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Optogenetic dissection of ictogenesis: in search of a targeted anti-epileptic therapy

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Abstract

For over a century, epileptic seizures have been characterized as a state of pathological, hypersynchronous brain activity. Anti-epileptic therapies have been developed largely based on the dogma that the altered brain rhythms result from an overabundance of glutamatergic activity or insufficient GABAergic inhibition. The most effective drugs in use today act to globally decrease excitation, increase inhibition, or decrease all activity. Unfortunately, such broad alterations to brain activity often lead to impactful side effects such as drowsiness, cognitive impairment, and sleep disruption. Recent advances in optical imaging, optogenetics, and chemogenetics have made it feasible to record and alter neuronal activity with single neuron resolution and genetically directed targeting. The goal of this review it to summarize the usage of these research tools in the study of ictogenesis (seizure generation) and propose a translational pathway by which these studies could result in novel clinical therapies. This manuscript is not intended to serve as an exhaustive list of optogenetic tools nor as a summary of all optogenetic manipulations in epilepsy research. Rather, we will focus on the tools and research aimed at dissecting the basic neuron-level interactions underlying ictogenesis.

Introduction

The International League Against Epilepsy (ILAE) defines a seizure as “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain.”[1]. This definition is necessarily broad because our knowledge of the neuronal network dynamics that underlie ictogenesis is incomplete, at best. Even such long-held tenets as a “hypersynchronous” seizure onset [2] have been challenged by intracranial and intracellular recordings demonstrating *decreased* synchrony at seizure onset [3–6]. Development of more targeted anti-epileptic therapies will require a better understanding of the basic mechanisms by which individual neurons interact to generate pathological rhythms.

Some differences in interpretation of seizure synchronicity likely come from the different spatial scales associated with electrophysiological measurements. For example, surface EEG recordings spatially average activity over many mm^3 of brain tissue, which detects a sharp

increase in regional field potential, but is insensitive to the correlation of spiking in individual neurons. Conversely, patch clamp recordings can precisely detect the coincident spiking in at most a few neurons, leaving open the possibility that multiple ensembles of neurons are synchronizing at different phases. The current state-of-the-art for “large-scale” single-unit recording in epileptic patients is the rapidly improving microelectrode array (e.g. Utah Array), wherein action potentials can be recorded simultaneously in >100 individual neurons, and classified as putative interneurons based on sorting of extracellular recordings from 100 platinum-tipped silicon probes [5]. However, physiological changes in ionic composition and/or recording artifacts make it impossible to use current spike-sorting techniques to identify individual neurons during seizure [7].

While electrophysiology remains the most widely used approach to studying ictogenesis in human epilepsy patients, imaging of genetically encoded fluorescent probes can enhance recordings in animal models of seizure in a number of ways. 1) From a scale standpoint, two photon mesoscopy is capable of imaging activity (with genetically encoded calcium indicators such as GCaMP6) in >10,000 individual neurons with cellular resolution across a volume spanning 5mm diameter x 1mm deep: nearly an entire hemisphere of mouse neocortex [8]. 2) Genetic targeting of fluorescent probes, for example using crelox technology [9], enables selective recording from precisely defined populations of cells. 3) There is a variety of probes, enabling measurement of calcium using green [10], red [11] and ratiometric [12] fluorescent proteins; chloride [13], extracellular glutamate [14], and vesicular release [15] to name a few. Other genetically encoded probes, which are under active development and promise to substantially advance epilepsy research in the near future, include those for recording membrane potential [16–19] and potassium concentration [20].

While this is not meant to be a comprehensive survey of optical probes and targeting strategies used in epilepsy research [c.f. 21], this review will highlight some of the ways in which imaging is beginning to reveal the neuronal dynamics that take place ictally, pre-ictally, and interictally. These data can be used to form hypotheses about which cells are most critical in initiating a seizure by looking at when they fire relative to seizure onset or by inferring “functional connectivity” from correlated activity. These hypotheses can then be tested using optogenetic or chemogenetic constructs to alter activity in putative ictogenic cell populations. Here we highlight some of the ways in which optogenetics have begun to augment imaging findings and speculate on how this approach might lead to a more efficient antiepileptic therapy.

Imaging seizure onset and spread

As in many fields of neurophysiology, the combination of two-photon imaging and genetically encoded calcium indicators has enabled recordings, in individual neurons with cell-type specificity, of epileptiform activity in awake, behaving animals [22,23]. However, *in vivo* imaging of seizure activity is complicated by both movement artifact (in convulsive seizures) and the fact that seizures are rare in chronic animal models of epilepsy. Furthermore, excited fluorophores tend to produce reactive oxygen species, which lead to reduced fluorescence and potentially harm cells: photobleaching and phototoxcity

respectively [24,25]. To avoid these effects, imaging sessions are typically limited to <10min. Since the likelihood of capturing a seizure during a few-minute-long imaging session is low, *in vivo* imaging studies to date have been limited to interictal spikes (which are frequent) or seizures that are induced chemically or optogenetically [23,26]. While induced seizures are a suitable way to study the spread of seizure activity *in vivo*, interpretation of ictogenesis in induced seizures (*in vivo* or *in vitro*) is difficult since the tissue itself is not epileptic per se.

Recently developed microscopy techniques have the potential to augment *in vivo recordings.* For example, head-mounted miniature microscopes enable stable recordings from awake, behaving animals [27 and the open source version at miniscope.org], increasing the feasibility of recording seizure activity *in vivo*. Ongoing development of this technology will enable untethered [28] and two-photon [29] imaging from epileptic rodents. While head-mounted microscopes increase the feasibility of capturing the *temporal* seizure onset, advances in large field-of-view two-photon microcscopes (aka mesoscopes) improve the likelihood of capturing the spatial zone of seizure onset [i.e. seizure focus, 8].

For *in vitro* studies of ictogenesis, organotypic hippocampal slice cultures have recently been recognized as an experimentally useful model for studying epileptogenesis and ictogenesis, as they spontaneously generate seizures at a rate of 10–20 seizures/hour after a ~1 week latent period following the “injury” of slice preparation [30,31]. While organotypic slices are a suitable model for studying how local neuronal circuits reorganize to become seizure-prone following injury, they neglect the 3-dimensional complexity and long-range projection of an intact brain.

Synchronization and propagation in epileptic networks

Calcium imaging in animal models of seizure has added new insight into the nature of hypersynchrony in epileptiform activity. For example, dentate gyrus granule cells were much more likely to fire synchronously during interictal-spike like activity in slices prepared from chronically epileptic mice vs control slices [32]. Interestingly, the interictal spikes observed in this tissue resulted from many different ensembles of neurons synchronizing. In organotypic hippocampal slice cultures, calcium imaging revealed a highly variable interictal spike propagation pattern dictated largely by GABAergic inhibition [33]. Conversely, in an *in vivo* mouse model of acute seizure, seizure propagation patterns were highly regular, although GABAergic inhibition introduced substantial variance in the propagation velocity [23]. Longitudinal studies in organotypic slice cultures also showed consistency in seizure onset patterns, but ictogenic synchronicity increased over time [i.e. seizure onset became more synchronous throughout epileptogenesis, 31].

Network architecture

One of the limiting factors in drug development for the treatment of neurological disorders has been the inability to record simultaneously activity in many neurons. The recent surge in the development of imaging technologies has enabled such large-scale recordings from genetically defined populations of cells. It is, however,not entirely straightforward to look at (e.g.) 500 calcium traces from cells in epileptic tissue and gain intuition about how a seizure

starts. One approach might be to average signals from common cell types to show that for example interneurons activate first during seizure onset in a brain slice model of epilepsy [34]. However, this approach is relatively superficial and disregards the detailed interactions between individual neurons which are likely to be important in generating rhythms. One approach to gaining intuition about network dynamics from such high-dimension datasets is the analysis of functional network architecture in which neurons are determined to be "functionally connected" when their activity is sufficiently correlated [35–37]. These data have begun to test hypotheses formed from computational models that certain network topologies are particularly well-suited to sustaining seizures [38–41]. The three most studied topologies, small-world, scale-free, and rich-club networks, are all defined by a small subset of elements. In small-world networks, a small percentage of edges in a regular (locally) connected network are randomly re-wired, efficiently shortening the path between any two nodes [41]. Scale-free networks are defined as having a degree (where degree = # of connections/node) distribution that follows a power law, which means a small number of nodes have a very large number of connections [42]. In a rich-club network, the most highly connected nodes are also interconnected with each other at a higher-than-expected rate [43]

Brain networks can be quantified over broad range of modalities (genetic, anatomical, functional) and spatial and temporal scales [44]. In human epilepsy, functional network architecture has predominantly been measured at a larger spatial scale (e.g. using fMRI and EEG), in which nodes represent brain regions on the scale of mm^3 rather than individual neurons. For example, experimental data from resting state fMRI suggests that functional connectivity in patients with medial temporal lobe epilepsy is closer to a random topology compared to control subjects, whose network topology is shifted slightly closer to the small-world network end of the functional connectivity spectrum [45]. And, pre-surgical intracranial EEG from patients with intractable epilepsy revealed local "rich-clubs" (highly interconnected regions), surgical removal of which, resulted in better post-surgical outcomes [46]. Interpretation of such large scale measures of network architecture in such a complex system can be complicated by the fact that functional connectivity changes with brain state [36,47]. Resting state fMRI recordings, which indicated random connectivity, were taken between seizures, whereas the iEEG data showing rich-club networks was recorded peri-ictally. At the cellular level, calcium imaging data from rodent brain slices revealed similar transitions in functional connectivity with sparse, random connectivity between seizures and scale-free connectivity just before and after seizure [31].

The role of interneurons in ictogenesis

The putative existence of a small population of network elements that are important for ictogenesis is exciting, as they represent a highly selective target for antiepileptic therapies. Further characterizing these components will be critical to developing such therapies. In one such study, although not explicitly recording epileptiform activity, Bonifazi et al. [48] used multicellular calcium imaging of synchronous giant depolarizing potential (GDP) events in acute hippocampal slices from juvenile (P6-P8) mice to identify a small subset of cells whose activity was highly correlated with nearly all other recorded cells. By performing functional network analysis online, they were able to target putative hub cells for patch clamp recording and confirm that stimulating high connectivity, but not low connectivity,

cells was sufficient to significantly alter the interval of GDPs. These hub cells were determined to be predominantly interneurons and had significantly longer axonal arbors than low connection cells, although this could result from better preservation of interneuron neurites in the axis of slicing.

Perhaps related to their proposed role as hubs, interneurons have been shown in numerous electrophysiological studies to increase firing pre-ictally and have a depolarizing, ictogenic effect in epileptic networks [49–53]. Paired intracellular recordings and multicellular calcium imaging from acute and organotypic hippocampal slices showed that elevated firing in interneurons, not principal neurons, comprised pre-ictal bursts of activity [34]. A chloride-sensitive fluorescent protein, Clomeleon, [54] exposed an ictal elevation in pyramidal cell chloride concentration beginning with the preictal bursts and peaking at a concentration that would make GABA synapses frankly depolarizing [34]. Photometric measurements in which calcium indicators were expressed in different subpopulations of interneurons confirmed activation of parvalbumin (PV), somatostatin (SOM), and vasoactive intestinal peptide (VIP) expressing interneurons all prior to principal cell recruitment [26]. Together these data support a model in which interneurons act as hub cells that, during synchronous firing in epileptic tissue, elevate postsynaptic chloride to make GABA transiently excitatory, which may contribute to ictogenesis.

Targeted manipulation of activity to alter ictogenesis

As described above, imaging is sufficient to demonstrate a correlation between neuronal activity and seizure onset, but to demonstrate that a population of cells is *necessary* for ictogenesis (and thus a suitable drug target), one must selectively manipulate activity in that population. One way this can be achieved is through the use of genetically encoded constructs that increase or decrease activity. Unlike most pharmacological agents, recently developed optogenetic and chemogenetic proteins can be specifically targeted to populations of cells defined genetically, using promoters and/or recombinase activity; and spatially, based on injection site of viral vectors and (with optogenetics) illumination profile. Furthermore, optogenetic tools enable manipulations of neural acitivity with millisecond temporal resolution.

Inhibition of principal neurons

The earliest attempts at manipulating seizure activity using genetically targeted constructs follow the dogma that epilepsy is a disease of too much excitation and/or too little inhibition. As such, two primary approaches have been taken: introduce inhibitory vectors in principal cells or excitatory vectors in interneurons. For example, Kätzel et al. [55] transduced principal neurons in the primary motor cortex of rats with the inhibitory DREADD $hM4D_i$ [56], a synthetic G-protein coupled inwardly rectifying potassium channel that is exclusively activated by the exogenous clozapine-N-oxide (CNO). Behavioral seizures induced by focal application of picrotoxin or pilocarpine (administered in the same region) were reduced by approximately 40% following systemic administration of CNO [55]. Simlarly, electrically-induced epileptiform activity in organotypic slice cultures was suppressed by activating $hM4D_i$ receptors [57]. Interestingly, in the latter example, $hM4D_i$ receptors were expressed

under control of the synapsin promoter, which results in expression in both interneurons and principal cells. It is unclear whether CNO application shifted the balance of excitation and inhibition.

Expression in principal cells of light-sensitive inhibitory opsins, such as the light-driven chloride pump halorhodopsin, provides the added dimension of temporal control of activity (i.e. principal cells are only inhibited when the light is on). This enabled development of real-time feedback controllers which detect seizures online and inactivate principal cells, with the goal of truncating seizures [58–60]. In a photothrombic neocortical stroke model of epilepsy, inhibition of principal cells in the thalamus consistently truncated spontaneous seizures [59]. Models of temporal lobe epilepsy (TLE), on the other hand, seem to represent a more treatment-resistant form of seizure. TLE seizures often have focal onset with secondary bilateral generalization associated with loss of consciousness. Thus, there is a theoretical window for optogenetic treatment that occurs during the focal onset electrographic seizure, before generalization and altered consciousness. In animal models of TLE, following intrahippocampal kainite injection, responsive inhibition of principal neurons in the hippocampus ipsilateral to injection reduced behavioral seizures by approximately 30% [58]. In this model, seizure duration could be shortened by inhibition of hippocampal principal neurons [57% duration reduction, 58], excitation of hippocampal PV + interneurons [43% reduction, 58], inhibition of dentate granule cells [66% reduction, 61], and activation or inhibition of PV+ neurons in the cerebellum [32% and 33% duration reduction, 62]. These results are encouraging that spatially and temporally limited interventions can impact seizure activity. It seems likely that TLE interventions in particular could be improved by technical advancements in early seizure detection and identification of progressively more specific subpopulations of cells to target.

Excitation of interneurons

A complementary approach to inhibiting excitatory cells is to excite inhibitory cells. Activation of channelrhodopsin expressed exclusively in interneurons suppressed chemically-induced seizures in acute hippocampal slices [63]. Similarly, in the Krook-Magnuson study above, channelrhodopsin-mediated activation of interneurons produced a reduction in seizure duration that was comparable to inhibition of pyramidal cells. In a study of seizures acutely induced by intrahippocampal kainite injection, Lu et al. [64] showed that the anticonvulsive action of optogenetic interneuron activation was subregion-specific: activating interneurons of the dentate gyrus disrupted seizures, while activating entorhinal cortex interneurons did not. In the aforementioned Khoshkoo et al. study, the antiepileptic potential of interneuron activation was explored with more specificity by expressing channelrhodopsin in Dlx12b+ (mostly PV and SOM interneurons) or VIP+ interneurons. Activation of VIP+ interneurons both shortened seizures and increased seizure threshold, whereas activation of the broad-spectrum Dlx12b+ interneurons only shortened seizures, without affecting seizure threshold [26].

One must exercise caution when using such tools as they do not always have the canonical effect. For example, in mice acutely treated with the convulsant 4-aminopyridine, ictal activation of PV+ interneurons would truncate a seizure, but interictal activation *initiated*

seizures 60% of the time [65]. Similarly, in seizures evoked by local NMDA application to acute slices, optogenetic activation of PV+ interneurons near the site of application ("the focus") prolonged seizures, while activation of interneurons distal to the "focus" inhibited propagation [66]. Although the authors of these studies speculate that the pro-convulsive role of interneurons was caused by rebound firing following the release of inhibition, it could also have been due to a post-synaptic accumulation of chloride caused by prolonged GABA receptor opening. Such an accumulation can reverse the direction of currents initiated by GABA receptor activation, causing GABA to be excitatory rather than inhibitory [34,67]. Inhibitory opsins can have similarly paradoxical effects. Extended activation of the inward chloride pump halorhodopsin, increased the probably of synaptically evoked firing immediately after the light was turned off [68]. Since this effect was not apparent when the outwardly directed proton pump was used to silence neurons, it was likely caused by a depolarizing shift in the GABA reversal potential induced by halorhodopsin-mediated chloride loading. In hyperexcitable (but not spontaneously seizing) brain slices, prolonged (1–10s) activation of halorhodopsin in pyramidal cells initiated seizures [69].

Experimental outlook

As highlighted above, genetic targeting of fluorophores and optogenetic actuators have made it possible to characterize and manipulate activity in broad, genetically defined classes of neurons (e.g. CamK2+, PV+, SOM+, etc.). However, functional network analysis suggests that, even within a class of neurons, there may be a high degree of variability in connectivity. Evidence of scale-free and rich-club networks suggest that there may be an elite class of hub neurons, whose genetic signature we do not yet know. Nearly a decade ago, Bonifazi et al. [48] used whole-cell patch clamp recordings to "close the loop" with functional connectivity by stimulating putative hub neurons. Since that time, a high throughput methodology for testing the relationship between functional connectivity and ictogenesis has yet to be developed.

Translational outlook

There are three clinically available classes of anti-eplileptic therapies: drugs/diet, surgical resection, and electrical stimulation. Currently available drugs are incapable of producing seizure freedom in more 30% of patients [70]. Furthermore, they tend to act on ion channels or neurotransmitter systems that are ubiquitous in the brain, which often produces serious neurological side effects. Surgical resection, removal of brain tissue thought to be essential to ictogenesis, might be an option if the suspect tissue is not located in eloquent cortex (areas responsible for critical functions such as language, movement, etc.). However, approximately 50% of surgical patients suffer a recrudescence of seizures within 5 years of resection [71,72]. Electrical stimulators fall into two categories: open-loop stimulators, which deliver continual doses of current to either the vagus nerve or the thalamus; and closed-loop stimulators, which deliver brief doses of current to the seizure focus when a seizure is anticipated. Closed loop stimulators represent a targeted spatial and temporal intervention, which should minimize the occurrence of side effects. However, all (closed and open-loop) clinically available electrical stimulators have a modest efficacy; reducing seizure frequency by 18–26% [73].

All three classes of anti-epileptic therapies stand to benefit from a more complete understanding of ictogenesis at the cellular level. In some cases, findings from optogenetic studies in animals could be directly applied to improving clinical techniques. For example, ictogenic network architectures identified from multicellular calcium imaging studies could be scaled up to EEG recordings in human patients to more effectively select targets for surgical resection. Or, with high density electrophysiological recordings, network analysis might be used to identify smaller target areas for surgical micro-resection. Similarly, imaging data characterizing pre-ictal changes in functional network architecture could be directly applied to improve electrical stimulators. Closed-loop stimulators are predicted to produce better seizure prevention if the seizure is detected early [74]. EEG-based seizure prediction for therapeutic intervention is an active field of research [75], which is starting to use functional network analysis to identify changes in network dynamics that are not readily visible in the amplitude of the EEG signal [76].

In other cases, the translational pathway will be less straightforward. Rather, it will be critical to establish a methodology for translating results from animal studies, which have high resolution, genetically targeted optical recordings and manipulation, to clinically feasible interventions. For example, if it is found that optogenetically exciting a small subset of PV+ interneurons is sufficient to suppress seizures, how would that be implemented in an epileptic human patient (where cre-lox targeting of an optogenetic construct is not currently feasible)? AAV viral vectors can be used to (seemingly safely) deliver optogenetic proteins to cells in humans [77], but promoter-based targeting to specific tissue and cell-types is largely constrained by the size of the DNA payload AAV can deliver. Recent work suggests that promoter fragments can be used as enhancers to confine expression to, for example, GABAergic interneurons [78]. Continued development of viral vectors with tropisms for increasingly more targeted populations of cells will be critical to the clinical translation of genetic therapies [79]. In addition to the development of new viral targeting strategies, simultaneous electrical stimulation and calcium imaging in animal models could be used to screen electrode configurations and stimulus paradigms for preferentially exciting PV+ interneurons with clinically available brain stimulators.

It is also conceivable that studies involving the multicellular optical recordings and manipulations summarized here will serve to identify novel targets for development of more selective pharmaceutical or genetic interventions. For example, if it turns out that a narrow subpopulation of neurons with particular network characteristics are essential to ictogenesis, then those cells could be characterized (e.g. using single cell RNASeq) to identify any unique features that might represent novel drug targets. For example, if inhibiting hub neurons is anti-convulsive, and hub neurons tend to overexpress NR2Dcontaining NMDA receptors, then perhaps an antagonist could be developed to selectively inhibit hub neurons. Or perhaps gene therapies could be developed to knock-down putatively overexpressed proteins. Devising strategies to translate discoveries made using the vast array of exciting new neuroscience research tools will be critical to developing a new generation of treatments for neurological disorders.

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