

# How do we use in vitro models to understand epileptiform and ictal activity? A report of the TASK I-WG4 group of the ILAE/AES Joint Translational Task Force

\*<sup>1</sup>Chris G. Dulla, †Damir Janigro, ‡Premysl Jiruska, §Joseph V. Raimondo , ¶Akio Ikeda, \*\*Chou-Ching K. Lin, ††Howard P. Goodkin, ‡‡Aristea S. Galanopoulou, §§Christophe Bernard , and ¶¶Marco de Curtis

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**Chris G. Dulla Ph.D.** is an assistant professor of neuroscience at Tufts University School of Medicine

## SUMMARY

**In vitro brain tissue preparations allow the convenient and affordable study of brain networks and have allowed us to garner molecular, cellular, and electrophysiologic insights into brain function with a detail not achievable in vivo. Preparations from both rodent and human postsurgical tissue have been utilized to generate in vitro electrical activity similar to electrographic activity seen in patients with epilepsy. A great deal of knowledge about how brain networks generate various forms of epileptiform activity has been gained, but due to the multiple in vitro models and manipulations used, there is a need for a standardization across studies. Here, we describe epileptiform patterns generated using in vitro brain preparations, focusing on issues and best practices pertaining to recording, reporting, and interpretation of the electrophysiologic patterns observed. We also discuss criteria for defining in vitro seizure-like patterns (i.e., ictal) and interictal discharges. Unifying terminologies and definitions are proposed. We suggest a set of best practices for reporting in vitro studies to favor both efficient cross-lab comparisons and translation to in vivo models and human studies.**

**KEY WORDS:** In vitro models, Ictal activity, Review.

The introduction of in vitro brain slices<sup>1</sup> opened a new era for the study of networks in the central nervous system. Because of the advantages offered by in vitro preparations in terms of stability and accessibility, work on acute brain slices quickly dominated neuroscience research. In vitro

brain slices were recognized immediately as an ideal simplified model to study epileptiform activity in experimental animal brain tissue,<sup>2</sup> and the approach has been rapidly extended to the study of human postsurgical tissue obtained in epilepsy surgery centers.<sup>3,4</sup> Questions that could not be

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\*Department of Neuroscience, Tufts University School of Medicine, Boston, Massachusetts; †Flocel Inc. and Case Western Reserve University, Cleveland, Ohio, U.S.A.; ‡Department of Developmental Epileptology, Institute of Physiology of the Czech Academy of Sciences, Prague, Czechia; §Division of Cell Biology and Neuroscience Institute, Department of Human Biology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa; ¶Department of Epilepsy, Movement Disorders and Physiology, Kyoto University Graduate School of Medicine, Kyoto, Japan; \*\*Department of Neurology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan; ††The Departments of Neurology and Pediatrics, University of Virginia, Charlottesville, Virginia; ‡‡Laboratory of Developmental Epilepsy, Saul R. Korey Department of Neurology, Isabelle Rapin Division of Child Neurology, Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, and Einstein/Montefiore Epilepsy Center, Montefiore Medical Center, Bronx, New York, U.S.A.; §§Aix Marseille Univ, INSERM, INS, Inst Neurosci Syst, Marseille, France; and ¶¶Epilepsy Unit, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milano, Italy

Address correspondence to Chris G. Dulla, Ph.D., Tufts University School of Medicine, 136 Harrison Ave, SC203, Boston, MA, 02111, U.S.A. Email: Chris.Dulla@tufts.edu

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## KEY POINTS

- In vitro approaches allow the investigation of electrical activity similar to that seen in electroencephalography (EEG) recordings from patients with epilepsy
- Because multiple in vitro models are used, the field could benefit from a unified understanding of how these models can be best utilized
- We describe the types of in vitro electrical activity that can be generated, focusing on best practices for recording, reporting, and interpretation

investigated in vivo were approached in the simplified in vitro slice preparations and complemented by later in vivo studies.

In vitro slice preparations, however, have limitations for the study of epilepsy. Ictal seizure-like events (SLEs) are not spontaneously observed in vitro (with the exception of hippocampal organotypic slices<sup>5,6</sup>). The contribution of the vasculature and blood–brain barrier to epileptiform activity is lost in slices. Finally, the slicing procedure damages the tissue, isolating it from extrinsic connections and reducing the local network available for study. In the last 2 decades, procedures to isolate larger brain regions in vitro have improved. Extended network slices (thalamo-cortical,<sup>7</sup> limbic,<sup>8</sup> and cortico-cortical slices<sup>9</sup>) isolated brain preparations (en bloc hippocampus,<sup>10</sup> and isolated whole brains<sup>11</sup>) have partially compensated for the above-mentioned drawbacks and allow the study of ictogenesis in larger networks, more similar to in vivo conditions. These types of preparations are often referred to as *ex vivo*, rather than in vitro, as they more closely represent in vivo conditions.

The induction of acute epileptiform discharges to study cellular and network activities can be promoted in acute brain slices either by adding a variety of ictogenic compounds to the bathing medium or by modifying the composition of the perfusion solution. Epileptiform activity can also be induced by intense and prolonged electrical stimulation. Moreover, the abnormal chronic epileptic networks that develop during epileptogenesis can be evaluated in brain slices obtained from experimental animals sacrificed at different times following the manipulation that starts the epileptogenic process. In addition, brain slices isolated from animal models of genetic epilepsies provide insights into the pathophysiology of the represented disease. In vitro preparations also allow pharmacologic experiments by simply adding compounds to the perfusion fluid, a privileged condition that is not accessible in vivo. Finally, organotypic slice cultures<sup>12</sup> have been used to study epileptogenesis and for long-term monitoring of epileptiform activities.<sup>6</sup>

In a previous report,<sup>13</sup> we discussed best technical and methodologic practices pertaining to in vitro electrophysiologic experiments. In this follow-up report, we do the same for how to record, report, and interpret spontaneous and

evoked epileptiform patterns recorded in in vitro experiments. We focus largely on the acute in vitro brain slice preparation and provide an overview of other in vitro systems including organotypic slice culture and whole brain preparations. Although cell culture preparations can generate epileptiform activity in individual neurons,<sup>14,15</sup> we do not address those systems here. In addition, we discuss the conditions under which epileptiform activities are observed in these in vitro systems. Diverse methods for inducing ictal-like activity in vitro have generated a large repertoire of epileptiform patterns highly reminiscent of events reported in vivo. We discuss the current criteria for defining in vitro activity that are posited to represent seizure-like patterns (i.e., ictal and interictal discharges). Based on these descriptions, we propose unifying terminologies and definitions, discuss the interpretation of in vitro activity patterns and their use in explaining and understanding human ictogenesis, and consider the associated opportunities, controversies, and limitations. Finally, we discuss current challenges facing researchers using in vitro models and propose steps forward to utilize in vitro models in the most advantageous ways possible.

## CLINICAL ICTAL AND INTERICTAL EEG PATTERNS

A variety of abnormal electrographic activities are described in the human epileptic brain including (but not limited to) interictal spikes, spike and slow wave discharges (isolated or in bursts), high-frequency oscillations (>80 Hz), low-voltage fast (LVF) activity, rhythmic discharges, bursting, post-ictal suppression, and hypersarrhythmia. Associations of such patterns with specific epileptic syndromes have been based on electroclinical associations using video–electroencephalography (EEG) studies, prevalence studies in cohorts of patients with epilepsy, or control subjects. The interpretation of these patterns strongly depends on the clinical context, as evidenced by EEG patterns previously presumed to be epileptic, now being considered benign variants.

In general, epileptologists divide abnormal EEG activity into ictal (occurring during a seizure) and interictal (occurring during the time between seizures). Clinical interpretation is based predominantly on pattern recognition and electroclinical correlations. Use of duration to distinguish ictal from interictal activity is also based on the type of epileptic seizure in question. For example, in video scalp EEG monitoring of focal-onset epilepsies, ictal events tend to be long in duration (e.g., focal seizures without impaired awareness: >3 s duration, median duration = 28 s, range of duration = 23 s; focal seizures with impaired awareness: >8 s, median duration = 78 s, range of duration = 46 s; tonic seizures: >8 s, median duration = 18.5 s, range of duration = 21.25; secondarily generalized seizures: >37 s, median duration = 130 s, range of duration = 37 s<sup>16</sup>),

although interictal events are more brief (<100 msec). In absence epilepsy, bursts >3 s are considered as potentially ictal. In myoclonic seizures or infantile spasms, ictal events can be even shorter in duration (a few milliseconds or seconds).<sup>17</sup> Similar strategies will be helpful in defining ictal versus interictal activity in vitro.

## IN VITRO ACTIVITY WITH RELEVANCE TO HUMAN EPILEPSY

As with human epilepsy, in vitro interictal-like activity can vary in frequency composition, duration, and interevent interval. Relevant variables include the brain region in which activity is generated, the model of epilepsy, stimuli evoking the activity, and recording conditions. In vitro preparations, by definition, have no behavioral correlate to differentiate ictal and interictal activity. Therefore, the standard is to define in vitro events based both on duration and electrographic features. To categorize in vitro activities, we focus on electrographic activities relevant to abnormal EEG findings seen in the human epileptic brain, or relevant in vivo animal models.

### In vitro activity relevant to spontaneous interictal-like spikes

A single EEG spike is defined as a transient clearly distinguishable from the background EEG lasting 20–70 msec.<sup>18,19</sup> EEG spikes can form either “polyspikes” (complex of spikes with no interim return to baseline) or spike trains (repetitive spikes with interim return to baseline EEG background). These events are distinct from sharp waves, which last longer (70–200 msec), but have similar clinical significance.<sup>18,19</sup> Benign variants of patterns resembling spikes or sharp waves have been reported (i.e., wickets, ctenoids), but correlation with clinical context is needed to consider them epileptic.

Events thought to model human interictal EEG “sharp waves,” “spikes,” or “spike trains” can be recorded in vitro using extracellular field potential recording in hippocampal and cortical brain slices and are referred to as “epileptiform bursts.” These events are characterized by a temporally close series of population spikes riding on depolarization envelopes in the field potential and are thought to be driven by the synchronous activity of local neuronal populations. Of note, population spikes are seen routinely in the hippocampus, whereas they are relatively rare in the cortex. It is notable that these network-driven “epileptiform bursts” need to be distinguished from bursts of action potentials recorded from a single neuron. Epileptiform bursts are population-driven events, whereas “burst-firing” neurons represent single neurons capable of firing brief flurries of action potentials due to their intrinsic membrane properties. In vitro “epileptiform bursts” vary in duration from approximately 100 msec to 1 s and can occur sporadically or can precede in vitro SLEs

(described next). Epileptiform bursts sustained by the activity of  $\gamma$ -aminobutyric acid (GABA)ergic interneurons have been described in acute hippocampal brain slices and in recordings from whole brain *ex vivo* preparations.<sup>20</sup> It has been proposed that such GABAergic spikes precipitate SLEs characterized by a low-voltage fast (LVF) activity onset.<sup>21,22</sup> Of interest studies performed in human slices show the occurrence of spontaneous interictal-like events with features similar to those recorded in the same patient before neurosurgery,<sup>23</sup> suggesting in vitro maintenance of the underlying circuitry that drives interictal activity in vivo. These types of studies are rare, however, so more study of how in vitro epileptiform spikes relate to in vivo EEG interictal spikes is necessary.

### Interictal-like activity evoked by electrical stimulation

Stimulus-evoked epileptiform activity can also be generated in vitro. Single electrical stimulation can generate high-amplitude, prolonged network responses in cortical slices cut from epileptic animals submitted in vivo to physical insults (such as controlled cortical impact,<sup>24</sup> fluid percussion injury,<sup>25</sup> cortical undercut,<sup>26</sup> and neonatal freeze lesions<sup>27</sup>) or chemical insults (e.g., tetanus toxin<sup>28</sup>). Similar stimulus-evoked in vitro epileptiform activity can also be generated in epilepsy-prone genetically modified animals, such as *Gria4* (glutamate receptor AMPA type subunit 4) mutants<sup>29</sup> and  $\text{Na}_v1.1$  heterozygous mice.<sup>30</sup> Although these models elicit behavioral seizures in vivo, in vitro electrophysiologic phenotypes typically involve interictal-like activity only.

Studying stimulus-evoked epileptiform activity is advantageous for a number of reasons. First, this activity can be generated under normal ionic conditions. Second, activity is reliably and repeatedly triggered, rather than occurring spontaneously without warning. Third, it can act as a proxy for abnormal activity in networks that go on to generate ictal activity in vivo (i.e., after traumatic brain injury). Fourth, it can be useful to probe network dynamics in various pathologic settings. Stimulus-evoked epileptiform activity is helpful to understand how epileptic circuits are organized in pathologic states but offers little insight into how interictal and ictal activity is spontaneously generated. In addition, the mechanisms that drive spontaneous interictal and ictal activity may be unique from those that contribute to stimulus-evoked epileptiform activity.

### In vitro activity relevant to seizure-like events

Ictal-like activity in vitro is often defined as an SLE. SLEs do not occur spontaneously in acute brain slices but can be evoked by pharmacologic or electrical stimulation. SLEs can vary in duration, frequency components, and progression patterns. A prototypical in vitro SLE, resembling activity seen in human temporal lobe epilepsy (TLE), is preceded by interictal activity, features a shift in the

extracellular field potential overlaid with some type of rhythmic activity, and is followed by a postictal suppression of activity. In vitro SLEs have been described as having a duration  $>5$  s, in in vitro hippocampal/entorhinal slices.<sup>31</sup> Shorter runs of epileptiform discharges have been called “recurrent epileptiform transients” (RETs). In order to use in vitro models to try to understand the progression of cellular and network events in a seizure, we must be able to study prolonged network activity. Thus, a minimum duration for SLEs has become widely accepted. It is important to note, however, that this is a minimum duration and that both in vitro SLEs and human seizures are often much longer and can vary greatly from seizure-to-seizure and patient-to-patient. The stimulus intensity and type, the region from which a recording is done, the animal characteristics, and the type of the specimen used for recording can all affect SLE characteristics. Such conditions should be standardized within a given study and be reported with sufficient detail to interpret the data. For example, it is not uncommon that when low intensity stimulus is used to induce SLEs, different slices from the same source may or may not manifest SLEs. When specific epileptic conditions are considered, the duration of the SLE discharge can be shorter. For instance, thalamocortical slices can be utilized to create oscillatory events that mimic generalized seizure patterns seen in absence epilepsy. In this case, electrical activity lasting 3–4 s may be helpful for understanding the mechanisms of spike-and-wave discharges, which this activity mimics.<sup>32</sup> Finally, it is important to emphasize that the goal of any in vitro experiment is to optimally simulate the properties of seizures seen in the human syndrome being modeled. In the ideal situation, the human EEG would be replicated in the in vivo EEG recording and in the in vitro slice preparation from the relevant animal model. Although this is rarely the case, in vitro models have enabled significant advances in our understanding of ictogenesis and network activity in epilepsy.

Basic scientists have used numerous preparations to induce and study SLEs that resemble focal or generalized seizure discharges, resulting in a number of different types of in vitro ictal-like activity.<sup>33</sup> Although exciting new approaches based on quantitative dynamics<sup>34</sup> promise future systems based on rigorous scientific categorizations (see Harnessing in vitro models), most in vitro studies utilize observational approaches similar to those used to interpret human EEG. Here we outline the most commonly described SLEs.

#### Hypersynchronous seizure-like event

Hypersynchronous SLEs are well studied, approximate ictal activity seen in TLE,<sup>35</sup> and are most commonly evoked by 4-aminopyridine (4-AP) or low  $Mg^{2+}$  solutions. Hypersynchronous SLEs can be recorded in hippocampal areas CA1 and CA3, in the entorhinal cortex, and in the olfactory bulb.<sup>36–38</sup> Hypersynchronous SLEs are preceded by burst

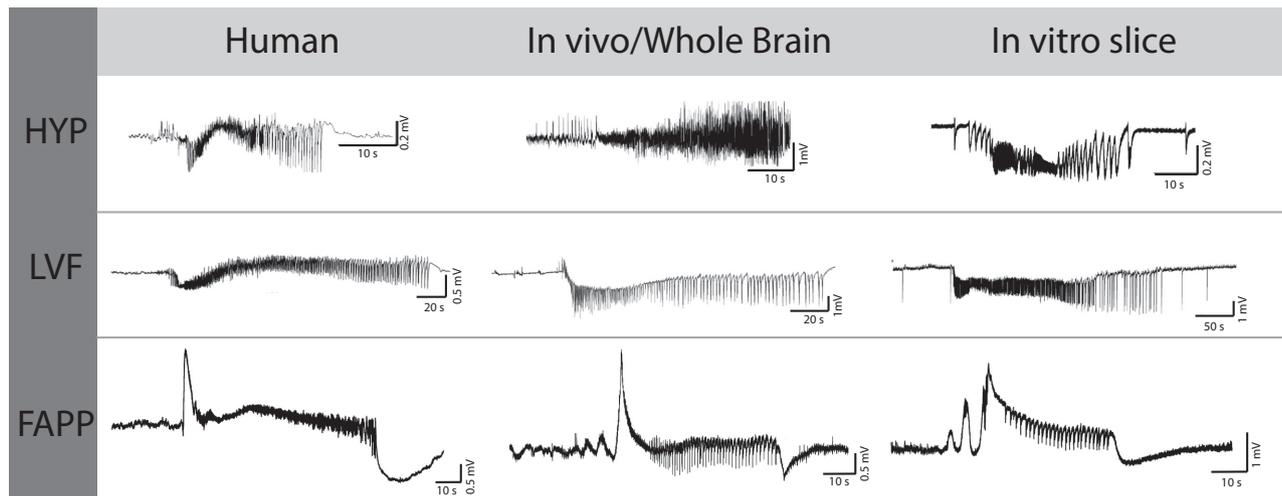
discharges (Fig. 1) that increase in amplitude over time. The transition to SLE is marked by a sudden negative shift in the field potential (appreciated when low frequencies are not filtered), overlaid by high-amplitude discharges at  $\approx 1$  Hz, that progresses into tonic activity (nearly constant small amplitude transients,  $\approx 20$ – $50$  msec in duration, occurring at approximately 8–10 Hz) followed by rhythmic bursting (large amplitude transients, 50–200 msec in duration, occurring at approximately 0.5–1 Hz). Postictal suppression of background activity occurs following hypersynchronous SLEs. This prototypical pattern can show variance: pre-SLE activity may or may not occur, repetitive bursting may be prolonged or absent, and the duration of tonic/bursting activities may vary. Each SLE phase represents different cellular and network activities, including synchronous neuronal activity, extracellular potassium accumulation, and synaptic depression. The onset of hypersynchronous SLEs is likely dominated by prevalent glutamatergic activation.<sup>35,39</sup>

#### Low-voltage fast seizure-like event

Another common SLE is the is LVF activity at onset, which mimics activity observed in human TLE and in extratemporal neocortical epilepsies. LVFs can be seen most easily, but not exclusively, when potassium channel antagonists like 4-AP are utilized. LVF activity is often initiated by a single leading spike that is followed by a period of low-amplitude gamma activity (35–100 Hz) riding on a negative field potential shift (Fig. 1). After the LVF onset, these SLEs progress into sustained tonic activity, followed by rhythmic bursting that gradually increases in amplitude and slows down in frequency.<sup>40</sup> LVF SLEs are followed by postictal suppression of activity. LVF onset likely represents a circuit mechanism different from the hypersynchronous SLEs. Preictal sentinel spikes and LVF activity have been proposed to be generated by hyperactivity of GABAergic networks.<sup>20,41</sup>

#### Fast-activity on plateau potential seizure-like event

A recent study using the in toto guinea pig brain preparation showed that piriform cortex generates SLEs characterized by fast-activity on a plateau potential (FAPP). FAPP SLEs are evoked by perfusion with the potassium channel antagonist 4-AP and are similar to ictal activity seen in patients with frontal neocortical epilepsies.<sup>42</sup> The piriform cortex generates fast activity nested within periodic slow oscillations at 0.1–0.5 Hz that precede the SLE. At SLE onset, there is a large amplitude extracellular upward plateau potential, with superimposed fast activity of small amplitude (30–60 Hz), followed by large-amplitude population spiking (Fig. 1). As with most SLEs, these events were followed by a postictal suppression. These SLEs are thought to occur due to accumulation of potassium in superficial cortical layers.



**Figure 1.**

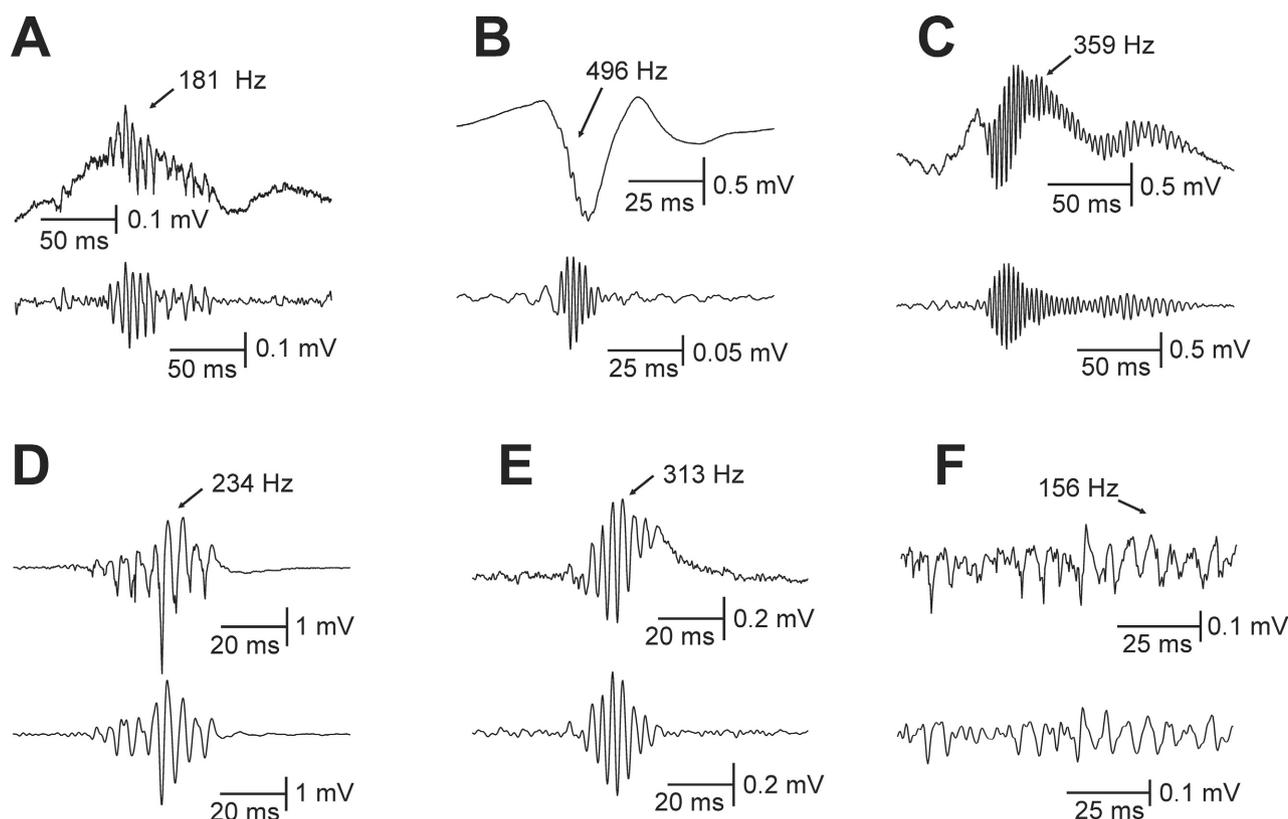
Seizure subtypes and their in vitro correlates in animal models. Representative ictal EEG subtypes recorded in human focal epilepsies with intracerebral electrodes (left) and their correlates recorded either in the limbic cortices of the whole brain in vitro or in vivo (middle) and in vitro slice preparations (right). **HYP**, hypersynchronous onset; **LVF**, low-voltage fast onset; **FAPP**, fast activity on a plateau potential. **Hypersynchronous.** Human seizure, left. Recorded using hippocampal depth electrodes in a patient with a malformation of the hippocampus using standard human EEG protocols. In these recordings, slow potentials are eliminated by high-pass filtering. In vivo rat brain, middle. In vivo recordings from hippocampal area CA3 in a pilocarpine-treated epileptic rat. In these recordings, slow potentials are eliminated by high-pass filtering. In vitro rat slice, right. In vitro extracellular field potential recordings from a rat perirhinal cortex in a bathing solution containing 4-AP. DC recording reveals significant negative-going shifts in the recording.<sup>35</sup> **Low-voltage fast onset.** Human seizure recorded in the temporal lobe with intracerebral electrodes, left. Seizure recorded in the entorhinal cortex of the in vitro whole guinea pig brain following arterial perfusion of 4-AP (50  $\mu$ M, middle panel). On the right, a seizure generated by 4-AP in the hippocampus of an in vitro rat slice is illustrated. **Fast activity on a plateau potential.** Human seizure recorded in the frontal cortex using intracranial depth electrodes according to a stereotactic procedure for exploratory epilepsy surgery is illustrated on the left. Seizure recorded in the piriform cortex of the in vitro whole guinea pig brain (middle) and from a tangential piriform cortex slice (right) during perfusion with 50  $\mu$ M 4-AP. Activity was recorded with glass capillary electrodes, at 3 KHz sampling in quasi-DC mode. (Modified from Avoli et al., 2016<sup>35</sup> and from Uva et al., 2017<sup>42</sup>.)

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### In vitro high-frequency oscillations

The brain has an inherent capacity to generate high-frequency oscillations (HFOs).<sup>43</sup> HFOs can be subclassified according to frequency profile, spatial distribution, and underlying cellular mechanism (Fig. 2), with different HFOs considered both physiologic and pathologic. Physiologic HFOs play an important role in cognitive functions, and the best-described representatives are gamma oscillations<sup>44</sup> or sharp wave ripples (Fig. 2A).<sup>45</sup> Pathologic oscillations have distinct cellular mechanisms, they do not occur in healthy brain tissue, and they are closely associated with epilepsy.<sup>46</sup> Pathologic HFOs (Fig. 2B–F) are currently subclassified into 3 main categories according to their frequency; gamma oscillations (30–100 Hz), epileptic ripples with frequencies between 80 and 250 Hz, and fast ripples (250–600 Hz). Pathologic HFOs are considered an electrographic marker of epileptogenic tissue.<sup>47,48</sup> In vitro techniques have substantially contributed to our understanding of the cellular and network HFO mechanisms.<sup>39,49</sup> HFOs can be recorded in slices from the normal brain under epileptic conditions, that is, induced by perfusion with high-potassium (Fig. 2D), low-magnesium, low-calcium

artificial cerebrospinal fluid (ACSF), or 4-AP.<sup>49,50</sup> It is now accepted that these oscillations arise from synchronized action potentials generated by a population of principal cells. Fast ripples (FRs), with their high specificity to epileptic tissue, have garnered great interest from epileptologists (Fig. 2B). FRs have been recorded in hippocampal-entorhinal slices from animals with chronic epilepsy following kainic acid- or pilocarpine-induced status epilepticus, or after intrahippocampal injection of tetanus toxin (Fig. 2E).<sup>51</sup> It is believed that FRs are generated by the out-of-phase action potential firing of small neuronal populations, each generating spikes at a much lower frequency.<sup>52</sup> This out-of-phase firing then results in a multiplication of the frequency, which in extracellular recording manifests as an oscillation in the FR band. Specific low-amplitude HFOs can be recorded using extracellular recording in vitro and in vivo in the CA1 region between seizures, or at the onset of SLEs in hippocampal in vitro slices using low-calcium, low-magnesium (Fig. 2F),<sup>53</sup> or high-potassium<sup>54</sup> models. This type of HFO is not dependent on abnormal network structure and requires only an actively firing population of neurons.<sup>55</sup> Particularly in humans, the ability to



**Figure 2.**

Examples of high-frequency oscillations. **A**, Physiologic sharp-wave-ripples recorded in the CA1 region of the hippocampus in the normal animal. Top trace represents raw data and bottom trace band-pass (100–1,000 Hz) filtered signal. **B**, Pathologic fast ripples recorded in a patient who was implanted with depth electrode located in the hippocampus. **C**, Fast ripples generated in the dorsal hippocampus of the animal with chronic epilepsy induced by intrahippocampal injection of tetanus toxin. **D**, Burst of ripple activity generated in CA3 in normal hippocampal slice after perfusion with high-potassium ACSF. **E**, Fast ripples recorded in vitro in the CA3 region of the hippocampal slice from the tetanus toxin model of epilepsy. Fast ripples were induced by perfusion of the slice with high-potassium ACSF. **F**, Low-amplitude ripple activity recorded between seizures in CA1 region perfused with low-calcium ACSF.

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discriminate between normal HFOs and pathologic ones can be challenging because they display spectral overlap. Multiple studies tried to discriminate physiologic and pathologic HFOs according to their spectral and spatial features,<sup>56</sup> relationship with sleep phases,<sup>57</sup> background activity, phase relationship with slow waves, or their response to antiepileptic drugs.<sup>58</sup>

### Slow oscillations

Slow or ultraslow oscillations (<0.1 Hz) usually require DC recordings to be detected. The underlying mechanisms include a change in ionic composition of the extracellular medium, for example the accumulation of K<sup>+</sup> likely due to compromised astrocyte buffering. A DC shift is seen consistently before or at seizure onset, both *in vivo* and *in vitro*.<sup>34,59,60</sup> Note that most recordings (in particular *in vivo*) are performed in AC mode, which explains why the literature is sparse on this topic.

### Ictal activity associated with generalized epilepsy

*In vitro* activity discussed earlier is based on models of focal seizures. Generalized seizures involve far-reaching brain circuitry, and slice models have inherent limitations. Nevertheless, *in vitro* thalamic or thalamocortical slices can generate spontaneous and stimulus-evoked slow (2–4 Hz) oscillations between the nucleus reticularis (nRT) and more central thalamic nuclei.<sup>61</sup> These oscillations are recorded mainly in horizontal thalamic *in vitro* slices,<sup>62</sup> and involve circuits involved in sleep spindles. Genetic mutations,<sup>29,63</sup> pharmacologic blockade of GABA receptors,<sup>64</sup> and modulation of thalamic neuron activity can predispose intrathalamic circuitry<sup>65</sup> to generate 6–10 Hz oscillations, similar to what is seen in spike and wave generalized epilepsy in rodents. These systems have proven extremely useful in understanding how thalamic neuron T-type calcium currents<sup>66</sup> participate in spike and wave generation.<sup>65,67</sup>

## GENERAL CATEGORIES OF MODELS AND THEIR BENEFITS/LIMITATIONS

In vitro SLEs can be generated in preparations from diverse species, including humans, and can occur in tissue from naive (presumably without epilepsy) and epileptic subjects. Here, we describe the most commonly used methods to induce SLEs (and see Table 1).

### Low Mg<sup>2+</sup>/high K<sup>+</sup> model

Epileptiform activity can be observed in a wide variety of brain areas following increase in extracellular K<sup>+</sup> (usually 5–6 mM) and removal of Mg<sup>2+</sup> from the artificial slice perfusion fluid.<sup>38,68–70</sup> This epileptiform activity is thought to occur through a reduction in surface charge screening, which shifts the conductance-voltage relationship of voltage-gated channels that are more likely to be activated,<sup>71</sup> coupled to the removal of the Mg<sup>2+</sup>-dependent block of N-methyl-D-aspartate receptors.<sup>38</sup> The reduced Mg<sup>2+</sup> model generates both interictal and ictal-like epileptiform patterns depending on both the brain region involved and the duration of Mg<sup>2+</sup> removal.<sup>38,68</sup> Early activity represents 50–300 msec population interictal discharges, and continued Mg<sup>2+</sup> withdrawal results in SLEs of several minutes. Intracellular recordings reveal large (>30 mV), extended depolarizations with action potential firing at onset, typically followed by rhythmic bursting or an afterdischarge phase resulting in the electrographic equivalent of a hypersynchronous onset pattern.<sup>35,68,72</sup> After 30–90 min of the zero-Mg<sup>2+</sup> solution, ictal events no longer occur and are

replaced by continuous rhythmic or afterdischarge activity,<sup>68</sup> proposed as an in vitro model of refractory status epilepticus.<sup>73</sup> Although ictal events show some sensitivity to treatment, this late-stage rhythmic bursting activity is resistant to standard antiseizure medications.<sup>74</sup>

### 4-Aminopyridine model

4-AP blocks voltage-gated potassium channels Kv1.1, Kv1.2, and Kv1.4, which are particularly important for repolarization of action potentials. In rat hippocampus, 4-AP causes interictal-like activity of 2 varieties: a short population discharge (~80 msec) and a longer lasting discharge (~500 msec). The first is dependent on glutamatergic transmission, whereas the latter is mediated by GABA and reflects synchronous firing of interneurons.<sup>21,75</sup> In parahippocampal and temporal cortex areas, 4-AP also elicits much longer ictal-like events (~100 s) characterized by either hypersynchronous or LVF activity onset patterns followed by distinct tonic and rhythmic phases.<sup>20,21,35</sup> These ictal events are sensitive to antiseizure medication<sup>76,77</sup> but can be rendered pharmacoresistant with the addition of bicuculline.<sup>76</sup>

### Low Ca<sup>2+</sup> model

The removal of Ca<sup>2+</sup> from the extracellular space reliably evokes epileptiform activity in vitro.<sup>78,79</sup> This might seem counterintuitive, as extracellular Ca<sup>2+</sup> is necessary to control vesicle fusion and synaptic release of neurotransmitter. Minimal extracellular Ca<sup>2+</sup> is thought to result in epileptiform activity by reducing surface charge screening.<sup>71</sup> In addition, without the desynchronizing effect of synaptic

**Table 1. Models of in vitro seizure-like events**

In vitro SLE model	Types of activity elicited	Brain region	Species	References
Reduced Mg <sup>2+</sup>	HYP SLEs, interictal	Hippocampal CA3	Rat, mouse, guinea pig	[38, 68–70]
4-aminopyridine	LVF SLEs, HYP SLEs, interictal	Hippocampal-entorhinal cortex, medial entorhinal cortex	Rat, mouse, guinea pig	[21, 42, 115]
Raised K <sup>+</sup>	Tonic SLEs, interictal	Hippocampus CA3/CA1	Rat, mouse	[84, 116]
Electrical stimulation	Tonic-bursting SLEs, interictal	Hippocampus	Rat	[83, 117]
Organotypic culture	Tonic-bursting SLEs, interictal	Hippocampus	Rat	[118]
Pilocarpine	LVF SLEs, interictal	Hippocampal-entorhinal cortex	Rat	[89, 90]
Reduced Ca <sup>2+</sup>	Tonic SLEs	Hippocampus	Rat	[78, 79]
Reduced Cl <sup>-</sup>	Tonic SLEs	Hippocampus CA3/CA1	Rat	[85, 119, 120, 121, 122]
GABA antagonists	Interictal	Hippocampus	Rat, mouse	[10, 87, 123–127]
Kainic acid	Interictal (brain slice), LVF SLEs (whole hippocampus)	Hippocampus	Rat, mouse	[88, 128, 129]

Manipulations that can evoke different forms of SLE, as well as brain regions and species that these events have been reported in.

noise, local field effects result in ephaptic coupling and enhanced synchronization of neurons.<sup>80</sup> In the low-Ca<sup>2+</sup> model, SLEs lasting tens of seconds correlate with profound neuronal depolarization and action potential firing that may organize in rhythmic bursting or afterdischarges. Low Ca<sup>2+</sup> SLEs are thought to provide a useful analogue of the ictal phase of epileptic seizures, as in vivo recordings of extracellular Ca<sup>2+</sup> in infrahuman primates have revealed that extracellular Ca<sup>2+</sup> drops to 100  $\mu$ M during seizures<sup>81</sup> and compromises synaptic transmission.<sup>82</sup> The reduced Ca<sup>2+</sup> model also represents a useful system for studying nonsynaptic mechanisms of seizure spread.

### Other acute models

Many other in vitro models of seizures exist (see Table 1), including electrical stimulation,<sup>83</sup> manipulation of the ionic concentration of the external medium (e.g., high K<sup>+</sup>,<sup>84</sup> low Cl<sup>-</sup><sup>85</sup>), application of a GABA<sub>A</sub> receptor inhibitor<sup>86,87</sup> (e.g., pentylenetetrazol, picrotoxin), or application of other chemicals such as kainic acid and pilocarpine.<sup>88–90</sup> When GABA<sub>A</sub> receptors are blocked, interictal spikes and afterdischarges are commonly observed, whereas SLEs are rare and short in duration compared to high K<sup>+</sup> or low Mg<sup>2+</sup> approaches.<sup>87,91</sup> Pilocarpine, a nonselective muscarinic agonist, efficiently promotes seizures in vivo, but generates only spontaneous epileptiform discharges in vitro at much higher concentrations,<sup>89,92</sup> or when extracellular K<sup>+</sup> is increased to 7.5 mM.<sup>93</sup> This suggests that the in vivo effect of pilocarpine is indirectly mediated by its peripheral effects, by its action on blood-brain barrier permeability, or both.<sup>89,92</sup> Kainic acid is also not as effective in vitro as it is in vivo,<sup>94</sup> and a role of serum albumin in facilitating kainic acid-induced seizure generation has been proposed.<sup>95</sup>

### Slice culture model

Organotypic hippocampal slice culture<sup>96</sup> has been developed to recapitulate posttraumatic epileptogenesis in vitro. In this model, interictal-like population spikes develop over a period of roughly 2 weeks in culture and are followed by the generation of spontaneous SLEs.<sup>97</sup> Organotypic cultures must be prepared from very young animals, and the development of epileptiform activity may be sensitive to variations in culture conditions. Recent reports, however, mitigate concerns regarding culture media.<sup>98</sup> Caveats of using organotypic slice culture include cell death, glial proliferation, and slicing- and culture-induced network reorganization. This approach is especially useful in identifying novel antiepileptogenic compounds, as cultures can be monitored and maintained for prolonged periods.<sup>97</sup>

### Special considerations for acquired and genetic models of epileptiform activity

When investigating epileptiform activity in genetic and acquired models of epilepsy, a number of additional factors

should be considered. With regard to animals with genetic mutations, control animals should be strain-matched and, if possible, should be littermates of experimental animals. For example, Cre-negative littermates from Cre/Lox approaches can serve as ideal controls. For acquired models, such as in traumatic brain injury, control animals should undergo a sham procedure that includes, at minimum, similar handling and anesthetic exposure. Finally, for both genetic and acquired models, there are often extended latent periods between insult and the first spontaneous seizures. This can require that in vitro experiments be performed in aged animals, which is often significantly more difficult. Therefore, experiments must be designed with careful consideration of the specific hypotheses to be tested and need to be interpreted in relationship to the timeline of disease progression. In addition, it is important to consistently report how in vitro recordings relate temporally to the development of spontaneous seizures in the animal model. For example, when in vivo insults are delivered to an animal (chemoconvulsant, injury, genetic lesion, and so on), there may be significant delays from the time of insult to the first spontaneous seizure. If in vitro recordings are made from animals during this latent period, they should be reported as such, and the data should be interpreted with regard to epileptogenic processes, not ictogenesis.

Finally, when experiments in slices obtained from epileptic animals are planned, it should be considered that seizure activity does not occur spontaneously in vitro. Seizure activity induced by proconvulsive drugs should be compared in slices obtained from naive controls and epileptic animals to evaluate changes in general SLE parameters, such as seizure threshold, seizure duration, and so on. More specific alterations of intrinsic membrane properties and local networks linked to epileptogenic and pathogenic mechanisms can be achieved with experiments focused on the study of membrane conductances, neuronal firing properties, and stimulus-evoked synaptic properties in both brain slices and acutely dissociated neurons obtained from genetic or acquired epilepsy models bathed in standard solutions. These types of in vitro studies have added significantly to our understanding of epileptogenic changes including augmented persistent sodium currents,<sup>99</sup> decreased inhibitory tone,<sup>100</sup> altered calcium conductances,<sup>101</sup> and decreased A-type potassium currents.<sup>102</sup>

## REPORTING EXPERIMENTAL PROTOCOLS

Because consistent and uniform reporting practices can improve the field's ability to compare in vitro results across laboratories, we recommend reporting the following: (1) species, strain, age, and sex; (2) animal supplier/vendor; (3) factors relating to animal breeding, housing, and transport; (4) the timing of tissue collection, method of sacrifice, and the use of anesthesia; (5) the method and equipment utilized

for brain slice preparation; (6) the composition of recording solutions including osmolarity, pH, and temperature; (7) the type of recording chamber used and the perfusion speed of ACSF. In a separate article prepared by this working group,<sup>13</sup> we examine each of the above technical factors in detail.

## RECORDING APPROACHES AND RELATED ISSUES

Multiple techniques can be used to quantify epileptiform activity *in vitro*. Electrical techniques afford better temporal resolution, whereas optical methods provide better spatial resolution. Electrical techniques include glass pipette electrodes and carbon fiber or metal-based electrodes. The latter category includes multielectrode arrays used to record local field potentials. Whole-cell patch-clamp or sharp intracellular recordings from single neurons can also be used to identify epileptiform activity. However, care should be taken when the recording of single-unit action potentials is utilized to infer epileptiform activity, as the physiologic bursting behavior of single neurons could be mistaken for interictal epileptiform activity.

Optical methods, including dyes and genetically encoded indicators of ion concentration, particularly  $\text{Ca}^{2+}$ ,<sup>103</sup> are growing in popularity.  $\text{Ca}^{2+}$  indicators enable the observation of both single cell and population activity. These indicators are useful for characterizing the spread of epileptiform activity as well as the recruitment of different cell types.  $\text{Ca}^{2+}$  imaging provides an indirect measure of electrical activity, as it requires  $\text{Ca}^{2+}$  entry into the cell via ionotropic or ionic channels or  $\text{Ca}^{2+}$  release from internal stores.

Voltage-sensitive dyes (VSDs) have been utilized to study epileptiform activity *in vitro* and to map with a high temporal resolution (milliseconds) the propagation of epileptiform discharges.<sup>104,105</sup> VSDs enter neuronal membranes and generate optical signals when the membrane potential depolarizes. VSD recordings are not accurate in describing polysynaptic events, since optical signals associated with depolarization of membranes of neurons (and possibly glial cells) largely outlast the duration of both synaptic events and neuronal firing that generate them. Novel advances have also introduced genetically encoded optical voltage sensors,<sup>106</sup> although their use in epilepsy research has been limited to date.

## CONFOUNDING ARTIFACTS THAT MAY APPEAR SIMILAR TO INTERICTAL/ ICTAL

Events can occur during an *in vitro* experiment that, to the untrained eye, mimic electrographic patterns associated with interictal or ictal activity. The first is spreading

depression (SD), which can be experimentally induced and can occur spontaneously. SD can occur when (1) slices are of poor health, (2) excitation is increased (i.e., high  $\text{K}^+$ , GABA<sub>A</sub> receptor antagonists in the perfusate), (3) or when oxygenation is disrupted. These events generally consist of a slow DC shift in the extracellular field potential lasting tens of seconds, during which synaptic and evoked activity is suppressed. When SD occurs in an *in vitro* epilepsy experiment, we suggest ending the experiment and moving onto a new slice.

Electrical artifact<sup>21</sup> can also mimic interictal activity. These artifacts include super high-frequency activity (>1 KHz), 50–60 Hz electrical noise and its harmonics, and events that occur with perfect regularity. Similarly, perfusion artifacts can look like interictal or ictal activity. Bubbles in the perfusion chamber, peristaltic pump artifacts, and electrical noise induced by the perfusion lines acting as antennae can all interfere with electrical recordings. Simple solutions exist including bubble traps along the perfusion line, ensuring that pump tubing is not cracked and stays properly lubricated, and including a metal component in the perfusion lines to allow electrical grounding.<sup>13</sup>

Filtering artifacts can also be of concern. Many field recording experiments utilize a high-pass filter (often 0.1 Hz) to ensure a stable baseline recording. If high-pass filtering is necessary, one must be aware that slow events and SD will be affected by filtering. If an abrupt DC shift occurs during a high-pass filtered recording, it is important to note that it will produce a signal resembling a large spike and wave event.<sup>34</sup> Hence, large, slow spikes at seizure onset may correspond to filtered DC shifts. On the other end of the spectrum, low-pass filters (1 KHz) are often used to eliminate high-frequency noise. These are of less concern because there is little evidence that biologically relevant activity occurs at these high frequencies. It is important to note that clinical EEG recording is normally low-pass filtered at 100 Hz, so there may be discrepancies between *in vitro* data recorded with a 1 KHz low-pass filter. False or artifactual HFOs are also of concern when signals are high-pass filtered. Fast electrical artifacts and “pointy” events like spikes can produce false HFOs as a direct consequence of the filtering.<sup>107</sup> Techniques have been defined to separate true from false HFOs.<sup>108</sup> Finally, it is important to note that both online (in the hardware) and offline filters distort signals.<sup>109</sup> In particular the phase of slow oscillations is strongly changed, which may pose problem when studying cross-frequency coupling.<sup>109</sup>

## HOW DOES IN VITRO ACTIVITY CORRELATE WITH HUMAN SEIZURES?

Correlating *in vitro* SLE patterns with human equivalents is not trivial. As discussed earlier, the minimum criteria to define an *in vitro* SLE is based on duration (>5 s). *In vitro*

SLEs that closely mimic human seizure patterns are likely the most relevant to understand epilepsy, given the assumption that human EEG patterns should be the starting point for devising in vitro models. SLEs that do not reproduce human patterns are still valuable if they aid in answering specific questions. For example, testing antiseizure drugs in in vitro preparations from resected temporal lobes of individuals with epilepsy is useful in testing drug efficacy, even if SLEs do not precisely mimic human EEG recordings. Undoubtedly, in vitro preparations offer the significant advantage of testing mechanisms and treatments in a controlled environment, while allowing for multimodal probing of a variety of biologic processes. Although these models provide a valuable tool in therapy discovery and screening, the conclusions of in vitro studies require validation in in vivo models.

Perhaps the largest challenge to using in vitro approaches centers on the fact that SLEs generally do not occur spontaneously. Although ictal and interictal events in human EEG occur spontaneously in a setting of chronic pathology, most in vitro studies take the normal brain and acutely evoke seizures. Therefore, in vitro SLEs may tell us more about how networks generate ictal activity than how seizures initiate in vivo. Studies using in vitro tissue obtained from animals with epilepsy, either genetic or acquired, may be more relevant to understanding the synaptic and cellular events that lead to seizures initiation. That caveat aside, if a given experimental model allows a better understanding of the neurobiology of epilepsy, then it is a “good” model. One must recognize, however, that these studies generally address only features of the human epilepsy, and do not replicate the epilepsy itself.

Of interest, evolving quantitative and dynamic systems models are forging new approaches to scientifically and quantitatively classify human seizures, animal seizures, and in vitro ictal activity. For example, a study based on quantitative dynamics of seizures recorded in different conditions (humans with temporal lobe epilepsy, in vitro SLEs recorded from the mouse CA1, and hyperthermia-induced SLEs recorded in zebrafish forebrain) proposed that there are 4 types of seizure onsets and 4 types of seizure offsets.<sup>34</sup> Approaches like these will help epileptologists and basic scientists unify the classification of seizures and could provide diagnostic or prognostic information, identify brain regions and mechanisms involved, and predict pharmacosensitivity. It is important to note that these approaches do not provide a mechanistic understanding of synaptic, cellular, and network changes that induce seizures.

Obviously in vitro models have significant limitations, which include SLE induction by powerful ionic and pharmacologic manipulations, limited networks (with the exception of the *ex vivo* whole guinea pig preparation), lack of normal blood flow, trauma associated with the preparation, and limited interaction with subcortical structures. Furthermore, findings from in vitro slice experiments

should be interpreted with the knowledge that the slicing procedure induces neurotrauma and associated morphologic and physiologic changes. These changes may be especially relevant when considering circuit-level changes, as the slicing procedure removes large portions of the relevant circuits and can damage the remaining circuit components. Finally, the nature of the recording itself, differential versus single-ended, is different in in vitro studies and human EEG recording. With these caveats in mind, it is remarkable that many EEG events can be generated using in vitro models. This capacity suggests that some basic principles of seizure generation or propagation are conserved in a reduced preparation. As further evidence for this concept, in vitro brain slices prepared from resected human tissue can replicate in vitro activity with sites of initiation and electrographic waveform similar to those seen in vivo.<sup>23</sup>

## HARNESSING IN VITRO MODELS TO UNDERSTAND HUMAN SEIZURES

There are multiple goals for in vitro studies of SLEs. Basic scientists strive to understand the mechanisms of seizure induction and propagation. Translational scientists aim to develop screening platforms to identify novel antiseizure and antiepileptogenic drugs. Clinicians want to understand what generates the ictal EEG events they are carefully trained to identify and interpret. With such diverse goals and training backgrounds, there are challenges and controversies to discussing, interpreting, and applying the value derived from in vitro tools. These issues, however, are far from insurmountable and highlight opportunities where the epilepsy community can work together to draw from each other's expertise to fill knowledge gaps.

The first challenge/controversy is the fundamental difference between human ictal activity and in vitro SLEs. Human ictal activity occurs spontaneously and sporadically. In vitro models require powerful pharmacologic manipulations to evoke them. A number of opportunities exist to overcome this challenge. Computational models can include results from in vitro studies to predict how molecular or cellular changes can affect large-scale brain activity. Multielectrode arrays allow surveying multiple points within an in vitro slice, thereby shedding light on how larger scale networks behave during ictal activity. In vitro screening approaches that identify potential therapeutic compounds can be integrated into a discovery pipeline that includes robust in vivo seizure and behavioral monitoring.

The next challenge/controversy to consider is that experimental manipulations that evoke seizures in animal models in vivo do not induce ictal activity in vitro. Conversely, manipulations that cause ictal activity in vitro, cause only interictal bursting in vivo. This suggests that (1) different mechanisms drive ictal onset *in vivo* and *in vitro*, (2)

experimental perturbations affect *in vitro* and *in vivo* differently, or (3) components of the networks that are essential for seizure generation may have been compromised in *in vitro* models. These differences are perhaps opportunities to distill the critical components of ictogenesis. Why do drugs that cause convulsive seizures *in vivo* generally fail to cause SLEs *in vitro*? What combinations of intrinsic neuronal properties, ionic imbalances, synaptic dysfunction, and/or minimal circuitry are required to drive ictal activity? Does network reorganization occur in chronic epilepsy that enables ictal activity or do the *in vitro* experimental manipulations we use to drive ictal activity replicate all required conditions of ictogenesis? It is interesting to note that the use of different organisms such as fly and zebrafish now enable the study of epilepsy mechanisms in intact animals (in particular genetically modified ones carrying human relevant mutations), with the arsenal of techniques available for *in vitro* studies.<sup>110,111</sup>

Finally, how do we accurately identify and name different types of ictal activity? This problem has been the basis for countless arguments and debates. Dynamic models can now inform how we categorize systems. Can we advance our “traditional” classifications of seizures and SLEs by incorporating classification based on dynamic principles and mathematical approaches?<sup>34</sup> Can the computational methods of classifying seizures incorporate features that may also predict clinically relevant questions, such as pharmacosensitivity or drug resistance, localization of seizure onset, or staging of the epilepsies? Moving forward, the combination of these approaches will be developed to categorize *in vitro* SLEs and will allow a new level of understanding of how *in vitro* assays parallel the human conditions.

In addition to these challenges, exciting opportunities exist to use new technologies to control neural circuits in new ways. Manipulating cellular and circuit activity using optogenetics and chemogenetics are enabling novel understandings of ictal and interictal activity. These methodologies enable cell type-specific activation and inactivation of neuronal activity with on a variety of temporal scales. Multiple recent reviews highlight their applications both *in vivo* and *in vitro* to better understand ictogenesis and cellular dysfunction associated with epilepsy.<sup>112,113</sup> Furthermore, viral approaches to introduce exogenous genes, such as those encoding potassium channels, capable of altering circuit activity are rapidly improving. A growing set of tools are rapidly evolving to manipulate cell and circuit activity with unprecedented levels of precision.

## CONCLUSIONS AND FINAL THOUGHTS

The use of *in vitro* models of SLEs to study epilepsy has led to advances in our understanding of epilepsy and ictogenesis, but has also raised questions regarding model validity, result interpretation, and the translatability of results.

To improve how we address these issues we outline a few suggestions and recommendations.

- Thorough reporting of all experimental approaches to allow replication of *in vitro* studies across laboratories is recommended.<sup>13,114</sup> Especially important are specific details regarding the animals used, tissue preparation procedures, manipulations used to generate epileptiform activity, and recording parameters used to collect data.
- *In vitro* reports must include a clear definition of the scientific question, choice of a model that allows direct investigation of that question, and interpretation of those findings within a framework defined by the model used.
- Although *in vitro* models can be extremely useful on their own to investigate mechanisms of epilepsy, the findings are strengthened when they are combined with *in vivo* models, especially when the aim is to identify novel epilepsy therapies.
- We recommend that the epilepsy research community acknowledges that human EEG events may be replicated *in vitro* only to some extent. *In vitro* preparations, however, are extremely useful for exploring the molecular and circuit-level mechanisms of seizures that mimic human patterns.
- We propose that the field recognize that *in vitro* experiments will allow drug screening approaches that simply cannot scale using *in vivo* approaches. Scaling up also requires significant resources and should be balanced by considerations of cost and time.

In summary, we hope to encourage the proper use of *in vitro* models for understanding epileptiform and ictal activity and that this article spurs a discussion of these issues that will benefit the epilepsy research field. The diversity of opinions is a strength of any research endeavor.

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