), manual patch-clamp methods yield relatively low throughput (Gonzalez et al., 1999). Likewise, the requirements for high technical expertise and high maintenance costs have limited the expansion of this technique into aspects of safety pharmacology beyond cardiac evaluation. Thus, given the drive within the pharmaceutical industry to develop new, higher throughput screens, several automated patch-clamp systems have been developed which simplify the patching procedure and generate data in an inexpensive, reproducible and high throughput manner (Dunlop, et al., 2008;; Terstappen, 2005; Terstappen et al., 2010; Yajuan, Xin, & Zhiyuan, 2012).

Automated patch-clamp systems utilized conventional glass micropipettes (*e.g.*, Apatchi-I Sophion Bioscience), FlyScreen (Flyion GmbH), AutoPatch (Xention Discovery Limited) and Dynaflow HT (Cellectricon)) or micro-fabricated planar electrode-based (patch-on-a-chip) technologies for patching either mammalian cells (*e.g.*, Qpatch (Sophion, Biosciences), NPC-16 Patchliner, SyncroPatch 96 and Port-a-Patch (Nanion Technologies GmbH), CytoPatch (Cytocentrics CCS), IonWorks (IonWorks HT and IonWorks Quatto; Molecular Devices), IonFlux (Fluxion Biosciences Inc) and PatchXpress (Molecular Devices)) or *Xenopus laevis* oocytes (*e.g.*, OpusXpress (Molecular Devices) and Roboocyte (IonGate)) (Dunlop et al., 2008; Terstappen, 2005; Terstappen et al., 2010; Wood et al., 2004; Xu et al., 2001; Yajuan et al., 2012). In practice these automated systems function by detecting ensemble responses in a whole-cell or perforated-patch recording, which is typically achieved by one of two methods: (i) directing a glass micropipette blindly, or through imaging, towards a cell until a giga-seal is achieved (or impaled in the case of oocytes; see below) or (ii) allowing cells to drift, or culturing cells onto a planar surface possessing micron-size holes from which loose or tight seal formation can occur (Py et al., 2011; Wood et al., 2004; Yajuan et al., 2012). When compared to manual patch-clamp techniques, automated systems significantly increase throughput by a factor of ten (*e.g.*, robotic glass pipette technologies) to upwards of 1000-fold (*e.g.*, planar electrode-based technologies) (Terstappen, 2005) generating greater than 10,000 data points/day (Fertig & Farre, 2010) (See Table 1).

Despite this high throughput success, current automated patch-clamp technologies lack the versatility and quality compared to manual patch-clamp. For instance, many automated systems are restricted to the whole-cell configuration thus limiting their data acquisition to ensemble responses — precluding single channel measurements. Several systems lack the ability for ultra-rapid solution exchange (*i.e.*, 1 ms; on par with synaptic vesicular release) or solution washout which may underestimate or inaccurately measure the biophysical properties (*e.g.*, activation, deactivation and desensitization kinetics) of a given response. The requirement of some automated platforms for specific cell lines, or cell suspensions, precludes the usage of intact tissue preparations thus restricting the assessment of neuronal network dynamics (Yajuan et al., 2012). Moreover, many automated systems sacrifice data quality for data volume by tolerating low resistance patches (MΩ *vs.* GΩ seals) and high leak currents (Fertig & Farre, 2010; Yajuan et al., 2012). Taken together, these limitations serve as ever-present hurdles for automated patch-clamp systems leading to the continued importance, validity and viability of the manual patch-clamp technique.

2.2. Impalement techniques

For large cells (e.g., *Xenopus laevis* oocytes), voltage-clamping the membrane in the whole-cell patch-clamp technique is very difficult. This is attributable to the large current produced within these cells that contributes to a significant voltage drop across the recording electrode which cannot be acceptability compensated. Consequently, impalement techniques have been developed which forcibly penetrate the membrane of large cells with sharp micropipettes (*i.e.*, in which tip resistances are tens or hundreds of MΩ). For example, the most commonly employed impalement technique is two-electrode voltage clamp (TEVC; Fig. 1B). In this technique, two electrodes penetrate the membrane where one acts as a dedicated membrane potential sensor (voltage-electrode) and the other as a current injector (current-electrode). Accordingly, the membrane is voltage clamped by a constant injection (or removal) of current through the current-electrode. The tips of the sharp electrodes are comparatively smaller to those used in the patch-clamp method enabling TEVC to measure the potential inside the cell with minimal disruption to the cytosolic ionic composition. *Xenopus* oocytes are also commonly used during TEVC since their large size permits impalement by multiple microelectrodes. Together with their relative lack of endogenous ion-channels and their excellent ability for high-fidelity homologous protein expression, *Xenopus* oocytes represent a popular ion-channel recording and drug screening system (Xu et al., 2001).

Automated TEVC systems are commercially available allowing for recordings from multiple oocytes either in a serial (*e.g.*, Roboocyte (Multi Channel Systems)) or parallel fashion (*e.g.*, OpusXpress (Molecular Devices)) (Terstappen, 2005; Wood et al., 2004; Yajuan et al., 2012). Furthermore, although their size precludes whole-cell patch-clamp, oocytes are amenable to the cell-attached patch-clamp method (as well as the inside- and outside-out configurations) and, as such, can be recorded from a planar electrode arrays (Klemic, Klemic, Reed, & Sigworth, 2002; Wood et al., 2004). However, development of automated systems for oocyte recordings has severely lagged behind their mammalian cell counterpart (Yajuan et al., 2012) for several reasons. First, due to their non-mammalian nature, oocytes may promote improper post-translational modifications (Wood et al., 2004). Second, lipophilic drugs tend to accumulate in the egg yolk of oocytes resulting in the generation of markedly different half maximal inhibitory concentration (IC_{50}) values between TEVC and patched mammalian cells (Wood et al., 2004; Xu et al., 2001). Third, oocytes are affected by seasonal variation (Xu et al., 2001) which may diminish data quality or throughput. Forth and finally, oocytes possess accessory follicular cells and a vitelline membrane which must be removed through digestive treatments before they can be effectively used for electrophysiology (Methfessel et al., 1986). As a result of these limitations, oocyte use is primarily restricted to academic or possibly investigative mechanistic studies and is not commonly utilized in drug safety pharmacology assessments of NCEs.

2.3. Extracellular recording techniques

One of the principle limitations of both the patch-clamp and impalement techniques is that they are intracellular recording systems and, as such, are limited to assessment of individual neurons. Hence, these systems preclude the accurate assessment of neuronal circuit dynamics. Although paired-recordings and field stimulations can elucidate the connective patterns between some neurons in the patch-clamp technique, these recordings are still limited to only a few neurons at a time. Furthermore, sharp- and patch microelectrodes are sensitive to mechanical and biophysical instabilities which may dramatically influence the duration of intracellular recording sessions. Thus, extracellular recordings have been developed to serve as a non-invasive, long-term, multi-cell method aimed at studying neuronal circuit connectivity, physiology and pathology (Spira & Hai, 2013). Extracellular recording techniques insert electrodes into tissue which, depending on the positioning and tip size, measure (with sub-millisecond time resolution) the extracellular field potential generated by an action potential discharge from either a single neuron (single-unit recording) or neuronal population (multi-unit recording). Therefore, extracellular recordings can be used to understand neuronal communication, information encoding, propagation, processing and computation of neuronal circuits (Obien, Deligkaris, Bullmann, Bakkum, & Frey, 2014).

One of the more notable methods of extracellular recordings is the use of substrate-integrated microelectrode arrays (MEA; Fig. 1C) (Obien et al., 2014). MEAs addresses neural network dynamics by sensing extracellular field potentials and have been adapted to function in a high throughput capacity for several tissue preparations such as primary cell cultures, stem-cell-derived neuronal cultures (Illes, Fleischer, Siebler, Hartung, & Dihne, 2007), mixed organotypic slice cultures (Berdichevsky, Sabolek, Levine, Staley, & Yarmush, 2009) and acute brain slices Fonck et al., 2015; Obien et al., 2014). Several commercial enterprises (*e.g.*, AlphaMED; Axion-Biosystems; Ayanda; Multi Channel Systems) currently offer marketed MEAs which typically consist of a grid of upwards to several hundred integrated electrodes which concurrently record the passive and stimulated electrophysiological activity of a tissue Obien et al., 2014; Py et al., 2011). Upon analysis of these recordings, a precise understanding of the focus and spread of electrical activity across the tissue preparation can be established. The utility of these arrays can be further increased by combining multiple arrays so that over 10,000 electrodes can be utilized and up to 200 compounds assessed per day (Johnstone et al., 2010; Py et al., 2011). MEAs also permit culturing of neurons directly onto the electrode arrays which may enable a continued use of MEAs for several days – a useful application for investigations of chronic drug exposure (Johnstone et al., 2010; Spira & Hai, 2013). Recent advances in nanotechnology have also begun to improve the signal-to-noise ratio for MEA recordings (Py et al., 2011; Spira & Hai, 2013), a persistent limitation for MEAs due to the low amplitude signals (*e.g.*, 10 - 500μV) generated by field potentials (Obien et al., 2014; Py et al., 2011).

3. Common *in vitro* CNS tissue models for electrophysiology

Safety pharmacology aims to predict the clinical risk profile of a potential new drug prior to first-in-human studies. Accordingly, it is imperative to select the most appropriate tissue and animal model for testing during electrophysiological investigations to ensure adequate predictability can be attained. This is a particularly important issue in CNS safety pharmacology since interspecies differences in ion-channel expression, drug metabolism and neuroanatomy can result in the manifestation of different drug effects between preclinical species and humans (Atack et al., 2011). To address these challenges, *in vitro* electrophysiology methods are amenable to a variety of tissue preparations suitable for studying adverse CNS effects. Isolated, *in vitro* whole brain preparations have been used in electrophysiological experiments (*e.g.*, Gnatkovsky, Librizzi, Trombin, and de Curtis (2008)) but are rather restrictive in terms of access to, and visibility of, the internal brain structures – limiting the use of several patch-clamp configurations. Accordingly, alternative preparations have been developed which provide a simplified model of the *in vivo* condition whose reduced complexity enables accurate and reliable methods for studying key neuronal processes on both the cellular and molecular level (Bal-Price & Hogberg, 2014). At the same time, some tissue preparations suffer several concessions due to their simplicity, limiting direct *in vivo* comparisons. Therefore, the process of tissue selection must be driven by the question being addressed, the cost, training and expertise required to successfully establish, validate and apply the model and the specific target endpoints.

3.1. Expression systems and immortalized cell lines

The most rudimentary *in vitro* electrophysiological models for CNS investigations are heterologous and recombinant expression systems which are cells/cell lines that can be maintained in culture for an extended period of time. The cells/cell lines typically used as heterologous (*e.g.*, *Xenopus* oocytes; (Methfessel et al., 1986)) or recombinant expression systems (*e.g.*, human embryonic kidney 293 (HEK-293) cells, Chinese hamster ovary (CHO) cells; (Khan, 2013; Thomas & Smart, 2005)) are easily maintained, amenable to manual and automated electrophysiological techniques and faithfully express high levels of desired protein within a short period of time. As such, these systems have been used extensively to evaluate the pharmacological properties and structure-function relationships of multiple CNS ion-channels. However, despite their simplicity and ubiquitous use, these cells lack many of the complexities associated with neuronal function within the intact brain (*e.g.*, network associations, glial interactions, and developmental regulation) — a disadvantage when attempting to predict CNS safety. Furthermore, these cells are of a non-neuronal origin and thus lack the same sophisticated level of cellular architecture, sub-cellular organization or biochemistry associated with native neuronal preparations (Thomas & Smart, 2005).

Early efforts to address these non-neuronal concerns focused on neuronal cells derived from mouse neuroblastoma C-1300 tumor (*e.g.*, N1E-115) (Spector, 1981) or the human SH-SY5Y neuroblastoma cell line (Puchacz, Buisson, Bertrand, & Lukas, 1994; Seward, Henderson, & Sadée, 1989; Toselli, Masetto, Rossi, & Taglietti, 1991). However, subsequent advances in molecular biology enable the use of neural stem cells (NSCs). NSCs are uncommitted cells with self-renewal potential and the ability to differentiate into cells of all neural lineages (Bal-Price & Hogberg, 2014; Weiss et al., 1996). These cells can be derived from several sources such as pluripotent embryonic stem cells isolated from the blastocyst, human umbilical cord blood, induced pluripotent stem cells and multipotent somatic progenitors derived from several tissues including the CNS (Bal-Price & Hogberg, 2014). NSCs derivation has occurred with both non-human (*e.g.*, mice, rats) and human tissue, the latter of which enables direct extrapolation to the human situation (Bal-Price & Hogberg, 2014). Electrophysiologically, these cells possess Na^+, K^+ and Ca^{2+} currents in accordance with the known patterns described for their *in vivo* neuronal counterparts, even at early stages of differentiation (Bain, Kitchens, Yao, Huettner, & Gottlieb, 1995; Cho et al., 2002; Jelitai, Anderova, Chvatal, & Madarasz, 2007; Risner-Janiczek, Ungless, & Li, 2011; Song, Stevens, & Gage, 2002). Furthermore, these cells are also capable of forming rudimentary, yet functional, glutamatergic and GABAergic synapses in culture (Toda, Takahashi, Mizoguchi, Koyano, & Hashimoto, 2000). Nevertheless, despite these benefits, several sources of NSCs (*e.g.*, embryonic or fetal neural stem cells) are limited in use due to ethical concerns. Additionally, those obtained from adults offer limited neural lineage potential and senesce after only a few passages (Jakel, Schneider, & Svendsen, 2004). Moreover, NSC cultures may possess mixtures of both undifferentiated and differentiated neurons, for which some neurons are developmentally immature, and thus hinder extrapolation of data to the adult *in vivo* condition (Jakel et al., 2004).

3.2. Dissociated neuronal primary cultures

Increasing in complexity, dissociated neuronal primary cultures represent another common tissue preparation used in electrophysiological investigations (Harry et al., 1998). These cultures are mechanically and enzymatically dissociated from various brain regions (*e.g.*, hippocampus, cortex, cerebellum, striatum, midbrain, superior cervical ganglion, etc.) and consist of either one predominant neuronal cell type, a co-mixture of different neuronal populations or mixed neuronal–glial cultures (Bal-Price & Hogberg, 2014; Harry et al., 1998). Dissociated neurons and astrocytes retain much of their functional capacity *in vitro* enabling these preparations to address many important processes observed in the *in vivo* condition such as network dynamics and neuronal-glial interactions (Bal-Price & Hogberg, 2014). However, the success of dissociated cultures is dependent upon specific requirements dictated by the neuron being cultured (Shahar, de Vellis, Vernadakis, & Haber, 1989). If these conditions are not met, isolated neurons may develop altered morphology and functions modifying their response to drugs when compared to the *in vivo* situation (Bal-Price & Hogberg, 2014; Costa, 1998). For instance, the age of the donor at harvest can largely influence the success of a culture (Harry et al., 1998) partly due to altered receptor expression between embryonic tissue and tissue isolated from more mature neurons (Lin et al., 2002). Furthermore, dissociated neurons cannot be maintained in culture for extended periods of time (Humpel, 2015) and thus are required to be freshly isolated on a regular basis.

3.3. Three-dimensional (3D) neuronal models

The 3D neuronal model represents the next level of complexity for CNS *in vitro* models. Like the two-dimensional (2D) preparations discussed above, 3D brain cell cultures can consist of a co-mixture of different neuronal and non-neuronal populations obtained from different sources such as cell lines (Labour, et al., 2012), dissociated neuronal primary cells (van Vliet et al., 2008) and stem cells (Giobbe et al., 2012). Interestingly, instead of being cultured in a traditional planar monolayer, 3D brain cultures are created up to 10 cell diameters thick within reaggregate or spherical cultures (*i.e.*, spheroids), hydrogel/scaffold cultures or rotary bioreactor cultures with cell aggregates or microcarriers (Lancaster & Knoblich, 2014; LaPlaca, Vernekar, Shoemaker, & Cullen, 2010). When grown in a 3D environment, neural cells demonstrate better survivability and behave differently when compared to traditional 2D-models due in large part to the closer physiological similarity to the *in vivo* condition of 3D-models (Fawcett, Housden, Smith-Thomas, & Meyer, 1989). As such, these models promote better development of native voltage-gated ion-channel functionality, resting membrane potentials, intracellular Ca^{2+} dynamics, Na^{+}/H^{+} exchange, enhanced neurogenesis and differentiation, synapse formation, neuronal mobility and axon myelination (Lancaster & Knoblich, 2014; Lancaster et al., 2013; LaPlaca et al., 2010; van Vliet et al., 2007). Importantly, these cultures are also compatible with various intracellular and extracellular (including MEAs) electrophysiological techniques (Huval et al., 2015; van Vliet et al., 2007); increasing their potential value over traditional 2D-models for safety testing. However, cells residing within the center of the 3D culture may experience greater levels of oxygen and nutrient deprivation due to diffusional transport limitations. Accordingly, 3D-models may suffer from culture-dependent alterations in gene expression, cell proliferation, viability, productivity and product quality not typically observed with 2D-models (LaPlaca et al., 2010).

3.4. Brain slice models

Brain slice models (*e.g.*, acute and organotypic slice cultures) represent the most accurate *in vitro* electrophysiology preparation of the *in vivo* CNS situation since they most closely retain the *in vivo*-like stereotypic and temporal organization and structural integrity of neuronal microcircuits (Gibb & Edwards, 1994). One of the most commonly used brain slice models in neuroscience is the acute brain slice (Colbert, 2006; Luhmann & Kilb, 2012). Acute brain slices are typically ≤ 450 μm thick and permit easy access to visually identifiable neurons and their dendritic and axonal compartments (Cho, Wood, & Bowlby, 2007). As such, brain slice models enable the application of virtually all intra- and extracellular electrophysiological techniques in a near *in vivo* situation, rapidly after euthanasia. Interestingly, these brain slices can be harvested from a donor of any age permitting drug safety assessments on neuronal activity and network processes at any developmental stage (Humpel, 2015). Unfortunately, factors such as slicing-induced cell damage, excitotoxicity, the lack of oxygen/glucose supply to neurons deep within the tissue and bacterial contamination, among others, limit the lifespan of acute brain slices to roughly 6 – 12 hours (Buskila et al., 2014; Fukuda et al., 1995).

Organotypic slice cultures – an extension of the acute brain slice method – are capable of maintaining a brain slice in culture, on a stable substratum, over prolonged periods of time (*e.g.*, ≥seven days) (Gahwiler, Capogna, Debanne, McKinney, & Thompson, 1997). This prolonged period in culture enables the re-establishment of long-distance connections, severed through the slicing procedure, to an *in vivo*-like connection pattern (Gahwiler et al., 1997; Humpel, 2015). However, unlike the acute brain slice which retains its relative shape and thickness, organotypic slice cultures flatten into a 3D structure approximately 3–4 cells thick. Nevertheless, this flattened structure retains the characteristic cytoarchitecture of neurons and glial cells as well as the anatomical structure of the tissue of origin (Gahwiler et al., 1997; Humpel, 2015). Notably, unlike acute brain slices, age is very important for organotypic slice cultures as it is well known that tissue obtained from embryonic or young (*i.e.* ≤postnatal day 11) donors possess greater survivability (Humpel, 2015). Remarkably, organotypic cultures generally show *in vitro* maturation with synapse development and spine morphology (Collin, Miyaguchi, & Segal, 1997; Gerfin-Moser, Grogg, Rietschin, Thompson, & Streit, 1995), protein expression (Bahr et al., 1995) and electrophysiological properties maturing in a pattern comparable to the *in vivo* situation (Gahwiler et al., 1997; Humpel, 2015).

3.5. Animal model considerations

The selection of an animal model is an important consideration since there are obvious interspecies differences (*i.e.*, mammalian *vs.* non-mammalian) that may promote improper translation of animal data to the human condition (Lynch, 2009). Furthermore, intra-species differences (*e.g.*, age and sex of an animal) must also be considered when selecting the appropriate animal model. *In vitro* electrophysiological studies can be conducted using tissue from a variety of animal species (*e.g.*, *Xenopus* oocytes, rabbit, rodent, zebrafish and non-human primate) if human equivalents (*e.g.*, cell lines) are deemed inadequate. Of these animal models, the primary experimental species used for CNS investigations are rodents, namely mice and rats (Manger et al., 2008). The development of transgenic mouse models has rapidly increased the utility of rodent models in CNS research providing a useful model for a variety of human neurological disorders (*e.g.*, Alzheimer's disease, amyotrophic lateral sclerosis, epilepsy) (Harper, 2010). These mutated mouse models enable the study of pharmacological drug effects in various disorders. Transgenic, humanized mice models have also shown good preclinical screening and safety testing potential as they express the exact protein observed in the human situation (Harper, 2010). Notwithstanding the fact rodent models have become an invaluable tool to study neurologic disease, they are nevertheless limited in their ability to recapitulate the full phenotype of any human disorder and disease (Lynch, 2009).

One consideration for the limited applicability of rodent models to humans are the anatomical and morphological differences observed between the rodent and human brain (Preuss, 2000). The use of rodent

Table 2
Recommended targets expressed within the CNS for receptor profile safety assessments.[a]

Class	Target	Roles within CNS function
G-protein coupled receptors	Adenosine receptor A_{2A} receptor	Sleep–wake cycle, motor function, cognition
	α_{1A}-adrenergic receptor	Learning and memory, addition, nociception
	α_{2A}-adrenergic receptor	Improve working memory, attention, nociception
	Cannabinoid receptor type 1 CB_1	Nociception, mood, memory, appetite
	Dopamine receptor D_1	Reward mechanism, addition, memory
	Dopamine receptor D_2	Reward mechanism, addition, memory, schizophrenia
	δ-opioid receptor	Nociception, mood, learning
	κ-opioid receptor	Nociception, drug abuse, addition
	μ-opioid receptor	Nociception, drug abuse, addition
	Muscarinic acetylcholine receptor M_1	Arousal, attention, learning and memory
	5-HT_{1A} receptor	Motor function, mood and emotion, cognition
	5-HT_{1B} receptor	Addiction, aggression, motor function
	5-HT_{2A} receptor	Learning and memory, cognition
Ion channels	nAChR; α4-subunit	Addiction, nociception
		Sedation, addiction, amnesia
	$GABA_AR$; α1-subunit BZD site	
	NMDAR; GluN1-subunit	Learning and memory
Enzymes		Depression
	Monoamine oxidase A	
	Phosphodiesterase 4D	Learning and memory, depression
Transporters	Dopamine transporter	Attention deficit hyperactivity disorder, schizophrenia
	Noradrenaline transporter	Mood, addiction, appetite
	Serotonin transporter	Anxiety, mood, anti-depressant

5-HT, 5-hydroxytryptamine (serotonin) receptor; nAChR, nicotinic acetylcholine receptor; BZD, benzodiazepine; CNS: central nervous system; $GABA_AR$, γ-aminobutyric acid type A receptor; NMDAR, *N*-methyl-d-aspartate receptor.
[a] CNS targets within the table were initially identified within Bowes et al. (2012).

models involves the sacrifice of the physiological complexity and function associated with the neuroanatomy of higher order mammalian species. To minimize these concessions, the use of higher order mammalian species within *in vitro* electrophysiology studies may be considered in the context of tissue slice models where the comparative value of non-human primate brain slices, for example, can serve to address drug safety in neuronal circuits with close anatomical and evolutionary ties to humans (Passingham, 2009; Preuss, 2000). However the use of higher order mammalian species, particularly nonhuman primates, may not always be feasible owing to ethical and logistical considerations.

4. Emerging models used in CNS safety pharmacology studies

Drug development is a rather expensive and lengthy endeavor with recent estimates placing the cost for new drug development at $2.6 billion (USD) (DiMasi, Grabowski, & Hansen, 2014) and requiring up to a decade for development and regulatory approval (Pangalos et al., 2007). This process has been reported to take even longer for pharmaceutical drugs targeting CNS disorders (Pangalos et al., 2007) since potential CNS drugs possess higher attrition rates and safety concerns compared to other therapeutic indications (Palmer & Alavijeh, 2012). The CNS is one of the most difficult areas to assess drug effects due to the diverse array of potential secondary neuronal and non-neuronal targets (*e.g.*, receptors, ion channels, enzymes and transporters) (Porsolt et al., 2002; Wakefield, Pollard, Redfern, Hammond, & Valentin, 2002). Furthermore, CNS drugs show the highest propensity for generating adverse CNS effects (Easter et al., 2009). With the CNS drug market forecasted to grow to $81.8 billion (USD) in 2015 (BCC Research, 2010), it has become imperative that more comprehensive CNS safety assessments are conducted early within drug development (Lindgren et al., 2008; Valentin & Hammond, 2008).

Traditionally, as discussed above, early preclinical safety screens have largely relied on behavioral assays (Irwin, 1968; Moser et al., 1995) or *in vivo* electrophysiological assessments of neuronal activity (e.g., EEG) (Authier et al., 2009, 2014; Kramer & Kinter, 2003). However, the nature and use of these methods prevent a thorough understanding of the mechanistic and molecular underpinnings contributing to observed changes in neuronal network function. This ensemble or 'black box' approach to pharmacodynamics can hinder the predictive power of these assays. Non-electrophysiological *in vitro* techniques (Table 1) may be used to investigate the molecular mechanism involved in a drug response, but these techniques lack the overall specificity, sensitivity and time resolution attainable through electrophysiological means. Thus it is unsurprising that several *in vitro* electrophysiological CNS techniques are beginning to emerge as interesting tools in the world of safety pharmacology.

4.1. Receptor profile safety screening

To increase preclinical predictability of adverse CNS effects, safety pharmacology has begun to frontload *in vitro* pharmacological profile screening early in safety testing (Bowes et al., 2012). Pharmacological profiling typically employs high-throughput radioligand binding assays owing to low costs, low compound requirements and rapid turnaround time (Armstrong et al., 2008; Easter et al., 2009). However, electrophysiological techniques are also suitable for *in vitro* pharmacological profiling and have been effectively used in early phase cardiac ion channel safety assessments (*e.g.*, hERG, Nav1.5, Cav1.2, Kv4.3) (Hancox, McPate, El Harchi, & Zhang, 2008; Moller & Witchel, 2011). However, equivalent screens for the CNS has lagged, in part, due to a limited consensus regarding appropriate neuronal targets. A recent review of the strategies and methodologies used at four major pharmaceutical companies (Bowes et al., 2012) recommended 44 molecular targets for *in vitro* pharmacological profile screens of which a total of 21 were direct CNS targets (Table 2). These targets are included on a recommended 'minimal panel' for safety testing which by no means represents an exhaustive list since several additional CNS ion channel targets could also be considered (Table 3). Since many of these targets (particularly ion channels) are amenable to manual and automated patch-clamp techniques, numerous drugs and their associated metabolites can be screened on several potential targets within a relatively short testing period (Terstappen et al., 2010). This approach can generate drug response profiles (*e.g.*, IC_{50} values) and highlight altered channel responsiveness which, at a minimum, can be used to raise concerns for future drug safety assessments (Wakefield et al., 2002). Furthermore, combining data obtained from receptor profile safety screens with other *in vitro* assays may indicate the need for specific CNS

Table 3
Additional CNS voltage- and ligand-gated ion channel considerations for receptor profile safety assessments.

Class	Ion channel type	Common isoforms or compositions
Voltage-gated calcium channels	L-type	Cav1.2; Cav1.3
	P/Q-type	Cav2.1
	N-type	Cav2.2
	R-type	Cav2.3
	T-type	Cav3.2
Voltage-gated potassium channels	A-type	Kv1.4
	Delayed rectifier	Kv7.2/ Kv7.3; Kv7.3/ Kv7.5
	Outward-rectifying	Kv10.1; Kv10.2
	Hyperpolization-gated, cyclic AMP-gated	HCN1-2
Voltage-gated sodium channels		Nav1.1
		Nav1.2
		Nav1.3
		Nav1.5 (*e.g.*, cerebellar isoform)
		Nav1.6
Ligand-gated ion channels	$GABA_AR$	α1β2/3γ2; α2β2/3γ2; α3β2/3γ2; α5β2/3γ2
	NMDAR	GluN1/GluN2A; GluN1/GluN2B
	AMPAR	GluA1/GluA2; GluA1/GluA4
	Kainate receptor	GluK2/GluK5
	Calcium-activated potassium channels	$K_{Ca}2.2$, $K_{Ca}2.3$

AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; $GABA_AR$, γ-aminobutyric acid type A receptor; NMDAR, *N*-methyl-D-aspartate receptor.

in vivo studies, based on known CNS pharmacology (Wakefield et al., 2002).

4.2. Liability testing

4.2.1. Seizure liability

Many pharmacological drugs have been associated with seizure liabilities, a potentially life-threating ADR representing one of the most frequent causes of injury or death in human clinical trials (Bass, Kinter, & Williams, 2004). Estimates suggest that 6% of new-onset seizures and up to 9% of status epilepticus cases are drug related (Chen, Albertson, & Olson, 2015). Drugs associated with this liability span a wide range of pharmacological classes and therapeutic areas (Table 4) (Easter et al., 2009; Fonck et al., 2015; Kumlien & Lundberg, 2010). Unsurprisingly, pharmaceutical drugs targeting CNS disorders show the highest propensity for drug-induced seizures; however, other therapeutic areas such as infectious, cardiovascular and the respiratory indications also present compounds with seizurogenic potential (Easter et al., 2009). This is a particularly important concern for the pharmaceutical industry underscoring the need for extensive safety testing amongst all therapeutic classes. This is no better illustrated than with Minaprine (Brantur, Cantor), an anti-depressive agent that was a reversible inhibitor of monoamine oxidase-A (MAO-A), which was withdrawn from the market in 1996 due to an increase in the clinical incidence of convulsions (Fung et al., 2001).

Convulsions observed in pre-clinical studies are often the first indication of the seizurogenic potential of a compound in development. However, not all seizures express convulsive behavior (Chang & Shinnar, 2011) requiring the use of EEG studies to pair clinical observations with electrical brain activity (Authier et al., 2009). Alternatively, abnormal seizure-like activity can be easily induced in an *in vitro* brain slice preparation by drugs known to induce seizures *in vivo*. The most common brain region for *in vitro* brain slice seizure-liability assessment is the hippocampus due, in part, to its (i) strong link to partial seizures (Schwartzkroin, 1994), (ii) retention of many *in situ* cytoarchitectural and microcircuitry properties (Lynch & Schubert, 1980), (iii) amenability to various *in vitro* electrophysiological techniques (Dingledine, Dodd, & Kelly, 1980) and (iv) its ability to be maintained, in culture, for several weeks (Gahwiler et al., 1997; Humpel, 2015). Additionally, unlike the reductionist approach applied to recombinant expression systems, hippocampal brain slices retain the impact of endogenous microcircuits (between neurons, glia and capillaries) and signaling pathways (*e.g.*, ion channels, GPCRs, kinases, *etc.*) that make it more effective at predicting unintended ADRs. In agreement with this, *in vitro* hippocampal slices show strong concordance with *in vivo* exposure values of various preclinical drug candidates and known seizurogenic agents (Easter et al., 2009; Easter, Sharp, Valentin, & Pollard, 2007; Fonck et al., 2015; Hablitz, 1984; Rostampour et al., 2002).

Seizurogenic activity is not restricted to the hippocampus with epileptiform activity observed in the neocortex (Kennedy & Schuele, 2012), amygdala (Hudson et al., 1993), entorhinal cortex (Vismer, Forcelli, Skopin, Gale, & Koubeissi, 2015), thalamocortical regions (Timofeev & Steriade, 2004) and possibly cerebellum (Harvey et al., 1996). Incidentally, seizure-like activity has also been recorded from neocortical (Voss & Sleigh, 2010) and thalamocortical (Gibbs, Zhang, Ahmed, & Coulter, 2002) brain slices. An interesting consideration would also be the use of intact inter-regional brain models (*e.g.*, cortico-hippocampal, hippocampal-entorhinal cortex), which maintain neuronal connectivity between brain regions (Leutgeb, Frey, & Behnisch, 2003; Luhmann & Kilb, 2012). This model is most effectively used within cultured conditions (Luhmann & Kilb, 2012) and could be useful in investigating the genesis and propagation of ictal discharges between cortical, subcortical and limbic systems.

4.2.2. Memory loss

Another use of hippocampal brain slice electrophysiological methods involves preclinical assessments of drug-induced cognitive deficits and memory loss as memory loss related to pharmaceutical use is a well-described phenomenon. Although not as widely used as it is within seizure liability testing, hippocampal slice electrophysiology has demonstrated great potential in studying learning and memory. Learning and memory are strongly associated with hippocampal function (Jarrard, 1993; Squire, 1992) and are molecularly mediated by both long-term potentiation (LTP) (Bliss & Collingridge, 1993; Bliss & Lomo, 1973; Nicoll & Malenka, 1999) and long-term depression (LTD) (Kemp & Bashir, 2001). LTP and LTD represent a long-lasting change in synaptic strength that may result in a potentiation or depression of synaptic function, respectively. These mechanisms have been extensively studied, both *in vitro* and *in vivo*, within multiple preclinical species (Fonck et al., 2015). For instance, electrophysiological experiments in rodent hippocampal slices have shown that compounds known to cause deficits in human memory *in vivo*, such as benzodiazepines (Tokuda, O'Dell, Izumi, & Zorumski, 2010) and glutamate receptor antagonists (Parsons, Stoffler, & Danysz, 2007), have also led to dramatic changes in the magnitude of LTP *in vitro*.

Table 4
Select drugs with known seizurogenic potential in humans.

Class	Drug	Relative seizure provocation frequency	Other comments
Anesthetic	Propofol	Common	Effects are well documented
	Enflurane	Rare	grand ma1 seizure patterns induced at concentrations of 3%–6%[a]
Antiarrhythmic	Lignocaine	Uncommon	Also an anesthetic; convulsion may occur with >75 μg/kg/min and blood concentrations above 9 mg/L[b]
	Mexiletine	Rare	
Antibiotic	Penicillins	Common in high doses	*e.g.*, penicillin G; penicillin induced seizures seen between 9 and 180 mg/kg[c]; 89–303 mg/L required to cause epileptiform activity in rat hippocampal slices[c].
	Cephalosporins	Common in high doses	*e.g.*, Cefazolin — shown to promote seizures at 100 mg[d]
Analgesic	Tramadol	Rare	
	Meperidine	Rare	High doses
Antihistamine	Diphenhydramine		Typical within withdrawal
	Hydroxyzine		Typical within withdrawal
Antipsychotic	Chlorpromazine	Rare	Observed with doses < 1000 mg/day[e]; cause seizurogenic activity in guinea pig hippocampal slices between 50 and 200 ng/ml[f]
	Clozapine	Common	>600 mg/day (4.4–14% risk of seizures)[a] some also observed < 300 mg/day[e]
Antidepressant	Bupropion	Rare	
	Minaprine	Common	Withdrawn from market
	Maprotiline	Uncommon	Seizures associated with doses > 225 mg/day[g]; Increased of 46% neuronal excitability in guinea pig hippocampal slices at 600 ng/ml[h]
Antimigraine	Sumatriptan	Rare	
Anxiolytic	Alprazolam	Rare	Typical within withdrawal
	Lorazepam	Rare	Typical within withdrawal
Anti-Parkinson	Pergolide	Rare	Withdrawn from market
Bladder	Oxybutynin	Common	Reduce bladder muscle spasms
Blood pressure	Carvedilol	Rare	
	Reserpine	Rare	May also be used to treat schizophrenia
Cancer	Temozolomide	Common	
	Vincristine	Rare	
	Busulfan	Uncommon	Anticonvulsant therapy should accompany high doses
Gastrointestinal	Ondansetron	Rare	May be used to anorexia
	Dronabinol	Rare	High Doses
Immune	Cyclosporine	Common	Elevated plasma levels (320 to 1590 ng/ml) associated with seizures[i]; seizures induced in hippocampal slice between 1000 and 10,000 ng/ml[j]
Neuroleptic	Chlorprothiexene	Common	
Dementia	Donepezil	Rare	
	Rivastigmin	Rare	
Respiratory	Theophylline	Common	Bronchodilator; shown to cause seizures as low as 10 to 20 mg/L[g]; Convulsive dose (CD50) in a rat hippocampal slice is 3 μM[k]
Skin care	Isotretinoin	Rare	

[a] Modica, Tempelhoff, and White (1990).
[b] Zaccara, Muscas, and Messori (1990).
[c] Grondahl and Langmoen (1993).
[d] Wallace (1997).
[e] Pisani, Oteri, Costa, Di Raimondo, and Di Perri (2002).
[f] Oliver, Luchins, and Wyatt (1982).
[g] Franson et al. (1995).
[h] Luchins, Oliver, and Wyatt (1984).
[i] Wijdicks, Plevak, Wiesner, and Steers (1996).
[j] Wong and Yamada (2000).
[k] Ault et al. (1987).

The versatility of hippocampal slices also enables long term (*e.g.*, organotypic culture) study of LTP/LTD (Lein, Barnhart, & Pessah, 2011) amenable to high throughput assessment (Kroker, Rosenbrock, & Rast, 2011). This is particularly important in the context of chronic exposure of pharmaceutical, industrial and/or agricultural chemicals on cognitive function (Altmann et al., 2002; Fonck et al., 2015; Sanders, Liu, Buchner, & Tchounwou, 2009). For example, anticholinergic (Cai, Campbell, Khan, Callahan, & Boustani, 2013), statins (Wagstaff, Mitton, Arvik, & Doraiswamy, 2003) and antianxiety medication (*e.g.*, benzodiazepines) (Curran, 1986) have all been shown to alter cognitive function in less than 60 days. In fact, a wide range of drug classes are known to illicit adverse cognitive effects in some fashion (*e.g.*, antiseizure, antidepressant, narcotic painkillers, dopamine agonists, beta-blockers, nonbenzodiazepine sedative-hypnotics and antihistamines) (Wood, 1984). Given this, the usefulness of an *in vitro* memory/cognitive dysfunction assay within the safety pharmacology arena cannot be overlooked.

4.2.3. Other possible liability tests

As our understanding of the safety testing evolves to incorporate an ever more sophisticated list of drug development liabilities, new assays and/or improvements of older assays may begin to take hold. For instance, drug-induced sleep disorders are a frequent concern since numerous drug classes (*e.g.*, antidepressants, antihistamines, antipsychotics and corticosteroids) have shown sleep disturbances. Accordingly, it has been suggested that *in vitro* brain slice electrophysiology may represent an important area for which *in vitro* screens may develop (Fonck et al., 2015). Nerve conduction velocity testing is often included in non-clinical studies (Arezzo, Litwak, & Zotova, 2011; Zotova & Arezzo, 2013) and serves as a quantitative assessment of possible impairment of impulse transmission in the central/peripheral sensory and motor pathways. However, limited *in vitro* models have been qualified for early screening of such effects. Sensory neuronal networks are also commonly used to assess potential pharmacological effects. For example, freshly isolated dorsal root ganglia represent an application of *in vitro*

electrophysiology to drug development (Serrano et al., 2012; Serrano et al., 2010); but there remains limited understanding of the translational value of such an assay.

4.3. Implementation of high-throughput techniques

Historically marred by low throughput and the requirement for highly trained personal, manual *in vitro* electrophysiology methods have seen slow and heterogeneous penetrance into the non-clinical drug development landscape. Over the last decade, several automated and semi-automated high-throughput platforms have addressed these concerns by simplifying the patching procedure and increasing the data acquisition capabilities of *in vitro* electrophysiology methods (Dunlop et al., 2008; Fertig & Farre, 2010; Terstappen et al., 2010; Wood et al., 2004). However, despite these advances, automated patch-clamp platforms have seen slow application and endorsement into the battery of safety pharmacology methods available to determine drug safety. In a recent market survey (HTStec Limited, 2014), 76% of current automated platform users responded that high-throughput systems are principally employed for assay development with only a quarter (25%) or half (55%) of the responders indicating use in GLP compliant or non-compliant safety assessments, respectively (Comley, 2014). This is partly due to the fact that current high-throughput automated patching systems are more applicable to primary screening and hits-to-leads (lead optimization) operations (Dunlop et al., 2008; Fertig & Farre, 2010) than safety testing. However, other logistical and economic factors may also be at play such as the cost of automated platforms and their associated consumables (Comley, 2014; Dunlop et al., 2008; Farre & Fertig, 2012). In fact, it was not until the development of automated systems capable of performing 384 and 768 parallel recordings that the cost per data point was actually below $1 (USD) (Comley, 2014).

Another issue is that most of the existing high-throughput platforms are designed to record from either mammalian cell lines and/or *Xenopus* oocytes (Dunlop et al., 2008; Wood et al., 2004). Thus, extrapolation of data obtained from these tissue preparations to the *in vivo* condition, where network connectivity is an undeniable influence, is quite limited – hampering predictive power. Recent advancements with automated patch-clamp techniques have begun to address this concern by expanding the high throughput capacity to preparations more applicable to the *in vivo* condition. For instance, MEAs and semi-automated *in vitro* hippocampal brain slice assays have been adapted for high-throughput analysis (Dunlop et al., 2008). These systems are capable of using mixed organotypic slice cultures or multiple acute brain slices (up to eight brain slices) providing *in vitro* safety models with a neuronal network perspective (Dunlop et al., 2008; Easter et al., 2007; Kroker et al., 2011; Obien et al., 2014). MEAs, for example, provide the possibility of long-term culture enabling chronic seizurogenic compound exposure; a useful technique for understanding how compounds that are prone to development of drug-induced seizures in humans change epileptiform activity over time. Furthermore, the automated patch clamp technique has been recently developed for simultaneous *in vivo* intracellular recordings from multiple neurons (Kodandaramaiah, Franzesi, Chow, Boyden, & Forest, 2012). This technique enables a technician, or robot, to rapidly obtain giga-seals on neurons of the same quality achieved by that of a skilled electrophysiologist (Kodandaramaiah et al., 2012). Although the full extent of the applicability of this technique is not yet been realized within safety pharmacology, its potential *in vivo* applicability with *in vitro*-type data quality is quite promising.

5. Conclusions

High drug attrition rates within pharmaceutical development due to adverse CNS effects (Arrowsmith & Miller, 2013; Fung et al., 2001; Hamdam et al., 2013; Palmer & Alavijeh, 2012; Pangalos et al., 2007) has led to the growing support for the requirement to perform more comprehensive CNS safety testing prior to clinical studies (Lindgren et al., 2008; Valentin & Hammond, 2008). Accordingly, CNS safety testing has begun to be frontloaded with *in vitro* assays (Bowes et al., 2012), calling for the re-evaluation of the merits for implementing *in vitro* electrophysiology testing early in this process. This rapid implementation into early-phase safety pharmacology warrants a call to ensure that these assays are met with a strict monitoring program from which it can be determined whether their implementation is justified by their predictive ability. In light of this, many recent studies have begun to highlight the success of *in vitro* electrophysiological CNS safety assays at reproducing *in vitro–in vivo* concordance with respect to certain preclinical drug candidates (Easter et al., 2007, 2009; Fonck et al., 2015; Kroker et al., 2011); demonstrating the translational potential of these assays in safety pharmacology. However, more studies are required to fully address and understand the nuances between *in vitro–in vivo* systems before full implementation within safety pharmacology can occur. It should be noted that, like most areas of medical science, a single approach will not be suitable to all programs. As such, *in vitro* electrophysiological methods should not be considered a replacement for *in vivo* assessments particularly because it is impossible to address the presence of certain adverse CNS effects *in vitro* (*e.g.*, delirium, confusion, hallucination, depression, schizophrenic and/or paranoid reactions, etc.). Instead, when paired with more traditional safety pharmacology methods, *in vitro* electrophysiology provides screening assays from which a greater understanding of preclinical drug liabilities can emerge. *In vitro* electrophysiological methods represent an area with opportunities for refinement as the field solidifies its foundation to support drug safety testing.

Conflict of interest

None of the authors have any conflict of interest, other than their employment in either a contract research organizations or pharmaceutical company. No information is presented in this paper that advocates for, or promotes, commercial products from any of their organizations.

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