

On-Demand Seizures Facilitate Rapid Screening of Therapeutics for Epilepsy

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The authors modified a common method to induce epilepsy in mice to provide an improved approach to screening new drugs for epilepsy. This is an **important** goal because of the need to develop drugs for patients who are refractory to current medications. The authors' method evokes seizures to circumvent a low rate of spontaneous seizures and the approach was validated using two common anti-seizure medications. The strength of evidence was **solid** in that some validation was provided, but **incomplete** because the method for quantification, definition of seizures, and some other aspects of the paper were not clear or absent.

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Abstract

Animal models of epilepsy are critical in drug development and therapeutic testing, but dominant methods for pharmaceutical evaluation face a tradeoff between higher throughput and etiological relevance. For example, in temporal lobe epilepsy, a type of epilepsy where seizures originate from limbic structures like the hippocampus, the main screening models are either based on acutely induced seizures in wild type, naïve animals or spontaneous seizures in chronically epileptic animals. Both types have their disadvantages – the acute convulsant or kindling induced seizures do not account for the myriad neuropathological changes in the diseased, epileptic brains, and spontaneous behavioral seizures are sparse in the chronically epileptic models, making it time-intensive to sufficiently power experiments. In this study, we took a mechanistic approach to precipitate seizures “on demand” in chronically epileptic mice. We briefly synchronized principal cells in the CA1 region of the diseased hippocampus to reliably induce stereotyped on-demand behavioral seizures. These induced seizures resembled naturally occurring spontaneous seizures in the epileptic animals and could be stopped by commonly prescribed anti-seizure medications such as levetiracetam and diazepam. Furthermore, we showed that seizures induced in chronically epileptic animals differed from those in naïve animals, highlighting the importance of evaluating therapeutics in the diseased circuit. Taken together, we envision our model to

advance the speed at which both pharmacological and closed loop interventions for temporal lobe epilepsy are evaluated.

Introduction

Epilepsy, a set of neurological disease syndromes characterized by recurrent, spontaneous seizures, is a debilitating condition that affects millions of people worldwide (1, 2). Even with the development of over forty anti-seizure medications (ASMs) over the course of the last century, between 15 – 30 % of patients are unable to achieve seizure freedom (3–5). The percentage of patients with drug resistant seizures has remained constant despite the introduction of multidrug therapies and newer ASMs classes (6).

The lack of screening in etiologically relevant models may be one reason for the disconnect between the increasing types of medications and the stubbornness of drug resistant seizures to treatment (7–9). Many patients with drug resistant seizures have temporal lobe epilepsy (TLE), a type of epilepsy where seizures originate from temporal lobe structures such as the hippocampus (10). The brains of TLE patients typically have structural and molecular changes, including hippocampal sclerosis, axonal sprouting, and receptor alterations (11–14). The resulting epileptic network functions differently from that of the healthy brain. This divergence may be why ASMs that work in a naïve, wild type animal screen are ineffective in epileptic patients.

Fortunately, TLE is well modeled in animals (15–18). The intrahippocampal kainate (IHK) model of epilepsy captures key markers of human TLE – animals exhibit hippocampal sclerosis, mossy fiber sprouting, and spontaneous limbic seizures (19, 20). Despite its lengthy history, the IHK model was only recently introduced into the Epilepsy Therapy Screening Program (ETSP) pipeline (8, 21). There were many factors contributing to this delay. Spontaneous seizures, by their very nature, are sparse, irregular, and unpredictable (22–24). Compared to testing ASMs in wild type animals by acutely inducing seizures via convulsants or kindling, testing therapeutics in etiologically relevant models is often a much more lengthy and unwieldy process.

To address the challenges of using etiologically relevant models for therapy screening, we asked whether we could harness the epileptic circuit to generate seizures on demand. Of particular interest to seizure initiation and propagation is region CA1 of the hippocampus. The CA1 is the main output of the hippocampus in the canonical tri-synaptic circuit (25). *In vitro*, electrical activation of excitatory inputs to the CA1 can generate seizure-like bursting (26). In animals, calcium imaging demonstrates that CA1 principal cells are highly active during acute convulsant induced seizures (27). In addition, changes in the CA1 cellular population during epileptogenesis, such as an increase in burster cells, makes the CA1 predisposed to rapid firing that can elicit epileptic events (28). Thus, we hypothesized that hyperexcitation of CA1 principal cells would activate etiologically relevant mechanisms and initiate seizures in epileptic animals.

Specifically, we investigated whether selective optogenetic activation of CA1 principal cells could precipitate time-locked seizures in freely moving epileptic animals (**Fig. 1**). We used a modified version of the IHK model that preserves CA1 structure, making it more similar to human TLE. To validate the model, we first compared the induced activity to spontaneous seizures. Next, we compared seizures induced in epileptic animals to those in wild type, naïve animals to ascertain whether unique therapeutically relevant features are evident in the epileptic brain. Finally, we attempted to shut down induced seizures with known anti-seizure medications. We present evidence that hypersynchronous excitation of CA1 principal cells can induce focal to bilateral tonic-clonic seizures in mice, and that these seizures can be used to evaluate the therapeutic efficacy of both pharmacological and time-sensitive treatments.

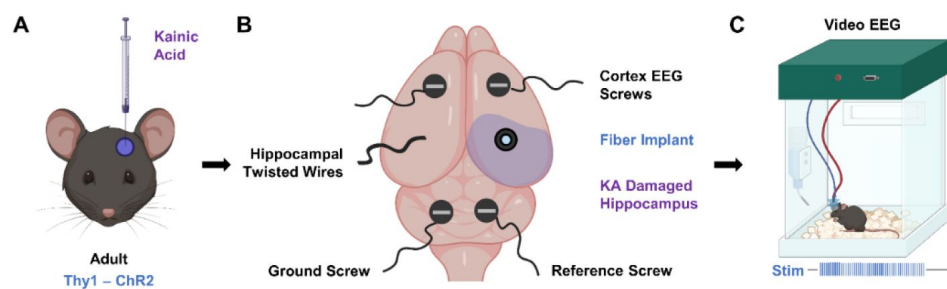


Figure 1

Experimental schematic for on-demand seizure induction in epileptic animals.

(A) Chronic epilepsy was induced in Thy1-ChR2 mice via intrahippocampal kainic acid injection into the CA3. (B) Mice were implanted with EEG recording apparatus consisting of two cortical screws, one set of insulated braided wire targeting the hippocampus, a ground screw, and a reference screw. A fiber was positioned so that the tip illuminated the CA1. (C) 10 Hz of 473 nm light delivered into the CA1 activated Thy1-ChR2 neurons and induced seizures on demand *in vivo*.

Results

CA3 kainate injection preserves CA1 cell layer targeted during on-demand seizure induction

The on-demand seizure induction procedure in mice expressing excitatory channelrhodopsins in CA1 pyramidal neurons (Thy1-ChR2 mice) is a three-part process (**Fig. 1**). First, we induced chronic epilepsy in the mice via CA3 IHK injection (**Fig. 1A**). An electroencephalogram (EEG) implantation surgery followed, during which two recording screws were inserted into the cortex, and two additional screws – a ground and a reference – were inserted into the cerebellum (**Fig. 1B**). An implanted optical fiber targeting the CA1 ipsilateral to the IHK injection allowed for optical excitation at the seizure focus, while a braided wire targeting the CA1 contralateral to IHK injection provided information on inter-hippocampal activity (**Figs. 1B** & **2A** middle). After the mice recovered for a week, they underwent 24-hour continuous video/EEG monitoring, during which 10 Hz, 473 nm optical stimulation was applied to induce seizures on demand (**Fig. 1C**).

The canonical injection site for IHK models is the dorsal CA1 (29); however, the destruction of the CA1 cell layer due to IHK injection is fundamentally incompatible with our approach for inducing seizures. Kainate, a glutamate agonist, induces hyperexcitation of cells in the vicinity of the injection site, leading to cell death and gliosis (30). The damage is clearly visible in the CA1, and the sclerosis is hypothesized to be the center of seizure generation (31) (**Figure 2A** top). Thy1 expression in the CA1 localizes to principal cells (32), so it is critical to preserve the health of the CA1 cell layer. To do so, we moved the IHK injection site to the medioventral CA3, which is 0.7 mm posterior, 1.4 mm lateral, and 1.6 mm ventral to the canonical CA1 injection site. CA3 IHK animals have a sparse CA1 layer (**Fig. 2A** middle) compared to naïve animals (**Fig. 2A** bottom), while CA1 IHK animals often do not have a visible CA1 layer (**Fig. 2A** top).

Comparative analysis of brain slices extracted from the CA1 IHK ($n = 6$) and the CA3 IHK ($n = 10$) mice showed greater CA1 preservation in the CA3 IHK group (**Fig. 2B**). Nissl staining using cresyl violet highlighted neural structures and stained the CA1 principal cell layer. Slices extracted at AP – 2.0 mm from bregma showed that the CA1 cell layer was present in only one third of CA1 IHK animals. In contrast, the CA1 cell layer was present in all CA3 IHK animals (**Fig. 2B** top). In both groups, the CA1 layer tended to be thinned when present. However, the CA1 in 1 CA3 IHK animal did not appear to be thinned (**Fig. 2B** middle). All animals in the CA3 IHK and the CA1 IHK groups exhibited CA3 damage and morphological changes in the dentate gyrus (**Fig. 2A, 2B** bottom). Thus, CA3 damage was not unique to the CA3 IHK group; it was already present in animals with the canonical CA1 IHK injection.

While preserving the CA1 structure was important to our model, we wanted to ensure that shifting the IHK injection site to the CA3 did not alter the number of spontaneous seizures an animal experienced per day. Thus, we acquired continuous video-EEG recording on the same CA1 IHK and CA3 IHK animals and tracked spontaneous seizures. We found that the average number of spontaneous seizures a day was similar between the two groups. The CA1 IHK animals experienced an average of 1.6 spontaneous seizures a day while the CA3 IHK animals experienced an average of 1.7 spontaneous seizures a day (**Fig. S1**). The nonparametric two tailed Mann-Whitney test, which compared the difference between the median daily spontaneous seizure count of the CA1 IHK (1.0) and CA3 IHK (1.6) animals, also failed to find a significant difference between the two groups ($p = 0.635$) (**Fig. 2C**).

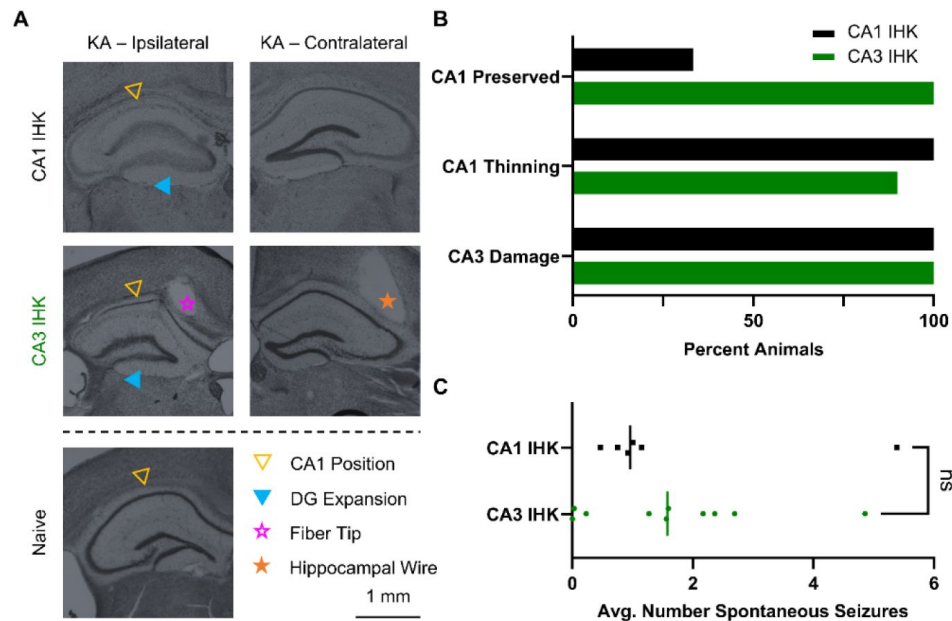


Figure 2

Intrahippocampal kainic acid (IHK) injection into the CA3 reduced CA1 damage without impacting average spontaneous seizure count.

(A) Cresyl violet staining of hippocampal slices from IHK injected animals visualized IHK induced damage to the hippocampus. The CA1 layer was eliminated in CA1 IHK animals (top). In the CA3 IHK animals, the CA1 layer was present but thinned (middle). Both CA1 IHK and CA3 IHK animals displayed expansion of the dentate gyrus structure and extensive CA3 damage when compared to naïve animals (bottom). (B) Two thirds (4/6) of CA1-IHK animals did not have a visible CA1 cell layer, while the rest (2/6) had a thinned cell layer. In contrast, all CA3 IHK (10/10) animals had a visible CA1 cell layer. All 10 CA3 IHK and 6 CA1 IHK animals had extensive damage to the CA3 coupled with expansion of the dentate gyrus (DG). (C) Two tailed Mann-Whitney test comparing the average number of spontaneous seizures per day showed no significant difference ($p = 0.635$) between CA1 IHK and CA3 IHK animals.

Optogenetically induced seizures resemble spontaneous seizures in epileptic mice

We hypothesized that high frequency activation of principal cells in region CA1, the major output of the hippocampus, could acutely induce seizures in epileptic animals. Since the optogenetic stimulus excites cells that are naturally active during seizures (27 [↗](#), 33 [↗](#), 34 [↗](#)), we theorized that the induced seizures should mechanistically resemble spontaneous seizures.

In epileptic, freely moving Thy1-ChR2 mice ($n = 10$), we recorded spontaneous seizures, as exemplified in **Figure 3A** [↗](#). One week prior to stimulation, animals experienced on average $1.0 (\pm 1.8 \text{ standard deviation})$ behavioral spontaneous seizures per day (Fig. S1A). To induce behaviorally salient seizures in epileptic animals, we optically activated Thy1-ChR2 expressing neurons in the CA1 ipsilateral to the IHK injection site using 10 Hz, 473 nm light. Seizures were induced several times a day at one-hour intervals. The threshold laser power and stimulation duration, defined as the minimum necessary for consistent ($> 66\%$) induction of afterdischarges lasting a minimum of 5 s, varied between the animals, with an average threshold laser power of $9.14 \pm 4.75 \text{ mW}$ and an average stimulation duration of $6.30 \pm 1.64 \text{ s}$ (Fig. S2 A&B). Using the threshold stimulus, activity longer than 5 s was induced approximately $88.7 \pm 8.8 \%$ of the time in the epileptic animals (Fig. S2C). Average duration of induced activity was $30.98 \pm 4.69 \text{ s}$ (Fig. S2D). After optical stimulation began, animals experienced 1.8 ± 3.5 spontaneous behavioral seizures per day on average (Fig. S1A). A two tailed Wilcoxon rank sum test on the distribution of daily spontaneous seizure counts before and after stimulation did not find that the difference of the two groups' medians diverged from 0 ($p = 0.1336$).

We next sought to determine whether EEG signals differed between spontaneous seizures and induced activity. First, we analyzed standard EEG features (35 [↗](#)) such as band power at three different frequency ranges, line length, and area, in overlapping 500 ms sections (Fig. S3). These features were normalized to a short pre-stimulation baseline period, after which a K nearest neighbor classifier was employed to determine the seizure durations (Fig. S4). Subsequently, we divided the activity into three segments (beginning, middle, ending). To account for the variations in electrographic signals among different animals, we used a linear mixed effect model, with the animal set as the random effect, to analyze changes in the feature space. This methodology allows us to compare features across activities of varying durations and across different animals.

Spontaneous behavioral seizures underwent stereotyped changes throughout the three segments (**Fig. 3A** [↗](#)). Visually, we noted that the beginning third of the seizure was characterized by an increase in spiking activity. The middle third was characterized by higher frequency activity and behaviorally resembled the tonic phase of a tonic-clonic seizure (36 [↗](#)). The final third was characterized by bursts and behaviorally resembled the clonic phase of a tonic-clonic seizure. Next, we quantified the feature space changes within the 337 spontaneous seizures from 8 animals using the linear mixed effect model (**Fig. 3C** [↗](#)). In the beginning third, there was a significant increase in the area ($p < 1e^{-4}$), all band power frequencies ($p < 1e^{-4}$), and line length ($p < 1e^{-4}$) over baseline values. A further increase occurred in all three features ($p < 1e^{-4}$) between the beginning and middle third of the seizure before decreasing between the middle and final third of the seizure ($p < 1e^{-4}$). The elevated values in the final third were still significantly higher than baseline ($p < 1e^{-4}$).

Like in the spontaneous seizures, induced seizures (**Fig. 3B** [↗](#)) underwent three visually distinct phases. In the beginning, there was a general increase in spiking amplitude and the emergence of higher frequency activity that was occasionally accompanied by behaviors such as freezing. The middle portion resembled the tonic phase of a seizure – the animal displayed forelimb clonus, shaking, or stiffening of the tail. In the final third, bursts began to emerge on the EEG signal, and the animal entered the clonic phase of the seizure, during which it uncontrollably reared,

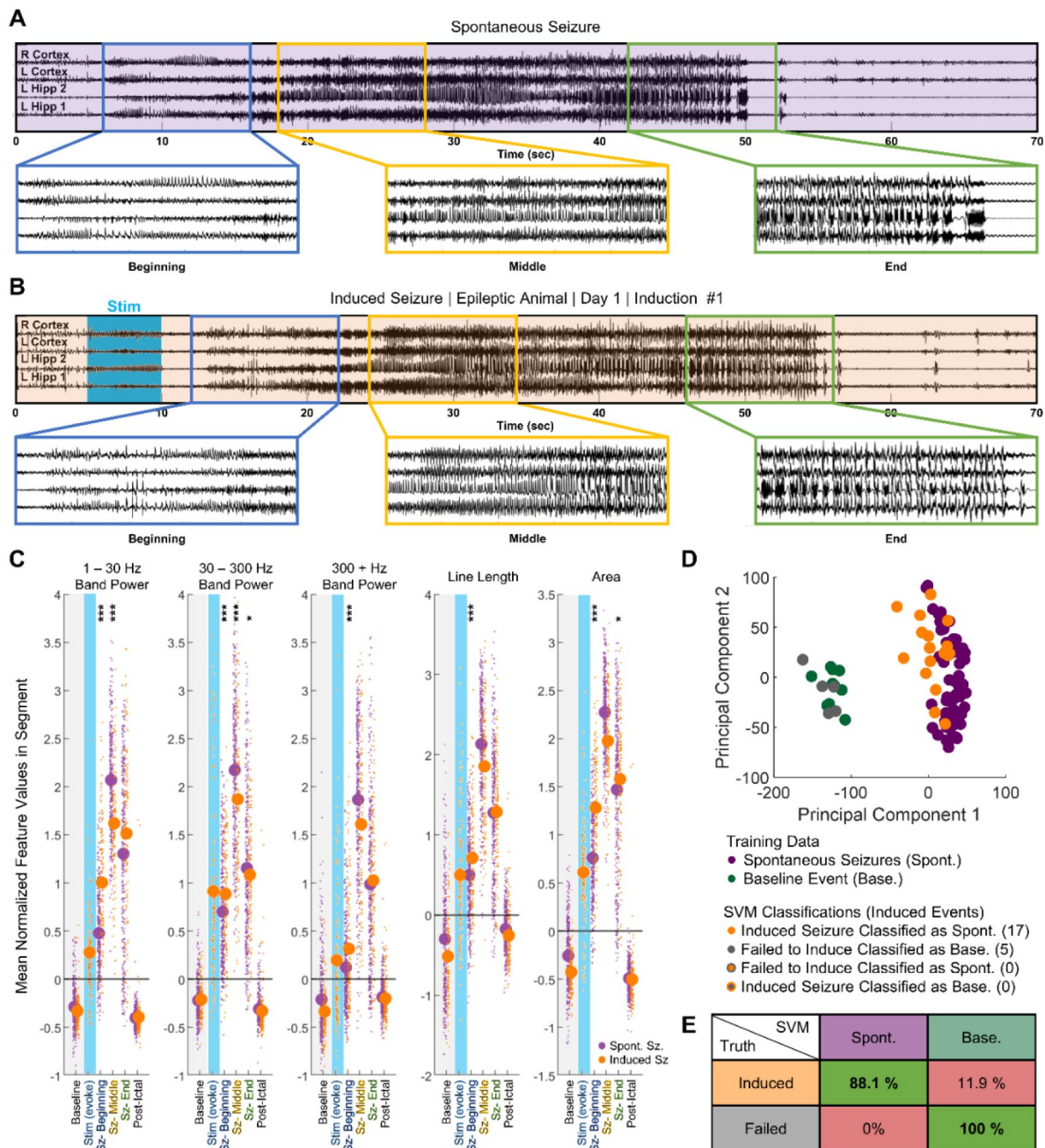


Figure 3

Induced seizures resembled naturally occurring spontaneous seizures in chronically epileptic animals.

(A) Example of electrographic signal from spontaneous seizure in epileptic animal, with 3 segments of 10 s enlarged for clarity. (B) Electrographic signal from first induction in the same animal. (C) Change in features from baseline (gray) to computationally defined segments (first tercile – beginning, second tercile – middle, and final tercile – end). In the first tercile, the extent of increase in 1 – 30 Hz band power, 300 + Hz band power, line length, and area significantly differed between 138 induced ($n = 10$) and 337 spontaneous behavioral seizures ($n = 8$). Differences become less significant by the middle and the final third. Linear Mixed Effect Model: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (D) Linear Support Vector Machine (SVM) classified inductions from the animal in (A) and (B). Successfully induced seizures were more closely associated with spontaneous seizures than with either baseline activity or optogenetic activations that failed to induce seizures. (E) Compiled table of SVM accuracies across epileptic animals ($n = 8$). Induced seizures were classified as similar to spontaneous seizures in 88.1% of all successful activations. Failed activations were classified as baseline 100% of the time.

backpedaled, jumped, or fell on its side (Fig. S5). Occasionally, optogenetic stimulus induced seizures of lower severity (Fig. S6) or failed to cause afterdischarges (Fig. S7). In the feature space, induced seizures followed a similar trend as spontaneous seizures (**Fig. 3C**). Line length, area, and band power significantly increased between baseline and the beginning third ($p < 1e^{-4}$). A further increase occurred between the beginning and the middle third ($p < 1e^{-4}$). Between the middle and final third, a significant decrease in area ($p < 1e^{-4}$), line length ($p < 1e^{-4}$), and 30 Hz + band power ($p < 1e^{-4}$) occurred. However, unlike in spontaneous seizures, the decrease in 1 – 30 Hz band power between the middle and final third in induced seizures was not significant ($p = 0.10$).

We then compared the progression of spontaneous and induced seizures in the feature space. All confirmed spontaneous seizures had a Racine score of 3 or greater. To ensure a fair comparison, we used two criteria to filter the induced seizures: 1) afterdischarges must have lasted a minimum of fifteen seconds and 2) Racine score of the seizure had to be greater than or equal to 3. 138 induced seizures from 10 animals fit these criteria. As shown in **Fig. 3C**, we used a linear mixed effect model to compare the changes in the band power, line length, and area of each segment to the baseline values. In the beginning third, the extent of increase across many features significantly differed between the induced and the spontaneous seizures. Specifically, the increase in area was significantly higher for induced seizures ($p < 1e^{-4}$), as were the increases in line length ($p < 1e^{-4}$), 1 – 30 Hz band power ($p < 1e^{-4}$), 30 – 300 Hz band power ($p < 1e^{-4}$), and 300 – 1000 Hz band power ($p < 1e^{-4}$). By the middle third, significant differences remained in the 1 – 30 Hz band power ($p < 1e^{-4}$) and 30 – 300 Hz band power ($p < 1e^{-4}$). However, the relationship was now inverted – the 1 – 300 Hz band power increased more for spontaneous seizures than for induced seizures. The changes in other features, such as 300 – 1000 Hz band power ($p = 0.3986$), line length (0.7769), and area ($p = 0.2223$), were no longer statistically significant. In the final third, the increase in area ($p = 0.0354$) and 30 – 300 Hz band power ($p = 0.0225$) were slightly significant. The increase in 1 – 30 Hz band power was no longer significantly different ($p = 0.083$), and the increases in 300 – 1000 Hz band power ($p = 0.3368$) and line length ($p = 0.1376$) remained not significant.

To further quantify the similarity between spontaneous and induced seizures, we trained individual two-class support vector machine (SVM) classifier for every animal. The goal of the SVM was to classify whether the induced activity was more alike to spontaneous seizures or baseline signal. A sample classification output for one animal is shown in **Fig. 3D**. In this animal, all optogenetic activations that resulted in a minimum afterdischarge length of 5 s were classified as spontaneous seizures by the classifier. Meanwhile, activations that failed to induce seizures or caused afterdischarges lasting less than 5 s were classified as baseline. When we integrated the results from all 8 SVM classifiers, we found that activations that induced afterdischarges lasting longer than 5 s were classified as spontaneous seizures 88.1 % of the time, whereas 11.9 % of the time they were inaccurately classified as baseline. Meanwhile, activations that failed to induce afterdischarges longer than 5 s were classified as baseline 100 % of the time (**Fig. 3E**). Taken together, the results show that synchronizing CA1 principal cell activity in epileptic mice generated seizures that resembled naturally occurring spontaneous seizures, both behaviorally and on EEG.

Induced activity significantly differs between epileptic and naïve animals

During epileptogenesis, neural networks in the brain undergo various changes ranging from the modification of membrane receptors to the formation of new synapses (37–39). We hypothesized that these changes are critical for successful on-demand seizure induction. To test our hypothesis, we attempted to optically induce seizures in naïve, wild type Thy1-ChR2 mice and compared the induced activity in naïve mice to the induced activity in epileptic mice.

Initial seizure provocations in naïve animals ($n = 7$) typically caused low frequency, high amplitude spiking activity (**Fig. 4A**). While long lasting, these afterdischarges did not result in a behavioral manifestation. Animals displayed independent movement, exploration, or grooming during the afterdischarges (Fig. S8A). Behavioral differences between initial naïve and epileptic inductions did not result from changes in the seizure induction parameters – the naïve threshold laser power (6.17 ± 1.58 mW) and stimulation duration (5.67 ± 1.03 s) both did not significantly differ from that of the epileptic animals ($p > 0.05$) (Fig. S2A, S2B). Rather, the observed behavior in the naïve animals was likely due to a lack of etiologically relevant changes in the healthy brain.

After 4 consecutive days of up to 5 optogenetic activations per day at a frequency of 1 per hour, we noticed that applying the same optogenetic stimulus resulted in visibly more higher frequency activity and bursting (**Fig. 4B**). In addition, all 7 animals displayed stereotypical behavioral signs of seizure, including shaking, tail stiffening, rearing, wild running, and uncontrolled jumping (Fig. S8B). The increased likelihood of behavioral seizures in later inductions is reminiscent of the ‘kindling model’ of epilepsy, where repeated administration of subthreshold electrical stimulation eventually causes the animal to enter a ‘kindled’ state. In the kindled state, the previously subthreshold electrical stimulation can induce seizures (40).

To visualize the ‘kindling effect’ of the optogenetic stimulation, we evaluated the rate at which both electrographic activity and seizure-like behaviors were induced over 10 experimental days (**Fig. 4C** top). The rate of inducing an electrographic event was defined to be the percentage of times the activation stimulus induced afterdischarges lasting a minimum of 5 s. A two-sided pairwise t test determined that, on all 10 days, the rate of inducing an electrographic event did not significantly differ between the epileptic and naïve animals ($p > 0.05$). After integrating data across all stimulation days, it was determined that the overall rate of inducing an electrographic event in naïve animals was approximately 86.0 ± 6.0 %, which also did not significantly differ from that of the epileptic animals ($p = 0.8506$, Fig. S2C). However, the average duration of induced activity in naïve animals was 20.87 ± 2.19 s, which was significantly shorter than the average duration of induced activity in epileptic animals (30.98 ± 4.69 s, $p = 0.0005$, Fig. S2D).

We next analyzed the percentage of electrographic events that exhibited a behavioral manifestation. This comparison between naïve and epileptic animals included behaviors such as visible forelimb clonus, uncontrolled shaking, tail stiffening, uncontrolled rearing, wild running, loss of righting reflex, and jumping (**Fig. 4C** middle). We did not include behaviors in which the animal retained control over its body, such as exploring or grooming. A two-sided pairwise t test found that the percentage of electrographic events with a behavioral seizure manifestation significantly differed between the naïve and epileptic animals on the first ($p = 0.0056$), second ($p < 1e^{-4}$), and third ($p = 0.0088$) stimulation day. No significant difference in behavioral seizure manifestations was found on stimulation day 4 or later ($p > 0.05$). To further quantify the behavioral differences, we compared the Racine seizure score for the induced activity (**Fig. 4C** bottom). A two-sided pairwise t test found that the average Racine seizure score significantly differed between the naïve and the epileptic animals on the first ($p = 0.0087$), second ($p < 1e^{-4}$), third ($p < 1e^{-4}$), and fourth ($p = 0.024$) stimulation day. No significant difference in Racine seizure score was found on stimulation day 5 or later ($p > 0.05$).

Later activations and their differing behavior indicated that the animal entered a distinct state after multiple days of optical activation. Thus, we decided to perform separate feature space comparisons of the electrographic activity between naïve animals and epileptic animals for the initial activations (day 1 – 4) and the later activations (day 5 +) (**Fig. 4D**). To standardize the comparison between naïve and epileptic induced activity, a linear mixed effect model with the animal set as the random effect was used to evaluate the changes in feature values from the baseline segments to the beginning, middle, and ending terciles of the induced activity. The terciles of the 308 inductions in naïve animals were computationally determined by the same k -nearest neighbor classifier used in epileptic animals. To reduce noise from shorter events, only induced

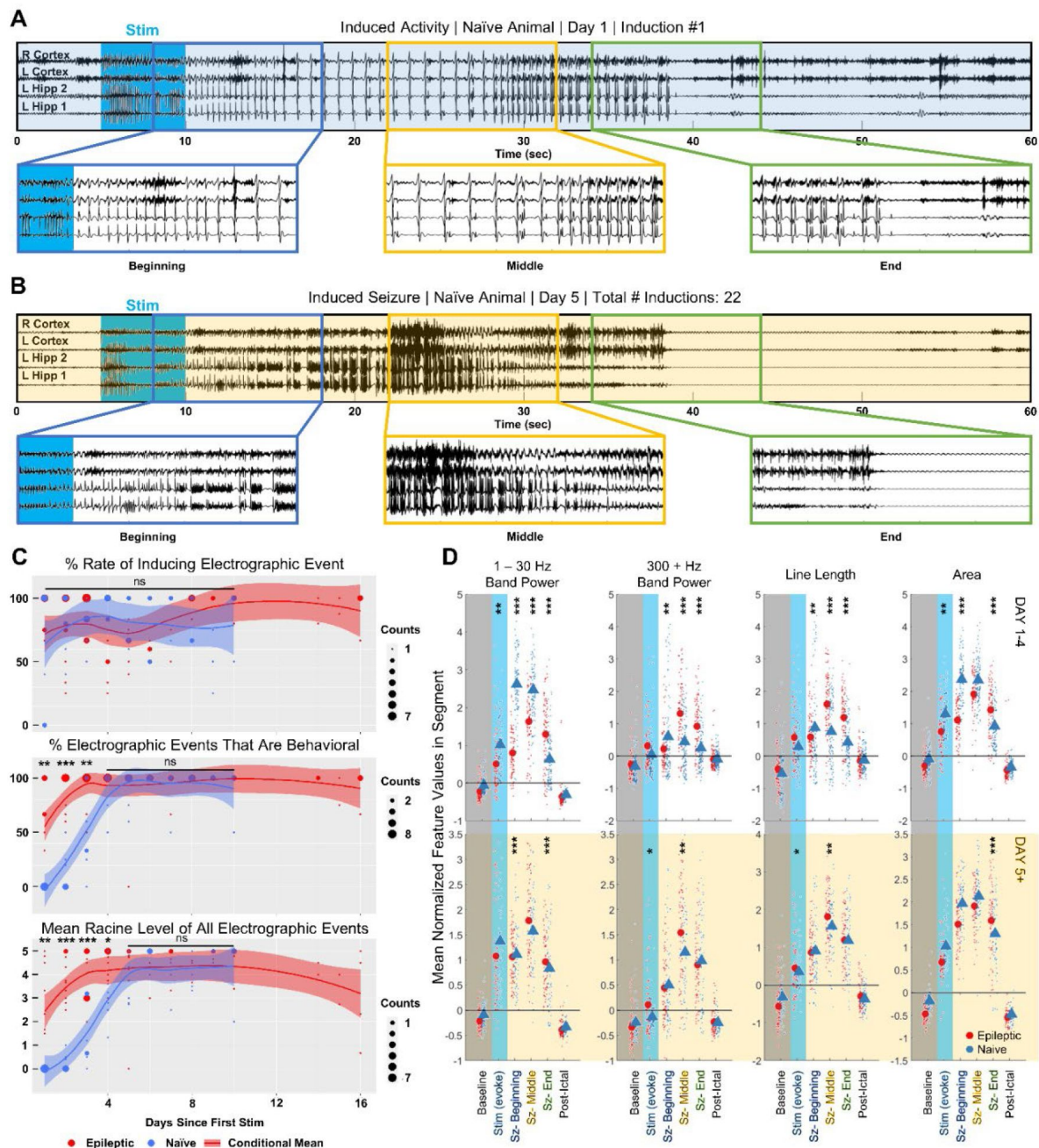


Figure 4

Inducing seizures in epileptic animals differs from optical kindling in naïve animals.

(A) Initial optogenetic activations in naïve animals induced low frequency afterdischarges. Representative EEG trace with 3 segments of 10 s at the start, middle, and end of seizure enlarged. (B) Following multiple days of stimulation, application of activation stimulus in the same animal induced Racine 5 seizures. (C) Rate of electrographic afterdischarges from optogenetic activation did not significantly differ between naïve and epileptic animals. Percent of behavioral electrographic events significantly differed between naïve and epileptic animals on stimulation day 1 through 3. Average Racine score of electrographic inductions significantly differed between naïve and epileptic animals on stimulation day 1 through 4. Pairwise T test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) In the first 4 days of stimulation, inductions in naïve (100 inductions, $n = 7$) and epileptic animals (87 inductions, $n = 10$) significantly differed in the feature space. Differences were reduced, but still existed, on stimulation day 5 or later (epileptic – 75 inductions, $n = 7$; naïve – 58 inductions, $n = 7$). Linear Mixed Effect Model: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

activity lasting more than 15 s were compared. The initial activations group was composed of 100 events from 7 naïve animals and 87 events from 10 epileptic animals. The later activations group was composed of 75 events from 7 epileptic animals and 58 events from 7 naïve animals.

Consistent with visual observation of initial activations, increases in the electrographic features versus baseline significantly differed between naïve and epileptic animals (**Fig. 4D** top). Overall, naïve induced activity tended to have higher area and more low frequency band power while epileptic induced activity tended to have more high frequency band power and higher signal complexity. The linear mixed effect model found significant differences in the increase of 1 – 30 Hz band power from baseline to the beginning third ($p < 1e^{-4}$), middle third ($p < 1e^{-4}$), and final third ($p < 1e^{-4}$). The difference in the 300 – 1000 Hz band power from baseline was also significant between the two groups at the beginning third ($p = 0.002$), middle third ($p < 1e^{-4}$), and final third ($p < 1e^{-4}$). A similar case existed for line length at the beginning third ($p = 0.008$), middle third ($p < 1e^{-4}$), and final third ($p < 1e^{-4}$) and for area at the beginning third ($p < 1e^{-4}$) and final third ($p < 1e^{-4}$). The increase in area from baseline to the middle third was barely insignificant ($p = 0.059$).

Differences in the feature space were reduced in later activations (**Fig. 4D** bottom). The differences in the increase of the 1 – 30 Hz band power between the two groups were reduced in the beginning third ($p = 0.0002$), middle third ($p = 0.051$) and final third ($p < 1e^{-4}$). The same applied to 300 – 1000 Hz band power (beginning third $p = 0.60$, middle third $p = 0.029$, final third $p = 0.097$), line length (beginning third $p = 0.21$, middle third $p = 0.002$, final third $p = 0.11$), and area (beginning third $p = 0.15$, middle third $p = 0.56$, final third $p < 1e^{-4}$). Despite reductions between feature values in the later activations group, not all significant differences between the naïve and epileptic animals were eliminated. The persistent differences highlighted by the linear mixed effect model show the importance of evaluating novel ASMs in epileptic animals.

Common anti-seizure medications could suppress induction of electrographic and behavioral seizures

To determine the utility of the induced seizure model for evaluating ASMs, we tested whether FDA approved and commonly prescribed ASMs, such as diazepam and levetiracetam (41), could reduce the rates of both electrographic discharge induction and behavioral seizure induction in epileptic animals. Diazepam, a benzodiazepine that interacts with GABA receptors, is commonly prescribed for first line control of convulsive seizures (42), whereas levetiracetam is an anticonvulsant that reduces overall network excitability through effects mediated by the synaptic protein SV2A (43). The experimental paradigm is shown in **Fig. 5A**. Prior to inducing seizures with the optogenetic stimulus, the baseline spontaneous seizure frequency was recorded in 9 epileptic animals. The next 2 – 5 days were used for determining and validating the consistency of the threshold stimulus. On the first day of ASM testing, optogenetic activation was performed hourly to establish the drug-free seizure induction success rate. Then, the animal received either a single intraperitoneal injection of 800 mg/kg levetiracetam or a single subcutaneous injection of 5 mg/kg of diazepam. The activation stimulus was applied to the animal approximately 10 minutes after ASM administration and was re-applied hourly. A washout period of 48 hours, if applicable, occurred between subsequent ASM administrations. After completion of drug testing, animals underwent a post-stimulation recording period of up to 7 days.

Application of diazepam significantly reduced the rates of electrographic discharge and behavioral seizure induction in 6 epileptic animals over 8 testing days (**Fig. 5B**). Typically, after diazepam administration, optogenetic activation did not induce electrographic activity. Even when afterdischarges were successfully induced, animals typically did not display seizure-like behaviors. Integrating data from all animals, prior to drug administration, electrographic discharge induction rate averaged 86.77 ± 22.3 % and the behavioral seizure induction rate averaged 83.65 ± 21.9 %. The paired one-tailed Wilcoxon signed rank test found that after drug administration, the electrographic discharge induction rate was significantly reduced to $44.17 \pm$

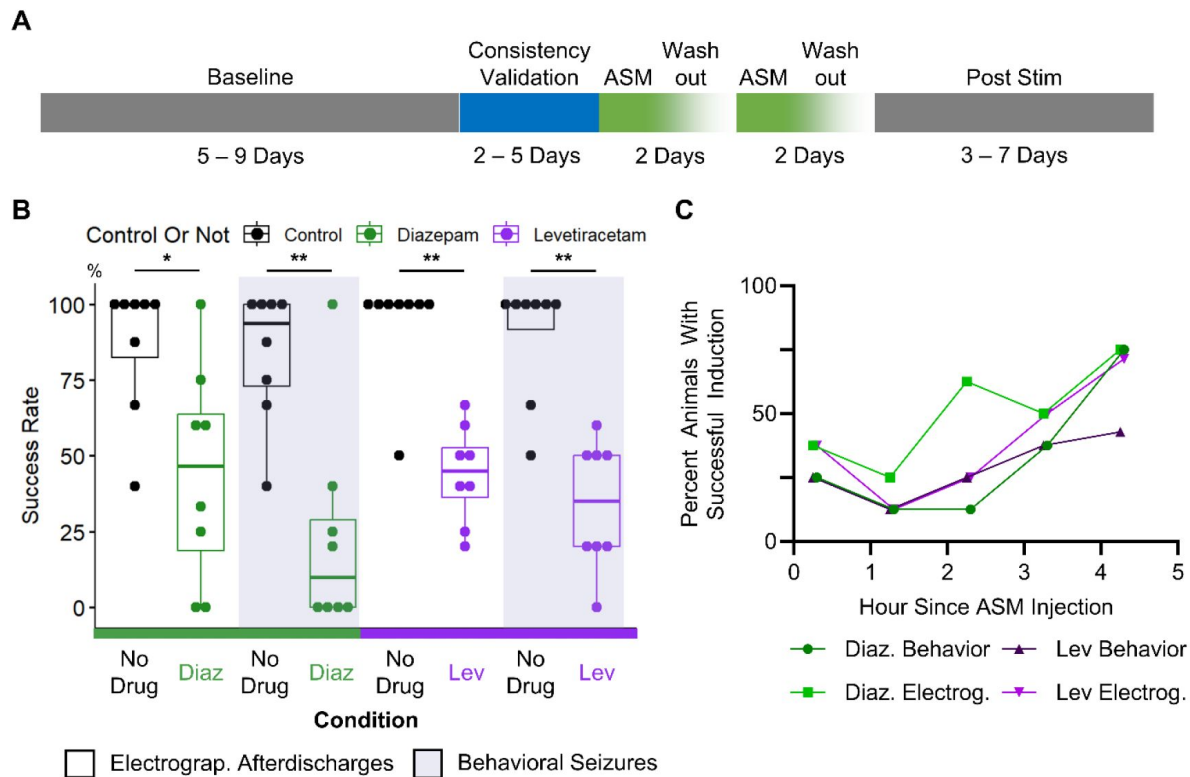


Figure 5

Induced seizures in epileptic animals responded to both diazepam and levetiracetam.

(A) Experimental timeline for testing ASM efficacy in on-demand seizure model. Stimulus for seizure induction was tested for consistency before ASM application. A 48-hour washout occurred between subsequent ASM applications. **(B)** Post ASM application, rates of inducing electrographic afterdischarges and behavioral seizures were significantly reduced. Paired One Tailed Wilcoxon Signed Rank Test, * $p < 0.05$, ** $p < 0.01$ **(C)** Probability of successful induction of activity (averaged across all epileptic animals) increased the more time has passed since ASM injection into the mouse.

35.8 % ($p = 0.016$) and the behavioral seizure induction rate was significantly reduced to $23.12 \pm 34.5\%$ ($p = 0.008$). One epileptic animal was a diazepam non-responder. Excluding the diazepam non-responder, no tonic-clonic seizures (Racine > 3) were induced 3 hours after drug administration in any of the 5 epileptic animals.

Application of levetiracetam significantly reduced the rates of electrographic discharge and behavioral seizure induction in 5 epileptic animals over 8 testing days (**Fig. 5B**). After levetiracetam administration, electrographic inductions were typically unsuccessful. In mice, levetiracetam has a short half-life of approximately 3.2 hours (44). In our study approximately 4 hours after the injection, tonic-clonic behavioral seizures (Racine > 3) were successfully induced in 2 out of 5 epileptic animals. Integrating data from all animals, prior to drug administration, average electrographic discharge induction rate was $93.75 \pm 17.7\%$ and the average behavioral seizure induction rate was $89.58 \pm 19.8\%$. A paired one-tailed Wilcoxon signed sum test found that after drug administration, electrographic discharge induction rate was significantly reduced to $43.96 \pm 16.1\%$ ($p = 0.004$), and the behavioral seizure induction rate was significantly reduced to $33.75 \pm 21.3\%$ ($p = 0.004$).

Quantifying the seizure induction rates after drug administration by hour showed that both drugs had maximal effect between the first and the second hour (**Fig. 5C**). As the drug was metabolized or excreted, the rate of successful seizure induction increased, eventually approximating 75% of all animals by the fourth hour. This was expected, as seizure induction rates should gradually return to pre-drug levels as the drug was cleared from the animal body. Taken together, these results showed that the induced seizure model has potential for evaluating ASM efficacy in preventing seizures and guiding calculations on ASM pharmacokinetics.

Discussion

In this study, we showed that selective activation of CA1 principal cells could induce on-demand seizures in both chronically epileptic and naïve animals. Induced seizures were reliably generated, reproducible, and bore striking similarities to spontaneously occurring seizures. Induced activity differed between epileptic animals and naïve animals, indicating that the epileptic network is critically different from that of the healthy brain. Finally, induced seizures could be suppressed with standard ASMs, proving the model has utility for assessing the effectiveness of potential therapies.

Seizure induction takes advantage of mechanistic changes in the hippocampus

The seizure induction procedure took advantage of naturally occurring changes in the epileptic brain to elicit seizures on demand, suggesting that hyperexcitation of principal cells could be a mechanism by which activity propagates out from the hippocampus and generalizes into behavioral seizures. The profound difference in the induced activity between naïve and epileptic animals further suggested that the epileptic circuit is a key facilitator of seizure generalization – only in the epileptic circuit could we provoke behavioral seizures from stimulation day 1.

Previous research suggests that changes in the CA1 during epileptogenesis contributes to increased bursting activity in chronically epileptic animals but stops short of establishing a causal relationship between the hyperactivity and generalized seizures (26, 28, 45, 46). In the pilocarpine model of chronic epilepsy, CA1 principal cells in epileptic animals were prone to firing synchronous bursts of spikes when excited with inputs that induced only single spikes in naïve animals (47). Inputs to the CA1 also change during epileptogenesis. The temporoammonic pathway, a direct connection from the entorhinal cortex to the CA1, switched from being a highly regulated, weak excitatory input in naïve animals to a powerful excitatory pathway in epileptic

animals (48 [↗](#), 49 [↗](#)). Electrical activation of the temporoammonic pathway was also sufficient to generate bursting activity in chronically epileptic brain slices (49 [↗](#)). Despite these pioneering studies, there was no direct investigation into whether seizures in the epileptic brain can initiate purely from CA1 hyperexcitation. In our work, we answer this question by showing that we can harness CA1 principal cells to induce generalized seizures on demand. We also show that these seizures are similar to spontaneous seizures in the EEG feature space; further suggesting that, in epileptic animals, this pathway is potentially active in seizure generation.

Behaviorally, induced seizures resembled tonic-clonic seizures in human patients (50 [↗](#)). Induced seizures were characterized by an initial tonic phase, where the dominant behavior was freezing, and the EEG showed continuous fast spiking activity. The tonic phase is followed by a clonic phase, where the animal displayed uncontrolled movements, such as backpedaling, rearing, and jumping. During the clonic phase, we observed clearly defined bursting and large amplitude single spikes. The gross similarity in the EEG and the behavior posits a question – could CA1 hyperexcitation be one of the mechanisms by which tonic-clonic seizures naturally start? Further studies with this model could answer questions about the initiation and the termination of such seizures, with clinical implications in both the epidemiology and the treatment of medication resistant seizures.

Application of model to testing multiple classes of ASMs, closed loop neuromodulation therapies, and studying cellular interactions

In this work, we found that seizures induced in epileptic animals could be blocked by applying two commonly used ASMs – diazepam and levetiracetam. These two medications were chosen because they act through different mechanisms – diazepam enhances GABA receptor efficacy and levetiracetam disrupts synaptic transmission (42 [↗](#), 43 [↗](#)). Diazepam historically performs well in many seizure models. However, levetiracetam is unique among many ASMs as it has little to no effect in induced seizure models such as the acute maximal electroshock seizure test and the maximal pentylenetetrazol seizure test. In our model, levetiracetam had a clear anti-seizure effect. We believe that the etiological relevance of our model allows levetiracetam to exert its effect on synaptic transmission and prevent seizure generalization. These experiments show that our induced seizure model could be used for testing multiple classes of ASMs.

Outside of pharmacologics, our model could be applied to evaluating time-dependent, closed loop treatment paradigms. The model allows for precise timing of seizure onset; thus, experimenters could rapidly test the effects of both open and closed loop electrical stimulation on seizure initiation, propagation, and termination. We envision this model to enable quick advances in determining optimal targets and tuning parameters for closed loop stimulation. There is an urgent need to explore the parameter space for closed loop seizure control, as the multitude of options makes it difficult to quickly optimize responsive neurostimulators for maximal seizure control (51 [↗](#)). A reliable, physiologically realistic on-demand seizure model could dramatically accelerate this process.

Alternatively, our model could be applied to evaluate circuit specific intervention strategies using cell-specific optogenetic or chemogenetic approaches (52 [↗](#)). By evaluating seizure induction rate while suppressing or activating certain neural populations, one can quickly assess the potential of the circuit specific intervention strategy to control seizures. Our experiments suggest that interfering with activity from the CA1 region may be one way to stop seizure progression. Diazepam and levetiracetam both reduced the likelihood of induced activity generalizing beyond the hippocampus, resulting in lower rates of electrographic activity induction and lower rates of behavioral seizure induction. As the CA1 is the main output of the hippocampus, deciphering ways to prevent hippocampal activity from generalizing could be impactful for clinical care. If abnormal hippocampal outputs could not leave the hippocampus, patients with seizures that originate in the hippocampus might not experience the debilitating effects of grand mal seizures.

Comparison to other seizure models used in pharmacologic screening

Other models that acutely induce seizures exist, but they either do not use chronically epileptic animals or do not have as rapid onset as the one presented in this work (29 [↗](#)). Some models utilize a kindling paradigm, requiring multiple days of successive activation before seizures can be reliably induced (40 [↗](#)). Other approaches are performed under anesthesia (53 [↗](#)) or utilize naïve animals (54 [↗](#)), both of which could introduce differences in neural activity when compared to studies in freely moving epileptic animals. Overall, prior studies we reviewed do not sufficiently address the challenges of efficiently evaluating epilepsy treatments in etiologically relevant models.

Conversely, our model allows for more robust powering of studies in etiologically relevant models. We target a potential seizure mechanism in the CA1 to reliably elicit seizures hourly in freely moving animals. Our model also generates both long-lasting afterdischarges and slow-onset tonic-clonic seizures. This is unlike models that rely on continuous stimulation throughout the seizure to generate behavioral events. In our model, ictal activity naturally propagates out of the hippocampus and evolves into generalized tonic-clonic seizures. Thus, seizures from our model are likely to be highly relevant for testing clinical treatments and developing novel approaches for epilepsy.

Long term, we envision this model could be integrated into a drug screening pipeline such as the Epilepsy Therapy Screening Program (ETSP) (8 [↗](#)). Currently, the ETSP utilizes a mixture of models, including acutely induced seizures in naïve animals and spontaneous recurrent seizures in chronically epileptic, IHK rodents. An intermediate step in the ETSP evaluates how ASMs affect hippocampal paroxysmal discharges (HPDs), which are frequent non-behavioral, focal electrographic seizures, to quickly produce dose-response curves with high confidence. However, HPDs are not generalizable across species – they are specific to the mouse model (55 [↗](#)). In addition, it is unclear whether medications that prevent HPDs would also be effective against generalized convulsive seizures or partial non-convulsive seizures in humans.

In line with modern medicine's aim to develop etiology-specific drugs as part of a precision medicine approach, it is crucial to incorporate evaluation of convulsive seizures into the screening process to discover drugs that can effectively stop these seizures. Our model is well positioned to supplant HPDs in the existing pipeline. On demand seizures allow for faster identification of new candidate ASMs and serve as an initial check as to whether the drugs' actions are sustained on seizures in the epileptic brain. However, prior to integration, additional scaling of the model and validation with more ASMs are needed. These experiments could better characterize the model and help determine whether the model can serve as a testbed for pharmacologics that specifically target refractory, drug resistant seizures.

In all, we present a biologically relevant, higher throughput on-demand seizure model that can be used to evaluate novel pharmacologics and time-sensitive treatment paradigms. This model takes advantage of a biologically relevant mechanism to generate long-lasting afterdischarges and slow-onset tonic-clonic seizures. With this model, we envision immediately increasing the speed of discovering and evaluating new clinical treatments for epilepsy and the identification of new mechanisms behind the initiation of tonic-clonic seizures.

Materials and methods

Study Design

The purpose of this study was to create a higher throughput model for evaluating ASMs in the diseased, epileptic brain. To do so, we tested whether specific optical activation of CA1 principal cells could induce generalized behavioral seizures in epileptic animals. Next, we compared these induced seizures to spontaneous seizures to establish the model's face validity. To show that successful induction was dependent on changes in the epileptic brain, we attempted to provoke seizures in naïve animals. Comparing the induced activity between naïve and epileptic animals allowed us to establish the model's construct validity. Finally, we attempted to use known ASMs to stop seizures. Diazepam and levetiracetam reduced likelihood of seizure induction, establishing that our model has predictive validity. Blinding was not utilized in this study.

Animal Experiments

All procedures are approved in accordance with the lab's Institutional Animal Care and Use Committee (IACUC) protocol at the Children's Hospital of Philadelphia. More details in supplemental materials and methods.

Epilepsy Induction

C57BL/6J-Thy1-ChR2-YFP mice (Jax #007612) underwent intrahippocampal injection of 50 nL, 20 mM kainic acid (Hello Bio) under anesthesia (coordinates: right CA3 AP – 2.7, ML + 3.0, DV – 3.2, right CA1 AP – 2.0, ML + 1.6, DV – 1.6). After onset of status epilepticus, 5 mg/kg diazepam was administered to reduce severity of seizures. Animals that died from epilepsy induction were excluded.

EEG and Fiber Implantation

Animals were implanted with A-M Systems Model 1700 adapters connected by wire to two cortical screws (AP + 0.5, ML $\pm$ 1.7), two cerebellar screws (AP – 5.5, ML $\pm$ 2.0, ground and reference), and a pair of twisted hippocampal depth wires (AP – 2.0, ML – 1.7, DV – 1.6). 400-micron diameter optic fiber cannula (RWD Life Science) was positioned at 2.0 mm (AP – 2.0, ML + 1.7) depth illuminating the damaged CA1. Dental cement (Lang Dental) secured the entire apparatus.

Video EEG and Optogenetic Stimulus

Animals were placed into a Plexiglass cage and connected to a Stellate Harmonie (Natus) recording system. A Master 8 controller that was set to 10 Hz, 25 ms pulses was used to control delivery of light from a Laserglow 473 nm laser. Laser power and stimulation duration depicted in Figs. S2A and S2B. Stimulation was performed every 1 – 3 hours over many days. Animals that had no light response at all – not even a stimulation artifact – were excluded.

Pharmaceuticals

On each testing day, a few optogenetic activations occurred prior to drug injection to determine the pre-drug induction success rate. Animals then received either one subcutaneous injection of 5 mg/kg diazepam (Dash Pharmaceuticals) or an intraperitoneal injection of 800 mg/kg levetiracetam (Sigma Aldrich). Up to six more activations occurred every 1 – 1.5 hours following ASM administration. One tailed Wilcoxon signed rank test was used to compare success rate before and after ASM administration.

Perfusion and Histology

Animals were deeply anesthetized and transcardially perfused with PBS and fixed in 4% paraformaldehyde in PBS. Cryoprotected brains were sliced and then stained with 0.1% cresyl violet (Sigma Aldrich) using the Nissl staining protocol. Slices were imaged on a Leica DMIRB microscope using the LasX software.

Statistical Analysis & Computational Processing

All code is available on Github.com at the following repository: https://github.com/yuzhangc/Evoked_Seizures

A. Preprocessing

EEG was filtered with a second order notch filter to remove 60 Hz line noise. The signal was then z-score normalized. A 4 Hz 6th order Butterworth high pass filter subsequently removed low frequency artifacts.

B. Feature Calculation

Features were extracted from 500 ms windows with 250 ms displacement. Full formula in supplements. Features calculated include line length, area, energy, zero crossing around mean, root mean squared amplitude, skewness, approximate entropy, Lyapunov exponent, phase locked high gamma, magnitude squared coherence, mean absolute deviation, and band power at three frequency ranges (1 – 30 Hz, 30 – 300 Hz, 300 + Hz). Features were z-score normalized for machine learning.

C. Induced Activity Length Determination and Calculation of Thirds

An unsupervised K nearest neighbor model was trained with z-score normalized input from one spontaneous seizure. The model had three output classes – two were classified as ‘seizure’ and the last one was ‘baseline.’ The model was then used to determine induced activity length across all events from all animals. Two tailed Wilcoxon rank sum test and/or two-sided pairwise t test was used to compare induction success rates. Wilcoxon rank sum test was used to compare the threshold duration and the threshold power distribution of the naïve and epileptic animals.

Features calculated in Section B were then segregated into 1 of 6 time points based on the length of induced activity: 1) before stimulation, 2) during stimulation, 3) initial/beginning third, 4) second/middle third, 5) final/ending third, and 6) post stimulation (30 seconds). Linear mixed effect models (using the lmer function in lmerTest package) was used to determine how the comparison (such as epileptic vs naïve or spontaneous vs induced) influenced the EEG features over time points.

D. Spontaneous Seizure Detection and Video EEG Behavioral Scoring

Spontaneous seizures were detected using custom-written Matlab code (56). Comparisons on daily spontaneous seizure frequency used a two tailed Mann Whitney test. Uncontrolled behaviors and the Racine scale (57) associated with the behaviors are as follows: (1) – clear freezing / flattening of the body, (2) – tail stiffening, forelimb clonus, uncontrolled shaking, (3) – rearing, (4) – wild run, and (5) – uncontrolled jumping, loss of righting reflex. If no behavior or movement was displayed, the score was 0. Behavioral manifestation was compared with a two sided pairwise t test.

E. Support Vector Machines

A new support vector machine for classifying induced activity was trained per animal. EEG features in (B) were calculated for baseline segments for training unique SVM classifiers. The baseline features were combined with spontaneous seizure features to form training inputs. Testing data consisted of features from induced activity. Accuracy and error rates were calculated based on ground truth, where a successful induction was defined as an electrographic event with afterdischarges lasting a minimum of 5 seconds.

Data and materials availability

Code is available on Github at the following repository: https://github.com/yuzhangc/Evoked_Seizures. Raw data and video clips are available upon request by contacting the corresponding author.

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Funding acquisition: BL, FV, HT

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Competing interests

Authors declare that they have no competing interests.

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Reviewer #1 (Public review):

Summary:

This important study by Takano et. al. describes a novel approach for optogenetically evoking seizures in an etiologically relevant mouse model of epilepsy. The authors developed a model that can trigger seizures "on demand" using optogenetic stimulation of CA1 principal cells in mice rendered epileptic by an intra-hippocampal kainate (IHK) injection into CA3. The authors discuss their model in the context of the limitations of current animal models used in epilepsy drug development. In particular, their model addresses concerns regarding existing models where testing typically involves inducing acute seizures in healthy animals or waiting on infrequent, spontaneous seizures in epileptic animals.

Strengths:

A strength of this manuscript is that this approach may facilitate the evaluation of novel therapeutics since these evoked seizures are demonstrated as being sufficiently similar to spontaneous seizures in these same mice which are more laborious to analyze. The data demonstrating the commonality of pharmacology and EEG features between evoked seizures and spontaneous seizures in epileptic mice, while also being different from evoked seizures in naïve mice, are convincing despite concerns regarding the biological significance of the differences in effect sizes of these features. The structural, functional, and behavioral differences between a seizure-naïve and epileptic mouse are complex and important issues. This study positively impacts the wider epilepsy research community by investigating seizure semiology and pharmaceutical responses in these populations.

Weaknesses:

While the data generally supports the authors' conclusions, a weakness of this manuscript lies in their analytical approach where EEG feature-space comparisons used the number of spontaneous or evoked seizures as their replicates as opposed to the number of IHK mice; these large data sets tend to identify relatively small effects of uncertain biological significance as being highly statistically significant. Furthermore, the clinical relevance of similarly small differences in EEG feature space measurements between seizure-naïve and epileptic mice is also uncertain. Finally, the multiple surgeries and long timetable to generate these mice may limit the value compared to existing models in drug-testing paradigms.

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Reviewer #2 (Public review):

Summary:

The authors have attempted to modify and adapt the IH-KA model in mice to provide an improved approach to screening for new ASDs by partially mitigating the problem of randomly occurring seizures and relatively low seizure frequency in the IH-KA model. The authors used KA micro-injections to selectively kill the hippocampal CA3 area as a way to induce temporal lobe "epileptogenesis" (TLE), and then used optogenetics to activate CA1 pyramidal cells specifically. This approach allowed the authors to trigger generalized seizures where the tonic-clonic pattern of electrical activity was reminiscent of actual tonic-clonic behavioral convulsions. Administration of levitracetam (LEV) and diazepam (DZP), two widely used ASDs with different mechanisms, reduced the probability of optogenetically activated epileptic seizures in IH-KA mice, thus seeming to provide evidence for a new approach to screen ASDs. A variety of problems and issues with the approach and the results lead to confounds that raise serious concerns about the conclusions.

Major strengths and weaknesses of the Methods and Results:

Strengths:

The authors have designed a method for triggering seizures, and the figures show bona fide electrographic seizures with concomitant convulsive behavioral components. The optogenetically evoked seizures in IH-KA mice had the electrical properties of actual seizures and the tonic-clonic components were readily apparent. These seizures appeared different from seizures evoked in naïve mice, and the authors attribute this difference to the epileptogenic process, but this may not be correct.

The ASDs (i.e., LEV and DZP) reduced the success rate of the optogenetically evoked seizures in IH-KA mice, thus suggesting the potential usefulness of the model for testing ASDs. The paper discusses whether the Epilepsy Therapy Screening Program (ETSP) will be able to use this modification of the IH-KA model in place of (1) ASD screening with acute seizures in naïve animals, where the brain has not undergone "epileptogenesis", (2) testing ASDs on hippocampal paroxysmal discharges (HPDs) in the IH-KA model, which has undergone epileptogenesis, or (3) spontaneous epileptic seizures in animal models of TLE based on systemic treatments that lead to acute convulsive status epilepticus that have later undergone epileptogenesis. This proposed version of the IH-KA model aims to address the former problem (#1, above) by using a mouse model of TLE, and to address the latter problems (#2 and #3, above) of the seemingly random occurrence of epileptic seizures and the low seizure frequency by using optogenetically "triggered" seizures.

Weaknesses

Although the figures provide excellent examples of individual electrographic seizures and compare induced seizures in epileptic and naïve animals, it is unclear which criteria were used to identify an actual seizure induced by the optogenetic stimulus, versus a hippocampal paroxysmal discharge (HPD), an "afterdischarge", an "electrophysiological epileptiform event" (EEE, Ref #36, D'Ambrosio et al., 2010 Epilepsy Currents), or a so-called "spike-wave-discharge" (SWD). Were HPDs or these other non-seizure events ever induced using stimulation in animals with IH-KA? A critical issue is that these other electrical events are not actual seizures, and it is unclear whether they were included in the column showing data on "electrographic afterdischarges" in Figure 5 for the studies on ASDs. This seems to be a problem in other areas of the paper, also.

The differences between the optogenetically evoked seizures in IH-KA vs naïve mice are interpreted to be due to the "epileptogenesis" that had occurred, but the lesion from the KA-induced injury would be expected to cause differences in the electrically and behaviorally recorded seizures - even if epileptogenesis had not occurred. This is not adequately addressed.

The authors did not test whether an apparent "kindling" effect, apparently seen in naïve controls, also occurred in animals micro-injected with kainic acid (KA). This effect could cause model instability that might result in variability in response to ASDs. It is not clear whether the number of optogenetically induced seizures in epileptic animals would affect the response to drugs. It is also unclear how much of an improvement the animal model in the present work is over other similar models of TLE, where electrically triggered seizures could simply be applied to one of them.

The authors offer little mention of other research using animal models of TLE to screen ASDs, of which there are many published studies - many of them with other strengths and/or weaknesses. For example, although Grabenstatter and Dudek (2019, Epilepsia) used a version of the systemic KA model to obtain dose-response data on the effects of carbamazepine on spontaneous seizures, that work required use of KA-treated rats selected to have very high rates of spontaneous seizures, which requires careful and tedious selection of animals. The ETSP has published studies with an intra-amygdala kainic acid (IA-KA) model (West et al., 2022, Exp Neurol), where the authors claim that they can use spontaneous seizures to identify ASDs for DRE; however, their lack of a drug effect of carbamazepine may have been a false negative secondary to low seizure rates. The approach described in this paper may help with confounds caused by low or variable seizure rates. These types of issues should be discussed, along with others.

While the paper may be relevant for the ETSP and contract research organizations (CROs), the paper was not written to attract the interest of biological scientists, even those in this specific

area of epilepsy research. It may be of low interest to other neuroscientists.

The outcome measure for testing LEV and DZP on seizures was essentially the fraction of unsuccessful or successful activations of seizures, where high ASD efficacy is based on showing that the optogenetic stimulation causes fewer seizures when the drug is present. The final outcome measure is thus a percentage, which would still lead to a large number of tests to be assured of adequate statistical power. Thus, there is a concern about whether this proposed approach will have high enough resolution to be more useful than conventional screening methods so that one can obtain actual dose-response data on ASDs.

The key issue the authors aim to address is the 30-40% of patients with DRE, but the real problem with DRE patients is not that these people have seizures with no effect of the ASDs; rather, although ASD may reduce seizure burden, these patients continue to have some remaining seizures even after high doses of ASDs, which often leads to adverse effects from the particular ASDs.

In several sections of the paper, the authors argue that two different groups are similar on the basis that no statistical difference was found between the two groups (i.e., $p > 0.05$); however, the failure to find a statistically significant difference, particularly with relatively small sample sizes, is not rigorous evidence that the two groups are actually similar - they are just "not significantly different."

It remains unclear that the optogenetically induced seizures in this model are better than similarly induced seizures in a naïve animal, and there is no evidence that the model will be useful for finding new ASDs to treat DRE.

Do the results support the conclusions?

Although the Results show examples of clear tonic-clonic seizures, it is not at all clear whether this approach is a significant improvement over previous methods used on animal models of TLE. The presented data from this method shows it provides an ability to detect the effect of widely used ASDs, but not that it will have the resolution to find better ASDs. The outcome measure of successful vs failed seizure inductions does not necessarily translate to a pathway for finding new ASDs for DRE, which often is a function of the side effects of the proposed new ASD. Although the recorded seizures in IH-KA rats differ in waveform from the ones in naïve mice, this could be due to the pattern of damage resulting from the micro-injection of KA or the density of expressed Chr2, which could be affected by sclerosis.

Impact and utility of methods and data.

The authors state that this approach should be used to test for and discover new ASDs for DRE, and also used for various open/closed loop protocols with deep-brain stimulation; however, the paper does not actually discuss rigorously or critically the background literature on other published studies in these areas or how this approach will improve future research for a broader audience than the ETSP and CROs. Thus, it is not clear whether the utility will apply more widely and how extensive a readership will be attracted to this work.

Final Conclusions:

Although this is an Interesting if not elegant new model for testing ASDs, it could be seen as a version of kindling (plus brain damage) in a rodent model, where some of the pathology of TLE is induced through focal injection of KA in the CA3 area of the hippocampus. Unfortunately, no evidence was presented that it will be any better than other models, although it could be faster and maybe easier than models based on spontaneous seizures. Although it has some similarities to the pathology of human TLE, the ablating part of the hippocampus does not account for the more widespread pathology that usually occurs

elsewhere in the brain, as studied with imaging and with anatomy in surgical specimens from patients with DRE.

Although this approach with seizure induction via an optogenetic approach adds specificity to the type of cell that is stimulated (i.e., CA1 pyramidal cells), it is not apparent why this provides a better or more effective tool than simple electrical induction of seizures in any TLE model. Most important, it remains unclear how this addresses any aspect of drug resistance. To improve the ASD discovery process, an important new model must make a significant reduction in seizure burden, and would ideally improve the percentage of patients that become seizure-free. It is not clear how this model will do that.

In the end, the authors have created a model with some of the pathology of TLE, where they can then induce actual seizures via specific optogenetic stimulation. So, although it is potentially elegant work, it remains unclear what new information this model will tell us about epilepsy, and most importantly DRE - or how it will improve treatment outcomes.

<https://doi.org/10.7554/eLife.101859.1.sa2>

Reviewer #3 (Public review):

Summary:

Chen et al. develop and characterize a new approach for screening drugs for epilepsy. The idea is to increase the ability to study seizures in animals with epilepsy because most animal models have rare seizures. Thus, the authors use the existing intrahippocampal kainic acid (IHKA) mouse model, which can have very unpredictable seizures with long periods of time between seizures. The authors employ an additional method to trigger seizures in the IHKA model. This method is closed-loop optogenetic stimulation of area CA1. There are several assumptions: area CA1 is the best location, triggered seizures are the same as spontaneous seizures, and this method will be useful despite requiring a great deal of effort. Regarding the latter, using a mouse model with numerous seizures (such as the pilocarpine model) might be more efficient than using a modified IHKA protocol that requires viral injection for optogenetics, fiber insertion requiring additional surgery, and accurate targeting to reliably trigger seizures on-demand. Aside from these caveats, the authors do succeed in studying seizures more readily in a mouse model of rare seizures. However, the seizures are evoked, not spontaneous. As currently presented, it is not clear how the triggered seizures can be used to investigate if antiseizure medication can reduce seizure burden as measured by seizure severity and seizures per day.

The authors modified the IHKA model to inject KA into CA3 instead of CA1 in order to preserve the CA1 pyramidal cells that they will later stimulate. To express the excitatory opsin channelrhodopsin (ChR2) in area CA1, they use a virus that expresses ChR2 in cells that express the Thy-1 promoter. The authors demonstrate that CA3 delivery of KA can induce a very similar chronic epilepsy phenotype to the injection of KA in CA1 and show that optical excitation of CA1 can reliably induce seizures. These are the strengths of the study.

While the authors show that electrophysiological signatures of induced vs spontaneous seizures are similar in many ways, the authors also show several differences and it is not clear if these differences are meaningful. Notably, the induced seizures are robustly inhibited by the antiseizure medication levetiracetam and variably but significantly inhibited by diazepam, similar to many mouse models with chronic recurrent seizure activity. I agree with the authors that this modified IHKA model will be of most value for higher throughput screening of potential antiseizure therapies, but with the caveat that the data may not generalize to other epilepsy models or humans. The authors evaluate the impact of repeated stimulation on the reliability of seizure induction and show that seizures can be reliably

induced by CA1 stimulation for as long as 16 days, but the utility of the model would be better demonstrated if seizures could be shown to be inducible over the range of weeks to months.

Strengths:

- (1) The authors show that the IHKA model of chronic epilepsy can be modified to preserve CA1 pyramidal cells (but at a cost of CA3 cells), allowing on-demand optogenetic stimulation of CA1 that appears to lower seizure threshold and thus trigger a seizure event.
- (2) The authors show that repeated reactivation of CA1 even in untreated mice can promote kindling and induction of seizure activity, indeed generating two mouse models in total.
- (3) Many electrophysiological signatures are similar between the induced and spontaneous seizures, and induced seizures reliably respond to treatment with antiseizure medications.
- (4) Given that more seizures can be observed per mouse using on-demand optogenetics, this model enhances the utility of each individual mouse.

Weaknesses:

- (1) Evaluation of seizure similarity using the SVM modeling and clustering is not sufficiently explained to show if there are meaningful differences between induced and spontaneous seizures. SVM modeling did not include analysis to assess the overfitting of each classifier since mice were modeled individually for classification.
- (2) The difference between seizures and epileptiform discharges or trains of spikes (which are not seizures) is not made clear.
- (3) The utility of increasing the number of seizures for enhancing statistical power is limited unless the sample size under evaluation is the number of seizures. However, the standard practice is for the sample size to be the number of mice.
- (4) Seizure burden is not easily tested.
- (5) It is unlikely that long-term adaptation to CA1-stimulated seizure induction is absent in these mice. A duration of evaluation longer than 16 days is warranted in light of the downward slope at days 13-16 for induced seizures in Figure 4C.
- (6) Human epilepsy is extensively heterogeneous in both etiology and individual phenotype, and it may be hard to generalize the approach.
- (7) No mention or assessment of mouse sex as a biological variable.

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Author response:

In this initial response to the public review, we outline our plan to address the major concerns raised. Below, we provide a general categorization of the suggestions and our corresponding responses

Weakness #1: Statistical Concerns - using the number of seizures (rather than the number of animals) may identify small effects that could be insignificant. Effect size should be taken into consideration.

Reviewer 1:

"While the data generally supports the authors' conclusions, a weakness of this manuscript lies in their analytical approach where EEG feature-space comparisons used the number of spontaneous or evoked seizures as their replicates as opposed to the number of IHK mice; these large data sets tend to identify relatively small effects of uncertain biological significance as being highly statistically significant."

Reviewer 2:

"In several sections of the paper, the authors argue that two different groups are similar on the basis that no statistical difference was found between the two groups (i.e., $p > 0.05$); however, the failure to find a statistically significant difference, particularly with relatively small sample sizes, is not rigorous evidence that the two groups are actually similar - they are just "not significantly different."

Reviewer 3:

"(3) The utility of increasing the number of seizures for enhancing statistical power is limited unless the sample size under evaluation is the number of seizures. However, the standard practice is for the sample size to be the number of mice."

Reviewer 3:

"(1) Evaluation of seizure similarity using the SVM modeling and clustering is not sufficiently explained to show if there are meaningful differences between induced and spontaneous seizures. SVM modeling did not include analysis to assess the overfitting of each classifier since mice were modeled individually for classification."

We understand the reviewers' concerns. In this work, we used linear mixed effect model to address two levels of variability –between animals and within animals. The interactive linear mixed effect model shows that most (~90%) of the variability in our data comes from within animals (Residual), the random effect that the model accounts for, rather than between animals. Since variability between animals are low, the model identifies common changes in seizure propagation across animals, while accounting for the variability in seizures within each animal. Therefore, the results we find are of changes that happen across animals, not of individual seizures. We will make text edits to enhance understanding of the linear mixed effect model.

To address the point raised about similarity, we will explain how the SVM classifier was trained. The purpose of the SVM is not to identify meaningful differences between induced and spontaneous seizures. Rather, it is to classify EEG sections as "seizures" or non-seizures, demonstrating the gross similarity between induced and spontaneous seizures despite minor differences. We will make text clarifications for the SVM model.

Weakness #2: Clinical and biological significance is unclear.
Reviewer 1:

"Furthermore, the clinical relevance of similarly small differences in EEG feature space measurements between seizure-naïve and epileptic mice is also uncertain."

Reviewer 2:

"While the paper may be relevant for the ETSP and contract research organizations (CROs), the paper was not written to attract the interest of biological scientists, even those in this specific area of epilepsy research. It may be of low interest to other

neuroscientists... The key issue the authors aim to address is the 30-40% of patients with DRE, but the real problem with DRE patients is not that these people have seizures with no effect of the ASDs; rather, although ASD may reduce seizure burden, these patients continue to have some remaining seizures even after high doses of ASDs, which often leads to adverse effects from the particular ASDs... It remains unclear that the optogenetically induced seizures in this model are better than similarly induced seizures in a naïve animal, and there is no evidence that the model will be useful for finding new ASDs to treat DRE."

Reviewer 3:

"(6) Human epilepsy is extensively heterogeneous in both etiology and individual phenotype, and it may be hard to generalize the approach."

Reviewer 2:

"The authors state that this approach should be used to test for and discover new ASDs for DRE, and also used for various open/closed loop protocols with deep-brain stimulation; however, the paper does not actually discuss rigorously or critically the background literature on other published studies in these areas or how this approach will improve future research for a broader audience than the ETSP and CROs. Thus, it is not clear whether the utility will apply more widely and how extensive a readership will be attracted to this work."

We appreciate the reviewer's concerns. We will revise the manuscript to better emphasize the potential significance of our approach. The on-demand seizure model can be applied to address biologically and clinically relevant questions beyond its utility in drug screening. For example, crossing the Thy1-ChR2 mouse line with genetic epilepsy models, such as Scn1a mutants, could reveal how optogenetic stimulation differentially induces seizures in mutant versus non-mutant mice, providing insights into seizure generation and propagation in Dravet Syndrome. Due to the cellular specificity of optogenetics, we also envision this approach being used to study circuit-specific mechanisms of seizure generation and propagation. Regarding drug-resistant epilepsy (DRE) and anti-seizure drug (ASD) screening, we agree with the reviewer that probing new classes of ASDs for DRE represents the critical goal. However, we believe a full exploration of additional ASD classes and/or modeling DRE lies outside the scope of this manuscript.

Weakness #3: Definition of Seizure is unclear

Reviewer 2:

"Although the figures provide excellent examples of individual electrographic seizures and compare induced seizures in epileptic and naïve animals, it is unclear which criteria were used to identify an actual seizure induced by the optogenetic stimulus, versus a hippocampal paroxysmal discharge (HPD), an "afterdischarge", an "electrophysiological epileptiform event" (EEE, Ref #36, D'Ambrosio et al., 2010 Epilepsy Currents), or a so-called "spike-wave-discharge" (SWD). Were HPDs or these other non-seizure events ever induced using stimulation in animals with IH-KA? A critical issue is that these other electrical events are not actual seizures, and it is unclear whether they were included in the column showing data on "electrographic afterdischarges" in Figure 5 for the studies on ASDs"

Reviewer 3:

"(2) The difference between seizures and epileptiform discharges or trains of spikes (which are not seizures) is not made clear."

Reviewer 2:

"The differences between the optogenetically evoked seizures in IH-KA vs naïve mice are interpreted to be due to the "epileptogenesis" that had occurred, but the lesion from the KA-induced injury would be expected to cause differences in the electrically and behaviorally recorded seizures - even if epileptogenesis had not occurred. This is not adequately addressed."

Thank you for pointing out the unclear definition of the seizures analyzed. We agree and will revise the text to clarify this issue. In this manuscript, we focused on tonic-clonic seizures. We analyzed animal behavior during evoked events, and a high percentage of induced electrographic events were accompanied by behavioral seizures with a Racine scale of three or above. Regarding epileptogenesis, our model is based on the IHK model, in which spontaneous tonic-clonic seizures occur a few to several days after KA injection. These mice are, by definition, epileptogenic. We will further clarify this methodology in the text.

Weakness #4: Similarity/Difference with Kindling Not Clear
Reviewer 2:

"The authors did not test whether an apparent "kindling" effect, apparently seen in naïve controls, also occurred in animals micro-injected with kainic acid (KA). This effect could cause model instability that might result in variability in response to ASDs. It is not clear whether the number of optogenetically induced seizures in epileptic animals would affect the response to drugs. It is also unclear how much of an improvement the animal model in the present work is over other similar models of TLE, where electrically triggered seizures could simply be applied to one of them."

Reviewer 3:

"(5) It is unlikely that long-term adaptation to CA1-stimulated seizure induction is absent in these mice. A duration of evaluation longer than 16 days is warranted in light of the downward slope at days 13-16 for induced seizures in Figure 4C."

We appreciate the reviewer's comments regarding the "kindling effect" as well as its similarity to the kindling model. We will carefully assess the data and address this in the revised manuscript. In electrical kindling, the activated cellular population is non-specific, including both excitatory and inhibitory neurons. In our model, we specifically activate predominantly excitatory neurons (Thy1-positive neurons), which we observed to participate in convulsant-induced seizures (as demonstrated in Thy1-GCaMP experiments). We consider this specificity an improvement over the kindling model, making our approach more biologically relevant.

Weakness #5: Time needed to generate model is significant. Unclear if animals were pre-selected
Reviewer 1:

"Finally, the multiple surgeries and long timetable to generate these mice may limit the value compared to existing models in drug-testing paradigms."

Reviewer 2:

"The authors offer little mention of other research using animal models of TLE to screen ASDs, of which there are many published studies - many of them with other strengths and/or weaknesses. For example, although Grabenstatter and Dudek (2019, Epilepsia)

used a version of the systemic KA model to obtain dose-response data on the effects of carbamazepine on spontaneous seizures, that work required use of KA-treated rats selected to have very high rates of spontaneous seizures, which requires careful and tedious selection of animals. The ETSP has published studies with an intra-amygdala kainic acid (IA-KA) model (West et al., 2022, Exp Neurol), where the authors claim that they can use spontaneous seizures to identify ASDs for DRE; however, their lack of a drug effect of carbamazepine may have been a false negative secondary to low seizure rates. The approach described in this paper may help with confounds caused by low or variable seizure rates. These types of issues should be discussed, along with others."

We appreciate the reviewer's insights. In an existing model investigating spontaneous tonic-clonic seizures (such as the intra-amygdala kainate injection model), the time investment is back-loaded, requiring two to three weeks per condition while counting spontaneous seizures, which may occur only once a day. In contrast, our model requires a front-loaded time investment. Once the animals are set up, we can test multiple drugs within a few weeks, providing significant time savings. Additionally, we did not pre-screen animals in our study. Existing models often pre-select mice with high rates of spontaneous seizures, whereas in our model, seizures can be induced even in animals with few spontaneous seizures. We believe that bypassing the need for pre-screening is a key advantage of our induced seizure model.

Reviewer 3:

"(7) No mention or assessment of mouse sex as a biological variable."

Thank you for pointing this out. Both female and male animals were included in this study: Epileptic cohort: 7 males, 3 females; Naïve cohort: 3 males, 4 females

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