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On-Demand Seizures Facilitate Rapid Screening of Therapeutics for Epilepsy

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eLife Assessment

The authors modified a common method to induce epilepsy in mice to provide an improved approach to screen new drugs for epilepsy. This is **important** because of the need to develop new 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**, making the study invaluable, but there were some limitations to the approach and methods.

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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 unwieldly 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, 29). 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 (**Figure. 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.

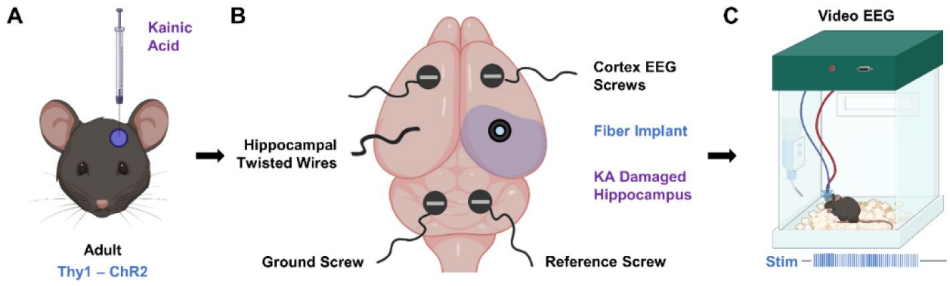

Figure 1

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

To avoid confusion and ensure clarity in the interpretation of the results presented in this manuscript, we define the following terms:

Afterdischarge: Afterdischarges, also referred to as induced activity or electrographic events, refer to long lasting (5s or more) electrical activity elicited following optical stimulation. This denotation includes both seizures and spikes. We use 'afterdischarges' interchangeably with 'induced activity'.

Seizures: We use the term 'seizure' specifically to describe generalized seizures that had behavioral components verified on video. Mostly these are tonic-clonic seizures with Racine Scale 3 or higher, but occasionally we observed milder seizures with Racine Scale 1 or 2.

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, 2A bottom right). 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 (30); 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 (31). The damage is clearly visible in the CA1, and the sclerosis is hypothesized to be the center of seizure generation (32) (**Figure 2A** top). Thy1 expression in the CA1 localizes to principal cells (33), 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 left), 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.

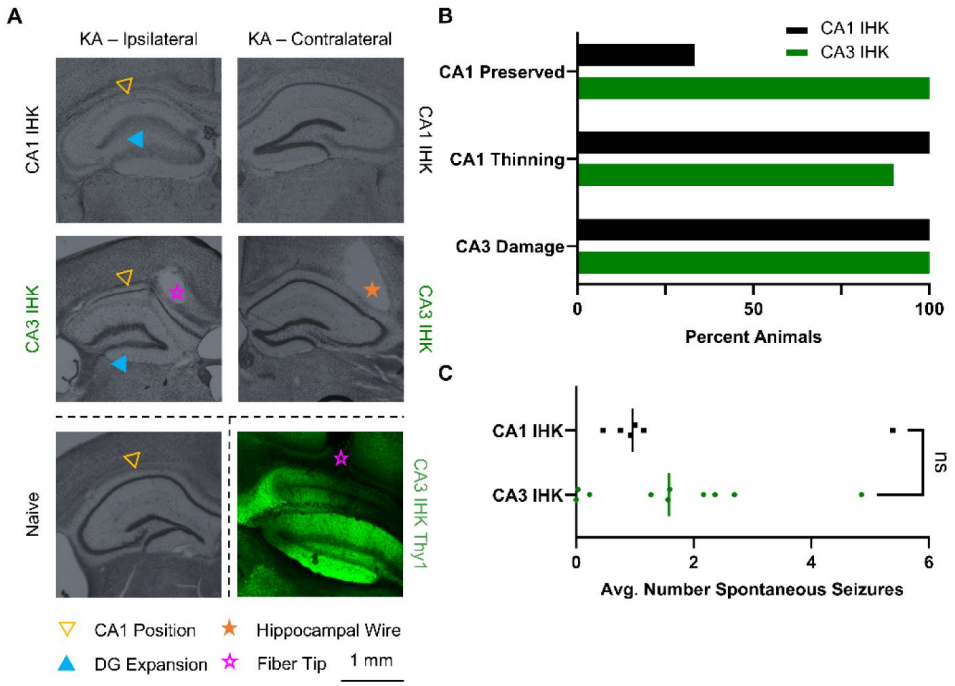

Figure 2

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 left). Fiber was implanted above cells expressing Thy1-ChR2 in the CA1 (bottom right). (**B**) Two thirds (4) of CA1-IHK animals did not have a visible CA1 cell layer, while the rest (2) had a thinned cell layer. In contrast, all CA3 IHK (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.

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 (Supplemental Figure 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**).

Optogenetically induced seizures resemble spontaneous tonic-clonic seizures in epileptic mice

In epileptic, freely moving Thy1-ChR2 mice (n = 10, 7 males, 3 females), we observed spontaneous generalized seizures, as exemplified in **Figure 3A**. Over one week of baseline recording, animals experienced on average 1.0 ± 1.8 (mean ± standard deviation) behavioral spontaneous seizures per day (Fig. S1A). We then optically stimulated Thy1-ChR2 expressing neurons in the CA1 ipsilateral to the IHK injection site using 10 Hz, 473 nm light 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 ± 4.75 mW and an average stimulation duration of 6.30 ± 1.64 s (Fig. S2B, S2C). The flowchart to determine the thresholds is described in Fig. S2A. Using the threshold stimulus, activity was induced approximately 88.7 ± 8.8 % of the time in the epileptic animals (Fig. S2D). Average duration of induced activity, mainly generalized seizures with behavioral components, was 30.98 ± 4.69 s (Fig. S2E). 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 seizures. First, we analyzed standard EEG features (34) 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). This approach allows us to compare features across seizures of varying durations. Our analysis also shows that features vary widely within each animal; to account for the high intra-animal variation, we used an interactive linear mixed effect model that accounted for intra-animal residuals to identify common inter-animal trends over time. This methodology allows us to identify changes across animals, changing the independent unit in the analysis to animals instead of seizures.

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 (35). 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

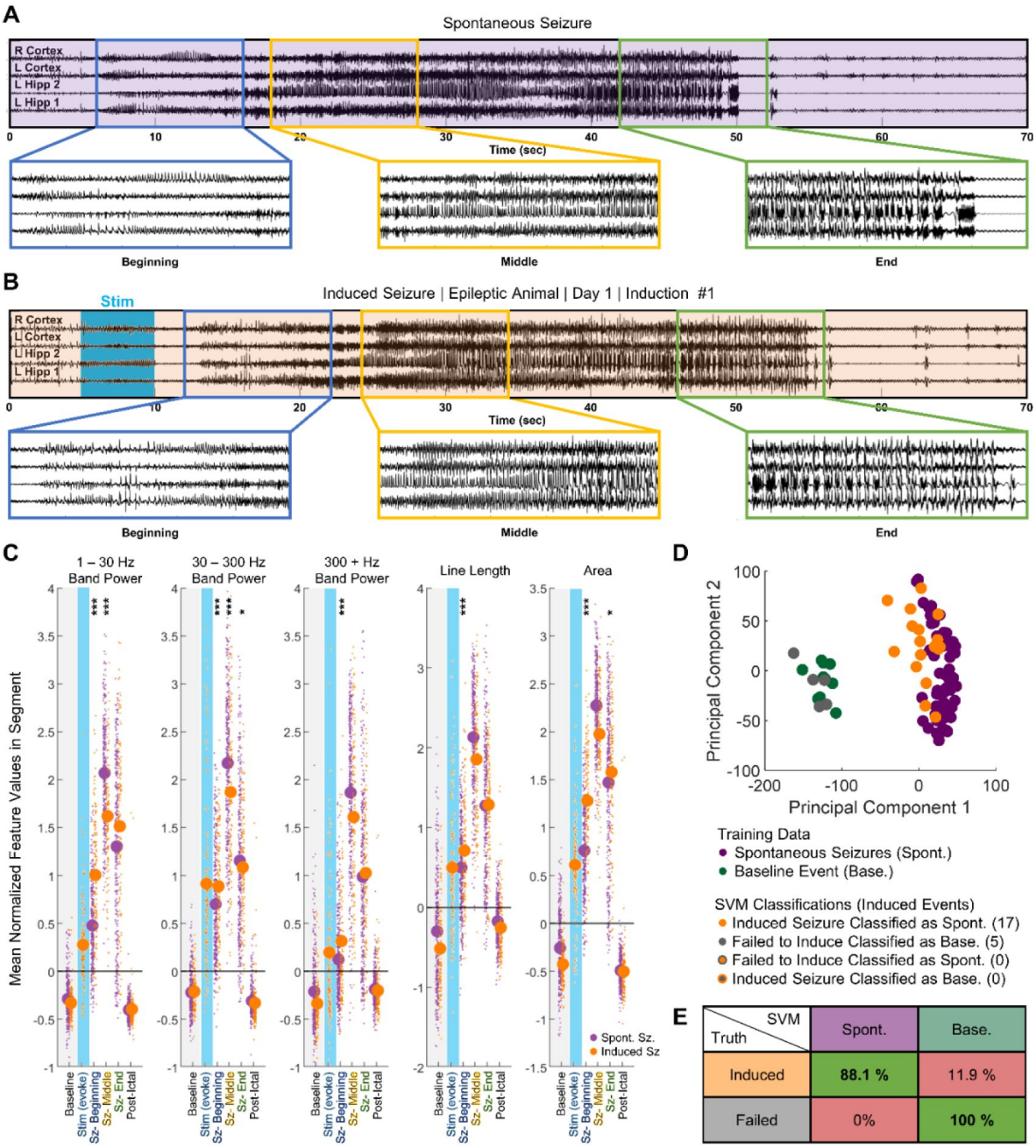

Figure 3

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 afterdischarges exceeding 5 seconds. (**E**) Compiled table of SVM accuracies across epileptic animals (n = 8). Induced activity was classified as similar to spontaneous seizures in 88.1% of all successful activations. Failed activations were classified as baseline 100% of the time.

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 ($p < 1e^{-4}$).

Induced activity predominantly consisted of generalized seizures (**Fig. 3B**). Like in the spontaneous seizures, most of induced seizures 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, backpedaled, jumped, or fell on its side (Supplemental Figure S5). Occasionally, optogenetic stimulus induced seizures of lower severity (Supplemental Figure S6), afterdischarges without seizure behavior, or failed to cause afterdischarges (Supplemental Figure 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 activity prior to comparison: 1) afterdischarges must have lasted a minimum of fifteen seconds and 2) Racine seizure score 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 ($p = 0.0001$). 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 ($p = 0.3866$), line length (0.7649), and area ($p = 0.1962$), were no longer statistically significant. In the final third, the increase in area ($p = 0.0259$) and 30 – 300 Hz band power ($p = 0.0160$) were slightly significant. The increase in 1 – 30 Hz band power was no longer significantly different ($p = 0.073$), and the increases in 300 – 1000 Hz band power ($p = 0.3245$) and line length ($p = 0.1172$) remained not significant.

To further quantify the similarity between spontaneous and induced activity, we trained individual one-class support vector machine (SVM) classifier to classify whether the induced activity was a seizure or not. The SVM is trained exclusively on data from spontaneous seizures and baseline activity; as such, the SVM is not designed to differentiate between induced and spontaneous seizures. Induced activity is then presented to the SVM, which classifies it as either in the category of spontaneous seizures or baseline activity. 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, inductions that failed to induce afterdischarges lasting more than 5 s were all 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, with the minor exception of EEG features during seizure onset.

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 (36–38). 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 optical provocations in naïve animals (n = 7, 3 males, 4 females) typically caused low frequency, high amplitude spiking activity (**Figure 4A**). While long lasting, these afterdischarges did not have a behavioral seizure manifestation. Animals displayed independent movement, exploration, or grooming during the afterdischarges (Supplemental Figure S8A). Behavioral differences between initial naïve and epileptic inductions did not result from changes in the optical induction parameters – the naive 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. S2B, S2C). 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 (Supplemental Figure 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 (39).

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. S2D). 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. S2E).

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, Table S1). 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

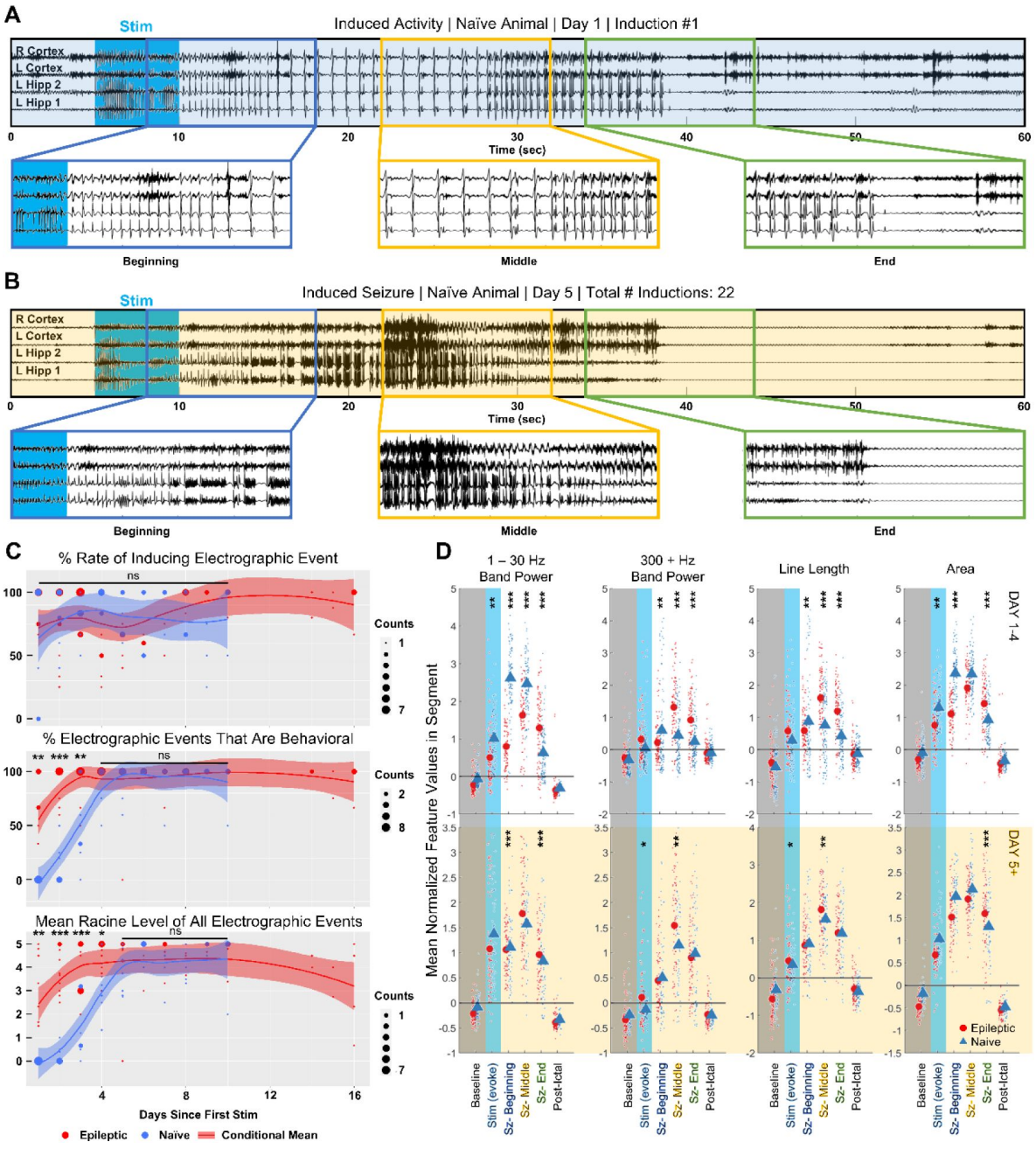

Figure 4

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 activity 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: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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, we followed a similar procedure to the analysis did in **Figure 3**. We used an interactive linear mixed effect model that accounts for the intra-animal variability to uncover the inter-animal changes in electrographic feature over three segments. 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 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 (40), could reduce the rates of both electrographic afterdischarge induction (electrographic activity lasting more than 5 seconds, including seizures) and behavioral seizure (generalized seizure with behavioral component) induction in epileptic animals. Diazepam, a benzodiazepine that interacts with GABA receptors, is commonly prescribed for first line control of convulsive seizures (41), whereas levetiracetam is an anticonvulsant that reduces overall network excitability through effects mediated by the synaptic protein SV2A (42).

The experimental paradigm is shown in **Figure 5A**. Prior to inducing activity 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 each day of ASM testing, optogenetic activation was performed hourly to establish the drug-free electrographic discharge induction and behavioral 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 8 assessments conducted on 6 epileptic animals (**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. In all animals, the severity of behavioral seizures is reduced after diazepam administration (Supplemental Figure S9A). 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 ± 35.8 % ($p = 0.016$) and the behavioral seizure induction rate was significantly reduced to 23.12 ± 34.5% ($p = 0.008$). One epileptic animal was a diazepam non-responder on one of two testing days. 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. Even in the diazepam non-responder, the Racine scale of induced seizures was lower after diazepam administration (Fig. S9A).

Application of levetiracetam significantly reduced the rates of electrographic discharge and behavioral seizure induction in 8 assessments on 5 epileptic animals (**Fig. 5B**, S9B). After levetiracetam administration, electrographic inductions were typically unsuccessful (Supplemental Figure S10A). In mice, levetiracetam has a short half-life of approximately 3.2 hours (43). 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 ± 17.7 % and the average behavioral seizure induction rate was 89.58 ± 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 ± 16.1 % ($p = 0.004$), and the behavioral seizure induction rate was significantly reduced to 33.75 ± 21.3 % ($p = 0.004$). Additionally, in all animals, seizure burden, measured as the severity of behavioral seizures decreased after levetiracetam administration (Fig. S9B).

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.

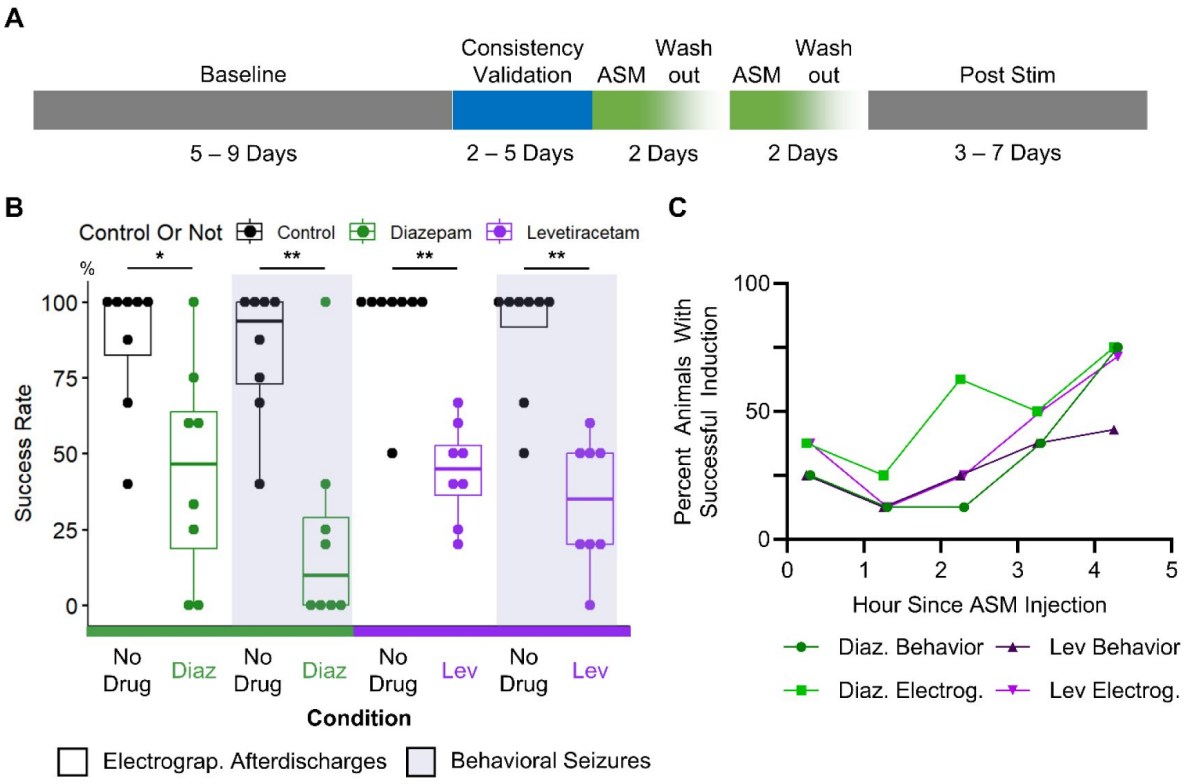

Figure 5

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 (any electrographic activity lasting more than five seconds) and behavioral seizures (generalized seizures with seizure behaviors) 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.

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 hyperactivity and generalized seizures (26, 28, 44, 45). 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 (46). 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 (47, 48). Electrical activation of the temporoammonic pathway was also sufficient to generate bursting activity in chronically epileptic brain slices (48). 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 (49). 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 (41, 42). Diazepam historically performs well in many seizure models. However, levetiracetam is unique among many ASMs in that it was originally missed by the standard ASM screens of the maximal electroshock seizure and subcutaneous pentylenetetrazol seizure tests. Its antiepileptic effect was only discovered using a kindling model of epilepsy, which has since been integrated into the ETSP pipeline (50, 51) 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 (52). A reliable, physiologically realistic on-demand seizure model could dramatically accelerate this process.

Alternatively, our approach can be harnessed to answer questions of biological and clinical significance. For instance, adaptation of our approach into rare disease models of epilepsy, such as the Scn1a mouse model used to study Dravet Syndrome (53), could produce fresh insights into how seizures spread in different models. Comparing seizure propagation across various models will also help elucidate how genetic mutations and cellular abnormalities influence seizure mechanisms across the heterogeneity of human epilepsy. Furthermore, circuit specific investigations can be conducted by integrating our model with cell-specific optogenetic or chemogenetic approaches (54). 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 (30). Some models utilize a kindling paradigm, requiring multiple days of successive activation before seizures can be

reliably induced (39). Other approaches are performed under anesthesia (55) or utilize naïve animals (56), 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 afterdischarges and tonic-clonic behavioral seizures. This is unlike models that rely on continuous stimulation throughout the seizure to generate behavioral events or models that pre-screen for animals that are predisposed to have spontaneous seizures (56, 57). In our model, all animals are used, and ictal activity naturally propagates out of the hippocampus and evolves into generalized tonic-clonic seizures. Our model is also unlike traditional kindling models because we target specific cellular populations instead of using non-specific electrical stimulus (39). 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 (58).

However, HPDs are not generalizable across species – they are specific to the mouse model (59). 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. First, on demand seizures do not require more time to prepare than HPDs. In the preparation phase, both methods require chronic epilepsy induction and an EEG implantation surgery. In the screening phase, on demand seizures produce significant time savings, as seizures can be evaluated immediately instead of waiting for HPD characterization and analysis. Furthermore, as on demand seizures are behavioral seizures, we predict that our model will respond to more categories of ASMs than HPDs, serving as a quick initial check as to whether the drugs' actions are sustained on seizures in the epileptic brain.

Some steps are still needed prior to integration - additional scaling of the model and validation with more ASMs are critical further studies. 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 afterdischarges and behavioral 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 ± 1.7), two cerebellar screws (AP – 5.5, ML ± 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. S2B and S2C. 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 anesthesized 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 6^{th} 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 naive 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 (60). Comparisons on daily spontaneous seizure frequency used a two tailed Mann Whitney test. Uncontrolled behaviors and the Racine scale (61) associated with the behaviors are as follows, and described in Table S1: (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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Author contributions

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Funding acquisition: BL, FV, HT Project administration: FV, HT, BL Supervision: HT, FV, BL

Writing – original draft: YC

Writing – review & editing: YC, HT, FV, BL

Additional files

Supplemental

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Editors

Reviewer #1 (Public review):

Summary:

This important study by Chen et. al. describes a novel approach for optogentically 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, despite having some features that were significantly different from spontaneous seizures, are suggested to be sufficiently similar to spontaneous seizures 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. The structural, functional, and behavioral differences between a seizure-naïve and epileptic mouse, which emerge due to the enduring changes occurring during epileptogenesis, are complex and important. Accordingly, this study highlights the importance of using mice that have underwent epileptogenesis as model organisms for testing novel therapeutics. Furthermore, this study positively impacts the wider epilepsy research community by investigating seizure semiology in these populations.

Weaknesses:

This study convincingly demonstrates that the feature space measurements for stimulus-evoked seizures in epileptic mice were significantly different from those in naïve mice; this result allows the authors to conclude that "seizures induced in chronically epileptic animals differed from those in naïve animals". However, the authors also conclude that "induced seizures resembled naturally occurring spontaneous seizures in epileptic animals" despite their own data demonstrating similar, albeit fewer, significant differences in feature space measurements. It is unclear if and what the threshold is whereby significant differences in these feature space measurements lead to the conclusion that the differences are meaningful, as in the comparison of epileptic and naïve mice, or not meaningful, as in the comparison of evoked and spontaneous seizures.

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Reviewer #2 (Public review):

The authors aimed to develop an animal model of temporal lobe epilepsy (TLE) that will generate "on-demand" seizures and an improved platform to advance our ability to find new anti-seizure drugs (ASDs) for drug-resistant epilepsy (DRE). Unlike some of the work in this field, the authors are studying actual seizures, and hopefully events that are similar to actual epileptic seizures. To develop an optimized screening tool, however, one also needs high-throughput systems with actual seizures as a quantitative, rigorous, and reproducible outcome measures. The authors aim to provide such a model; however, this approach may be over-stated here and seems unlikely to address the critical issue of drug resistance, which is their most important claim.

Strengths:

- The authors have generated an animal model of "on demand" seizures, which could be used to screen new ASDs and potentially other therapies. The authors and their model make a good-faith effort to emulate the epileptic condition and to use seizure susceptibility or probability as a quantitative output measure.

- The events considered to be seizures appear to be actual seizures, with some evidence that the seizures are different from seizures in the naïve brain. Their effort to determine how different ASDs raise seizure probability or threshold to an optogenetic stimulus to the CA1 area of the rodent hippocampus is focused on an important problem, as many if not most ASD screening uses surrogate measures that may not be as well linked to actual epileptic seizures.

- Another concern is their stimulation of dorsal hippocampus, while ventral hippocampus would seem more appropriate.

- Use of optogenetic techniques allows specific stimulation of the targeted CA1 pyramidal cells, and it appears that this approach is reproducible and reliable with quantitative rigor.

- The authors have taken on a critically important problem, and have made a good-faith effort to address many of the technical concerns raised in the reviews, but the underlying problem of DRE remains.

Weaknesses:

- Although the model has potential advantages, it also has disadvantages. As stated by the authors, the pre-test work-load to prepare the model may not be worth the apparent advantages. And most important, the paper frequently mentions DRE but does not directly address it, and yet drug resistance is the critical issue in this field.

- Although the paper shows examples of actual seizures, there remains some concern that some of the events might not be seizures - or a homogeneous population of seizures. More quantitative assessment of the electrical properties (e.g., duration) of the seizures and their probability is likely to be more useful than the proposed quantification in the future of the behavioral seizure stages, because the former could be both more objective and automated, while the behavioral analysis of the seizures will likely be more subjective and less reliable (and also fraught with subjectivity and analytical problems). Nonetheless, the authors point that the presence of "Racine 3 or above" behavioral seizures (in addition to their electrical data) is a good argument that many (if not all) of the "seizures" are actual epileptic seizures.

- Optogenetic stimulation of CA1 provides cell-specificity for the stimulation, but it is not clear that this method would actually be better than electrical stimulation of a kindled rodent with superimposed hippocampal injury. The reader is unfortunately left with the concern of whether this model would be easier and more efficacious than kindling.

- Although the authors have taken on a critically important problem, and have combined a variety of technologies, this approach may facilitate more rapid screening of ASDs against actual seizures (beneficial), but it does not really address the fundamentally critical yet difficult problem of DRE. A critical issue for DRE that is not well-addressed relates to adverse effects, which is often why many ASDs are not well tolerated by many patients (e.g., LEV). Thus, we are left with: how does this address anti-seizure DRE?

- The focus of this paper seems to be more on seizures more than on epilepsy. In the absence of seizure spontaneity, the work seems to primarily address the issues of seizure spread and duration. Although this is useful, it does not seem to be addressing the question of what trips the system to generate a seizure.

An appraisal of whether the authors achieved their aims, and whether the results support their conclusions:

- The authors seem to have developed a new and useful model; however, it is not clear how this will address that core problem of DRE, which was their stated aim.

- A discussion of the likely impact of the work on the field, and the utility of the methods and data to the community.

- As stated before in the original review, the potential impact would primarily be aimed at the ETSP or a drug-testing CRO; however, much more work will be required to convince the epilepsy community that this approach will actually identify new ASDs for DRE. The approach is potentially time-consuming with a steep and potentially difficult optimization curve, and thus may not be readily adaptable to the typical epilepsy-models neuroscience laboratory.

Any additional context you think would help readers interpret or understand the significance of the work:

- The problem of DRE is much more complicated than described by the authors here; however, the paper could end up being more useful than is currently apparent. Although this work could be seen as technically - and maybe conceptually - elegant and a technical tour de force, will it "deliver on the promise"? Is it better than kindling for DRE? In attempting to improve the discovery process, how will the model move us to another level? Will this model really be any better than others, such as kindling?

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Reviewer #3 (Public review):

This revised paper develops and characterizes 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. This approach is of clear utility to researchers who may need to observe many seizure events per mouse during screening of antiseizure medications. A key strength is also that more utility can be derived from each individual mouse. 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. 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, at least for the short term (up to 16 days). These are strengths of the study.

However, there are several limitations: the seizures are evoked, not spontaneous. It is not clear how induced seizures can be used to investigate if antiseizure medication can reduce spontaneous seizures. Although seizure inducibility and severity can be assessed, the lack of spontaneous seizures is a limitation. To their credit, the authors show that electrophysiological signatures of induced vs spontaneous seizures are similar in many ways, but the authors also show several differences. 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. One also wonders if using a mouse model with numerous seizures (such as the pilocarpine model) might be more efficient than using a modified IHKA protocol.

In this revised manuscript, the authors address some previous concerns related to definitions of seizures and events that are trains of spikes, sex as a biological variable, and present new images of ChR2 expression (but these images could be improved to see the cells more clearly). A few key concerns remain unaddressed, however. For example, it is still not clear that evoked seizures triggered by stimulating CA1 are similar to spontaneous seizures, regardless of the idea that CA1 plays a role in seizure disorders. It also remains unclear whether repeated activation of the hippocampal circuit will result in additional alterations to this circuit that affect the seizure phenotype over prolonged intervals (after 16 days). Furthermore, the use of SVM with the number of seizures being used as replicates (instead of number of mice) is inappropriate. Another theoretical concern is whether the authors are correct in suggesting that one will be able to re-use the mice for screening multiple drugs in a row.

Strengths:
- The authors show that the IHKA model of chronic epilepsy can be modified to preserve CA1 pyramidal cells, allowing optogenetic stimulation of CA1 to trigger seizures.
- The authors show that repeated optogenetic stimulation of CA1 in untreated mice can promote kindling and induce seizures, indeed generating two mouse models in total.
- Many electrophysiological signatures are similar between the induced and spontaneous seizures, and induced seizures reliably respond to treatment with antiseizure medications.
- Given that more seizures can be observed per mouse using on-demand optogenetics, this model enhances the utility of each individual mouse.
- Mice of each sex were used.

Weaknesses:
- Evaluation of seizure similarity using the SVM modeling and clustering is not sufficiently justified when using number of seizures as the statistical replicate (vs mice).
- Related to the first concern, the utility of increasing number of seizures for enhancing statistical power is limited because standard practice is for sample size to be numbers of mice.
- The term "seizure burden" usually refers to the number of spontaneous seizures per day, not the severity of the seizures themselves. Because the authors are evoking the seizures being studied, this study design precludes assessment of seizure burden.
- It seems likely that repeatedly inducing seizures will have a long-term effect, especially in light of the downward slope at day 13-16 for induced seizures seen in Figure 4C. A duration of evaluation that is longer than 16 days is warranted.
- Human epilepsy is extensively heterogeneous in both etiology and individual phenotype, and it may be hard to generalize the approach.

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Author response:

The following is the authors' response to the original reviews

> ***Reviewer 1 (Public review):***
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> *Weaknesses:*
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> *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.*

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 made text edits to clarify the use of the linear mixed effect model. (page6, second paragraph and page 11, first paragraph)

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

Thank you for the suggestion. We added a discussion in the 'Comparison to other seizure models…' section on pages 15 and 16. 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 also is a key advantage of our induced seizure model.

> ***Reviewer 1 (Recommendations for the authors):***
>
> *(1) Address why the EEG data comparisons were performed between seizures and not between animals (as explicitly described in the public review). Further, a discussion of the biological significance (or lack thereof) of the effect size differences observed is warranted. This is especially concerning when the authors make the claim that spontaneous and induced seizures are essentially the same while their analysis shows all evaluated feature space parameters were significantly difference in the initial 1/3 of the EEG waveforms.*

We made text edits to clarify the use of the linear mixed effects model (page 6, second paragraph, and page 11, first paragraph)

> *(2) The authors place great emphasis on the use of clinically/etiologically relevant epilepsy models in drug discovery research. There is discussion criticizing the time points required to enact kindling and the artificial nature of acute seizure induction methods. However, the combination IHK-opto seizure induction model also requires a lengthy timeline. A more tempered discussion of this novel model's strengths may benefit readers.*

Thank you for the suggestion. We added a discussion in the 'Comparison to other seizure models…' section on pages 15 and 16.

> *(3) The authors should further emphasize the benefit of having an inducible seizure model of focal epilepsy since other mouse models (e.g., genetic or TBI models) may have superior etiological relevance (construct and face validity) but may not be amenable to their optogenetic stimulation approach.*

Thank you for the suggestion. We revised the manuscript to better emphasize the potential significance of our approach. We added a discussion in the 'Application of Models...' section on page 15, second paragraph. 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.

> *(4) Suggestion: Provide immunolabeled imagery demonstrating ChR2 presence in Thy1 cells.*

Thank you for the suggestion. We added a fluorescence image showing ChR2 expression in Fig. 2A

> *(5) It might be prudent to mention any potential effects of laser heat on hippocampal cell damage, although the 10 Hz, ~10 mW, and 6 s stim is unlikely to cause any substantial burns. Without knowing the diameter and material of the optic fiber, this is left up to some interpretation.*

Thank you for the comments. In the Methods section, we listed the optical fiber diameter as 400 microns (page 17, EEG and Fiber Implantation section). Using 5–18 mW laser power with a relatively large fiber diameter of 400 microns, the power density falls within the range of commonly employed channelrhodopsin activation conditions in vivo. That said, we would like to investigate potential heat effects or cell damage in a follow-up study.

> *(6) There are instances in the manuscript where the authors describe experimental and analytical parameters vaguely (e.g. "Seizures were induced several times a day", "stimulation was performed every 1 - 3 hours over many days"). These descriptions can and should be more precise.*

Thank you for the comments. To enhance clarity, we added the stimulation protocol in a flowchart format in Fig. S2A, describing how we determined the threshold and proceeded to the drug test. Following this protocol, there was variability in the number of stimulations per day.

> *(7) In the second to last paragraph of the discussion, the authors state "However, HPDs are not generalizable across species - they are specific to the mouse model (55)." This statement is inaccurate. The paper cited comes from Dr. Corrine Roucard's lab at Synapcell. In fact, Dr. Rouchard argues the opposite (See Neurochem Res (2017) 42:1919-1925).*

Thank you for pointing out the mistake. On page 16, in the first paragraph, reference 55 (now 58 in the revised version) was intended to refer to 'quickly produce dose-response curves with high confidence.' In the revision, we cited another paper reporting that hippocampal spikes were not reproduced in the rat IHK model. R. Klee, C. Brandt, K. Töllner, W. Löscher, Various modifications of the intrahippocampal kainate model of mesial temporal lobe epilepsy in rats fail to resolve the marked rat-to-mouse differences in type and frequency of spontaneous seizures in this model. Epilepsy Behav. 68, 129–140 (2017).

> *(8) In the discussion, Levetiracetam is highlighted as an ASM that would not be detected in acute induced seizure models; the authors point out its lack of effect in MES and PTZ. However, LEV is effective in the 6Hz test (also an acute-induced seizure model). This should be stated.*

Thank you for the comments. We highlighted the discussion on LEV in the 'Application of Model to Testing Multiple Classes of ASMs...' section on page 14.

> *(9) The results text indicates that 9 epileptic mice were used to test LEV and DZP. However, the individual data points illustrated in Figure 5B show N=8 mice. Please correct.*

Thank you for the comments. A total of nine epileptic mice were used to assess two drugs, with the animals being re-used as indicated in the schematic. A total of eight assessments were conducted for DZP with six mice and eight assessments for LEV with five mice. Each assessment included hourly ChR2 activations without an ASM and hourly ChR2 activations after ASM injection.

> *(10) Figure 4D: Naïve mice are labeled as solid blue circles in the legend while the data points are solid blue triangles. Please correct.*

Thank you. We corrected the marker in Fig.4D.

> ***Reviewer 2 (Public Review):***
>
> *Weaknesses:*
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> *(1) 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.*

Thank you for pointing out the unclear definition of the seizures analyzed. We added sentences at the beginning of the Results section (page 3) to clarify the terminology we used.

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. We added Supplemental Figure S9, which shows behavioral seizure severity scores observed before and during ASM testing. We hope these changes address the reviewer's concern and improve the clarity of the manuscript.

> *(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 the comments. IHK-injected mice had spontaneous tonic-clonic seizures before the start of optical stimulation, as shown in Figure S1.

> *(3) 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. We added a discussion comparing our model with other existing models in the Discussion section (pages 15 and 16, 'Comparison to Other Seizure Models Used in Pharmacologic Screening' section). 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.

> *(4) 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.*

Thank you for the comments. In this revision, we added Supplemental Figure S9, showing the severity of behavioral seizures observed before and during ASM testing for each animal. We observed a reduction in behavioral seizure severity for each subject. We would like to explore using behavioral severity as an outcome measure in a follow-up study.

> *(5) 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 insights. We revised the manuscript to better emphasize the potential significance of our approach (page 15, second paragraph). 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 a critical goal. However, we believe that a full exploration of additional ASD classes and/or modeling DRE lies outside the scope of this manuscript, and we would like to explore it in a follow-up study.

> ***Reviewer 2 (Recommendations for the authors):***
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> *(1) The authors should explain why 10 Hz was chosen as the stimulation frequency.*

Thank you for the comment. A frequency of 10 Hz was determined based on previous work using anesthetized animals prepared in an acute in vivo setting. To simplify the paper and avoid confusion, we did not include a discussion on how we determined the frequency. Instead, we added a detailed description of how we optimized the power in a flowchart format in Supplemental Figure S2. We hope this improves reproducibility.

> *(2) After micro-injection of KA, morphological changes were observed in the hippocampus, but no comparison of Chr2 expression was made in naïve animals vs KA-injected animals. Presumably, the Thy1-Chr2 mouse expresses GFP in cells that express Chr2. Thus, it may be useful to show the expression of Chr2 in animals with hippocampal sclerosis. This may explain the lack of dramatic difference between stimulation parameters in naïve vs epileptic animals, as shown in supplemental Figure S2.*

Thank you for the suggestion. We added a fluorescence image of ChR2 expression in CA1, ipsilateral to the KA-injected site, in Fig. 2A.

> *(3) The authors state that "During epileptogenesis, neural networks in the brain undergo various changes ranging from modification of membrane receptors to the formation of new synapses" and that these changes are critical for successful "on-demand" seizure induction. However, it is not clear or well-discussed whether changes in neuronal cell densities that occur during sclerosis are important for "on-demand" seizure induction as well. Also, the authors showed that naïve animals exhibit a kindling-like effect, but it was unclear whether a similar effect was present in epileptic animals (i.e. do stimulation thresholds to seizure induction change as the animal gets more induction stimulations)? If present, would the secondary kindling affect drug-testing studies (e.g., would the drug effect be different on induced seizure #2 vs induced seizure #20)?*

Thank you for the suggestion. Since this is an important aspect of the model, we would like to address the kindling effect, the secondary kindling effect, and histopathology in a longer-term setting (several weeks) in a follow-up study.

> *(4) The authors show that in their model, LEV and DZP were both efficacious. The authors do not seem to mention that, over 25 years ago, LEV was originally missed in the standard ETSP screens; and, it was only discovered outside of the ETSP with the kindling model. The kindling model is now used to screen ASDs. The authors should consider adding this point to the Discussion. It remains unclear, however, if the author's screening strategy shows advantages over kindling and other such approaches in the field.*

Thank you for the suggestion. We added a discussion on LEV in the 'Application of Model to Testing Multiple Classes of ASMs...' section on page 14.

> *(5) P8 paragraph 2. The authors state values for naïve animals, but they should also provide values for epileptic animals since they state that the groups were not significantly different (p>0.05). It would be useful to show values for both and state the actual p-value from the test. This issue of stating mean/median values with SD and sample size should be addressed for all data throughout the paper. Additionally, Figure S2 should be added to the manuscript and discussed, as it has data that may be valuable for the reproducibility of the paper.*

Thank you for the suggestion. Figure S2 shows the threshold power required to induce electrographic activity for n = 10 epileptic animals (9.14 ± 4.75 mW) and n = 6 naïve animals (6.17 ± 1.58 mW) (Wilcoxon rank-sum test, p = 0.137). The threshold duration was comparable between the same epileptic animals (6.30 ± 1.64 s) and naïve animals (5.67 ± 1.03 s) (Wilcoxon rank-sum test, p = 0.7133).

> *(6) In addition to the other stated references on synaptic reorganization in the CA1 area, the authors should mention similar studies from Esclapez et al. (1999, J Comp Neurol).*

Thank you. We have included the reference in the revision.

> *(7) All of the raw EEG data on the seizures should be accessible to the readers.*

Thank you for the suggestion. We will consider depositing EEG data in a publicly accessible site.

> ***Reviewer 3 (Public review):***
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> *Weaknesses:*
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> *(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."*

Thank you for the comment. We made text edits to clarify the purpose of the SVM analysis. It was not intended to identify meaningful differences between induced and spontaneous seizures. Rather, it was used to classify EEG epochs as 'seizures' based on spontaneous seizures as the training set, demonstrating the gross similarity between induced and spontaneous seizures.

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

Thank you for pointing out the unclear definition of the seizures analyzed. We added sentences at the beginning of the Results section (page 3) to clarify the terminology we used. 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. We added Supplemental Figure S9 to show the types of seizures observed before and during ASM testing. We hope these changes address the reviewer's concern and improve the clarity of the manuscript.

> *(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.*

In this work, we used a linear mixed-effects model to address two levels of variability—between animals and within animals. The interactive linear mixed-effects 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 is 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 reflect changes that occur across animals, not individual seizures. We made text edits to clarify the use of the linear mixed-effects model.

> *(4) Seizure burden is not easily tested.*

Thank you for the comment. We added Supplemental Figure S9 to summarize the severity of behavioral seizures before and during ASM testing. This addresses the reviewer's comment on seizure burden. In a follow-up study, we would like to explore this type of outcome measure for drug screening.

> ***Reviewer 3 (Recommendations for the authors):***
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> *(1) Provide a stronger rationale to use area CA1. For example, the authors mention that CA1 is active during seizure activity, but can seizures originate from CA1? That would make the approach logical and also explain why induced and spontaneous seizures are similar.*

Thank you for the comment. We discussed it in the Discussion section (page 14, first and second paragraphs).

> *(2) Explain the use of SVM classifiers so it is more convincing that induced and spontaneous seizures are similar. Or, if they are not similar, explain that this is a limitation.*

We made text edits to clarify the purpose of the SVM analysis. It was not intended to identify meaningful differences between induced and spontaneous seizures. Rather, it was used to classify EEG epochs as 'seizures' based on spontaneous seizures as the training set, demonstrating the gross similarity between induced and spontaneous seizures.

> *(3)If feasible, extend the duration over which seizure induction reliability is assessed so that the long-term utility of the model can be demonstrated.*

Thank you for the suggestion. We would like to assess long-term utility in a follow-up study.

> *(4) The GitHub link is not yet active. The authors will be required to supply their relevant code for peer evaluation as well as publication.*

Thank you. The GitHub repository is now active.

> *(5) State and assess the impacts of 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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