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Ventral Hippocampal Formation Is the Primary Epileptogenic Zone in a Rat Model of Temporal Lobe Epilepsy

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Temporal lobe epilepsy is common, but mechanisms of seizure initiation are unclear. We evaluated seizure initiation in female and male rats that had been systemically treated with pilocarpine, a widely used model of temporal lobe epilepsy. Local field potential (LFP) recordings from many brain regions revealed variable sites of earliest recorded seizure activity, but mostly the ventral hippocampal formation. To test whether inactivation of the ventral hippocampal formation would reduce seizures, mini-osmotic pumps were used to continually and focally deliver TTX. High doses of TTX infused unilaterally into the ventral hippocampal formation blocked seizures reversibly but also reduced LFP amplitudes in remote brain regions, indicating distant effects. A lower dose did not reduce LFP amplitudes in remote brain regions but did not reduce seizures when infused unilaterally. Instead, seizures tended to initiate in the contralateral ventral hippocampal formation. Bilateral infusion of the lower dose into the ventral hippocampal formation reduced seizure frequency 85%. Similar bilateral treatment in the amygdala was not effective. Bilateral infusion of the dorsal hippocampus reduced seizure frequency, but only 17%. Together, these findings reveal that the ventral hippocampal formation is a primary bilaterally independent epileptogenic zone, and the dorsal hippocampus is a secondary epileptogenic zone in pilocarpine-treated rats. This is consistent with many human patients, and the results further validate the LFP method for identifying seizure onset zones. Finally, the findings are more consistent with a focal mechanism of ictogenesis rather than one involving a network of interdependent nodes.

Key words: epilepsy; hippocampus; ictogenesis; rat; seizure; temporal lobe

Significance Statement

To better understand how seizures start, investigators need to know where seizures start in the animal models they study. In the widely used pilocarpine-treated rat model of temporal lobe epilepsy, earliest seizure activity was most frequently recorded in the ventral hippocampal formation. Confirming the primary role of the ventral hippocampal formation, seizure frequency was reduced most effectively when it was inactivated focally, bilaterally, and continually with infused TTX. These findings suggest that the ventral hippocampal formation is the primary site of seizure initiation in this animal model of temporal lobe epilepsy, consistent with findings in many human patients.

Introduction

Temporal lobe epilepsy is common and can be difficult to treat effectively (Semah et al., 1998; Téllez-Zenteno and Hernández-Ronquillo, 2012). Fundamental questions persist, including the following: How does it develop? How does a seizure start? Animal models help address these questions, and investigating sites of seizure onset might be especially revealing. The goal of the present study was to identify where seizures initiate in epileptic pilocarpine-treated rats. Rats systemically treated with pilocarpine were introduced >35 years ago and are one of the most widely used models of temporal lobe epilepsy (Turski et al., 1983; Cavalheiro et al., 2006).

Previously, we evaluated local field potentials (LFPs) from many brain regions in pilocarpine-treated rats. The most common site of earliest recorded seizure activity was the ventral hippocampal formation (Toyoda et al., 2013; Wyeth et al., 2020). The rat ventral hippocampus is homologous with the human anterior hippocampus, which is the most common site of seizure initiation in patients with temporal lobe epilepsy (Quesney, 1986; Spencer et al., 1987, 1990; Sperling and O'Connor, 1989; Duckrow and Spencer, 1992; Spencer and Spencer, 1994; Masukawa et al., 1995; King et al., 1997; Wennberg et al., 2002).

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These findings suggest that the primary seizure onset site is similar in patients and pilocarpine-treated rats. Caveats remain, however. Onset site is defined by earliest seizure activity (Rosenow and Lüders, 2001), but recording electrode numbers are always limited, unavoidably resulting in sparse spatial sampling. Seizures might start in unrecorded areas. This problem could be exacerbated by the potentially small size of onset zones (Schevon et al., 2008; Stead et al., 2010), making them easier to miss. Second, electrographic seizure activity might be broadcast far from the generating region, which could hamper identification of the actual onset zone (Weiss et al., 2013; Smith et al., 2016). Third, even if an electrode were at the onset site, LFP recording might not be sufficiently sensitive to detect the earliest microseizure activity (Wenzel et al., 2019). Finally, the very notion of a focal seizure onset zone has been challenged in some cases of temporal lobe epilepsy. It has been proposed that variability in seizure onset sites and rapid spread of seizure activity are attributable to hyperexcitability across extended networks of limbic and subcortical structures, instead of a more restricted focus (Bertram, 1997, 2009; Bertram et al., 1998; Spencer, 1998, 2002; Bartolomei et al., 2004, 2017; Wendling et al., 2010; but see Smith and Schevon, 2016). Similarly, in pilocarpine-treated rats, sites of earliest recorded seizure activity are variable and seizures spread quickly (Toyoda et al., 2013; Wyeth et al., 2020).

Because of the limitations of relying solely on LFP recording of earliest seizure activity, the present study sought to determine the functionally defined epileptogenic zone in pilocarpine-treated rats. The epileptogenic zone is "the minimum amount of cortex that must be resected (inactivated or completely disconnected) to produce seizure freedom" (Lüders et al., 2006). Galvan et al. (2000) established a method to inactivate the dorsal hippocampus for days at a time by continual infusion of the voltage-gated sodium channel blocker TTX. We adapted the method. With this approach, it was possible to test whether inhibiting activity in the ventral hippocampal formation would block seizures in pilocarpine-treated rats. Results of this test address the following questions: How valid is the pilocarpine-treated rat model for human temporal lobe epilepsy? How reliable is LFP recording for identifying sites of seizure onset? Is ictogenesis in temporal lobe epilepsy focal, or is a network of functionally interdependent brain regions required?

Materials and Methods

All experiments were performed in accordance with the National Institutes of Health's *Guide for the care and use of laboratory animals* and were approved by a Stanford University Institutional Animal Care and Use Committee. Rats were maintained on a 14 h light, 10 h dark cycle, with lights turning on at 7:00 A.M. Food and water were available *ad libitum*. Rats were pair-housed but separated if fighting began. Female ($n = 14$) and male ($n = 23$) Sprague Dawley rats experienced status epilepticus when they were 40 ± 1 d old (mean ± SEM, range 33-52 d). To induce status epilepticus, pilocarpine hydrochloride (380 mg/kg, i.p.) was administered 20 min after atropine methyl bromide (5 mg/kg, intraperitoneal). Diazepam (10 mg/kg, i.p.) was administered 2 h after the onset of motor seizures and repeated as needed for the next 10 h to suppress convulsions. Lactated Ringer's solution (10 ml, s.c.) was administered during recovery.

To allow time for epilepsy to develop and for seizure frequency to plateau (Van Nieuwenhuyse et al., 2015), surgery occurred 6.4 ± 0.3 months after pilocarpine treatment (range 2.6-11.0 months). Rats were sedated with diazepam (5 mg/kg, i.p.), anesthetized with isoflurane (1.5%), placed in a stereotaxic frame, maintained on a heating pad with feedback control, given antibiotic (enrofloxacin, 10 mg/kg, s.c.), lactated Ringer's solution (10 ml, s.c.), and analgesics (carprofen, 5 mg/kg, s.c. and sustained-release buprenorphine, 0.3 mg/kg, s.c.), and prepared for aseptic surgery. Bipolar electrodes consisted of 25-µm-diameter H-formvar-coated stainless-steel wires (California Wire) glued together with tips 1 mm apart. Electrodes were directed toward the following regions bilaterally (anterior-posterior and medial-lateral stereotactic coordinates in mm referenced to bregma and to the brain surface): septum (including the bed nuclei of stria terminalis, bed nucleus of anterior commissure, and septohippocampal nucleus) (−0.7, 0.3, 5.9), amygdala (including the cortex-amygdala transition zone and amygdaloid-hippocampus) (−2.8, 4.4, 7.9), olfactory cortex (including the endopiriform nucleus, postpiriform transition area, and olfactory tubercle) (−2.8, 5.8, 7.4), dorsal hippocampus (dentate gyrus and CA1-3) (−4.6, 2.6, 3.1), ventral hippocampus (−5.5, 4.8, 7.1) and ventral subiculum (−6.6, 4.6, 7.5) (both part of the ventral hippocampal formation), and entorhinal cortex (medial and lateral parts) (−7.9, 5.0, 5.3). In some cases, instead of a bipolar electrode, an electrode in the dorsal hippocampus consisted of four or six wires with tips 0.5 mm apart. A reference electrode was placed in the cerebellum. Electrodes were connected to an interface board (EIB-36-9 drive, Neuralynx) on an aluminum ring affixed to the skull with cranioplastic cement and screws (#0-80 × 1/8). The ground consisted of screws in the skull caudal to lambda (~−10.1, ± 2.2). In addition, rats were implanted with bilateral cannulas for focal infusion. Cannulas consisted of 28-gauge stainless-steel tubing, a polycarbonate elbow, and 21-gauge polyvinylchloride tubing (Plastics One) connected to an Alzet mini-osmotic pump (model 2004, Durect) that was placed under the skin of the back. A bipolar LFP electrode, consisting of 25-µm-diameter H-formvar-coated stainless-steel wires (California Wire), was glued to each infusion cannula with one tip at the end of the cannula and the other extending 1 mm beyond. Targets for infusion were the ventral hippocampal formation, amygdala, or dorsal hippocampus. Rats were singly housed after surgery.

Recording began 8.0 ± 0.3 d after surgery (range 5-16 d). LFP and time-locked video recordings were obtained as rats rested in a cage. Recordings began at ~7:30 A.M. and lasted 10.5 ± 0.1 h/d (range 9.5-11.6 h/d) for 58.6 ± 2.4 consecutive days (range 35-104 d). Cumulative gaps in recording days totaled 2.2 ± 0.5% of the total recording period (range 0%-12%). Signals were buffered with a headstage (HS-36, Neuralynx), amplified, digitized, filtered (0.1–500 Hz), sampled (2000 Hz) (Cheetah Data Acquisition, Neuralynx), and saved for offline analysis.

Alzet mini-osmotic pumps (model 2004, Durect) delivered 0.25 µl/h. Initially, pumps contained vehicle consisting of 0.9% NaCl. After a first period of baseline recording (12.6 ± 0.6 d, range 8-25 d), rats were briefly anesthetized with isoflurane to replace a vehicle-containing pump with a pump containing 0.01-10 μM TTX (Alomone Labs, catalog #T-550) dissolved in 0.9% NaCl. TTX infusion into the hippocampus can cause persistent hyperexcitability and epilepsy if administered to rat pups starting at <17 d old, but not in adults (Galvan et al., 2000). Travel time for TTX to move from the pump, through the tubing and cannula, and into the brain was taken into account when analyzing data. After a period of infusing TTX (12.1 ± 0.8 d, range 3-27 d), pumps were replaced with a vehicle-containing pump or removed, and a second period of baseline recording was acquired (32.7 ± 2.1 d, range 10-64 d). The total time of baseline recording was 45.4 ± 2.2 d (range 20-80 d).

Recordings included video of the rat and the computer monitor displaying LFPs. Video recordings were reviewed to identify seizures and score seizure behavior according to Racine (1972). Seizures scored as Class 3 (forelimb clonus) or greater were considered convulsive. Seizures scored as less than Class 3 were considered nonconvulsive. Electrographic seizures (Fig. 1*A*) were evaluated by examining all LFP recordings together to mark the onset and offset (Neuraview, Neuralynx). Onset was the earliest persistent change that developed into clear seizure activity. Seizures were included for analysis if they were at least 10 s in duration. To determine sites of earliest recorded seizure activity, each channel was evaluated at high temporal resolution by a single investigator (P.S.B.) who was blind to electrode locations. This method was previously found to be more reliable than automated approaches (Toyoda et al., 2013). The number of seizure onsets evaluated per animal and condition (during baseline or TTX infusion) was 71 ± 8 (range, 16-312). To analyze the effect of TTX

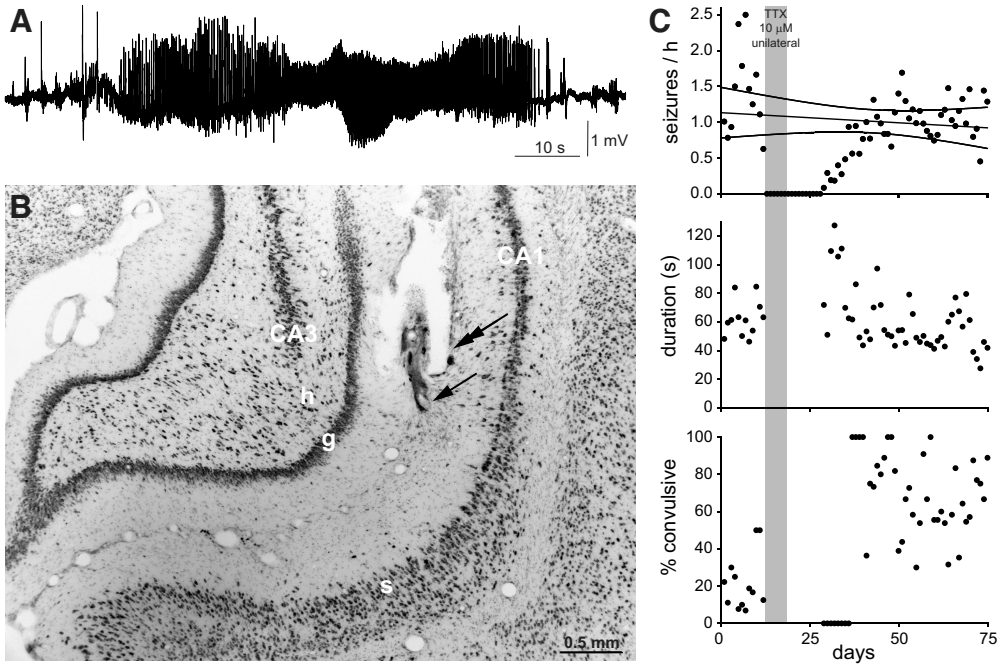

Figure 1. Unilateral infusion of 10 μM TTX at 0.25 μl/h into the ventral hippocampal formation reversibly blocked seizures in a pilocarpine-treated rat (Rat 1 in Fig. 4.) ***A***, Seizure recorded by an electrode in the right ventral hippocampal formation (electrode tract shown in ***B***). ***B***, Right ventral hippocampal formation and site of the cannula that delivered TTX (double arrow) and the attached electrode (arrow). h, Hilus; g, granule cell layer; s, subiculum. ***C***, Average daily seizure frequency, average daily seizure duration, and average daily percentage of seizures that were convulsive. Gray bar represents period of TTX infusion. The plot of average daily seizure frequency includes a regression line and 99% CIs for baseline values, including those before TTX infusion and after, starting when seizure frequency began to recover from zero.

infusion on neural activity, LFP amplitude was measured from the maximum to the minimum during a 1 min period of slow wave sleep for every electrode (NeuroExplorer, Nex Technologies). Slow wave sleep was recognized as periods of sleep when theta activity (4-12 Hz) was not evident and large amplitude slow waves (<4 Hz) occurred. LFP amplitude is high and interictal spikes are frequent during slow wave sleep (Lieb et al., 1980).

After recordings were complete, rats were killed with pentobarbital (>100 mg/kg, i.p.) and perfused at 30 ml/min through the ascending aorta for 1 min with 0.9% NaCl and 30 min with 4% formaldehyde in 0.1 M PB, pH 7.4. Brains were removed and stored in fixative at 4°C at least overnight, equilibrated in 30% sucrose in PB, and sectioned coronally using a sliding microtome set at 40 μm. Every other section was Nissl-stained with 0.25% thionine to visualize electrode and cannula locations (Fig. 1*B*). Electrodes and cannulas were placed according to stereotaxic coordinates, but actual locations varied. With *post hoc* histology, each electrode tip was located to a brain region as defined by the atlas of Swanson (1992). The border between the dorsal hippocampus and ventral hippocampal formation was defined as described previously (Wyeth et al., 2020). A Neurolucida system (MBF Biosciences) was used to three-dimensionally reconstruct brains and measure distances from electrodes to the cannula that infused TTX.

Experimental design and statistics. In the experimental design of the present study, the most important comparison was the effect of different concentrations of TTX and infusion locations on seizure frequency. Seizure frequency was analyzed as follows. First, for each rat, seizure frequency was calculated for each day by dividing the number of seizures by recording time. Next, baseline seizure frequency was calculated by averaging daily seizure frequencies during the baseline period. The same was done for the period of TTX infusion. Finally, the effect of TTX infusion on seizure frequency for each experimental group was determined by a paired *t* test. A one-tailed test was used to test the hypothesis that treatment with TTX would reduce seizure frequency. Normality was verified with a Shapiro–Wilk test. $p < 0.05$ was considered significant. Other seizure parameters (duration and percent convulsive seizures) were analyzed similarly. Comparisons between groups (female vs male, for example) were tested with unpaired two-tailed *t* tests. Normality was verified with a Shapiro–Wilk test. Equal variance was verified with $p < 0.05$ to reject. If normality or equal variance tests failed, nonparametric tests were used: Mann–Whitney rank sum or Wilcoxon signed rank. Sigma Plot 12 (Systat Software) was used for statistical analyses.

To identify sites of earliest recorded seizure activity, the observed number of earliest recorded seizure onsets was compared with that expected by chance based on the number of recording electrodes in a region. *z* scores were calculated according to the following formula:

$$z = \frac{p' - p}{\sqrt{\frac{pq}{n}}}$$

where p' = observed probability of earliest seizure onset, p = expected probability of earliest seizure onset if random (= number of electrodes in that brain region/total number of electrodes), $q = p - 1$, n = number of seizures. *z* scores ≥ 1.96 were significant ($p < 0.05$) (Daniel, 1987).

Results

A total of 18,716 seizures were analyzed, 506 ± 53 seizures/rat (mean ± SEM). Averaging across all experimental groups, baseline seizure frequency was 1.0 ± 0.1 seizures/h, baseline seizure duration was 62 ± 3 s, and baseline percentage of convulsive seizures was 65 ± 3%. There was no correlation between baseline seizure frequency (range 0.1-2.2 seizures/h) and age (range 139-378 d, $R^2 = 0.004$, $p = 0.71$, ANOVA). Baseline seizure frequency of female rats (0.7 ± 0.1 seizures/h, $n = 14$) was less than that of males (1.1 ± 0.1 seizures/h, $n = 23$, $p = 0.006$). The sex difference was not a problem for experimental design because each individual rat served as its own control. Baseline seizure duration of female rats (61 s, median) was similar to that of males (58 s, $p = 0.46$, Mann–Whitney rank sum test). Baseline percentage of convulsive seizures of female rats (70 ± 6%) was similar to that of males (61 ± 4%, $p = 0.21$, *t* test).

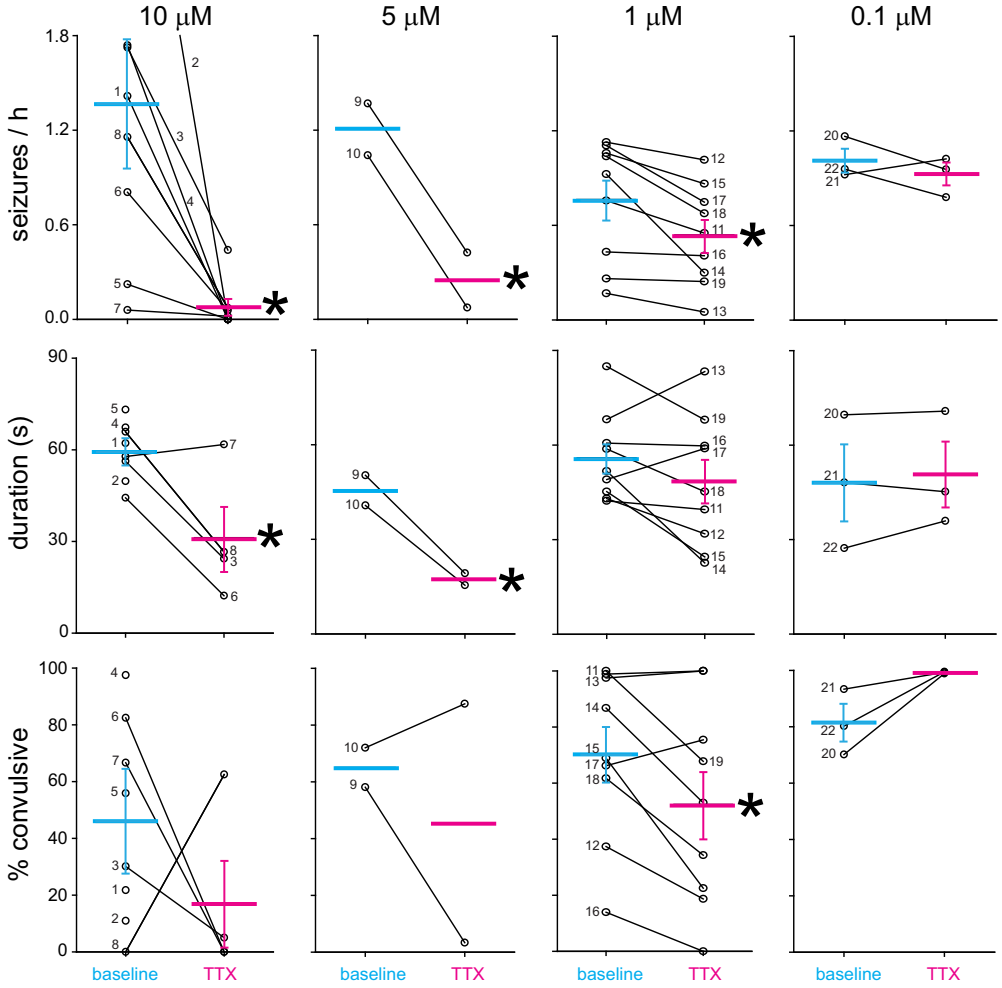

Figure 2. Dose-dependent effects of unilateral infusion of TTX into the ventral hippocampal formation on seizure frequency, seizure duration, and percentage of convulsive seizures. Horizontal lines indicate averages. Error bars indicate SEM. *$p < 0.05$, significant difference (paired t test). One rat in the 10 μM group had a baseline frequency of 3.8 seizure/h, which was off the scale of the y axis. Results of individual rats indicated (numbered in Fig. 4).

To test whether unilateral deactivation of the ventral hippocampal formation would block seizures, 10 μM TTX was infused at 0.25 µl/h. That concentration and infusion rate had been used previously to reduce LFP activity in the dorsal hippocampus (Galvan et al., 2000). In the ventral hippocampal formation, unilateral 10 μM TTX for 11.5 ± 2.6 d blocked seizures, and the effect could persist for a period after TTX infusion ceased (Fig. 1*C*). Because of the lingering effects of TTX infusion, only the pre-TTX baseline period was used for analysis. For rats in this group ($n = 8$), seizure frequency during the baseline period was 1.36 ± 0.41 seizures/h. Seizure frequency was reduced to 5% of baseline during TTX infusion (0.07 ± 0.05 seizures/h, $p = 0.008$) (Fig. 2). Seizures were blocked completely during the entire period of TTX infusion in 4 rats. Four other rats had some seizures during TTX infusion; and in those cases, seizure duration and behavioral severity could be compared with baseline. Seizure duration during baseline was 60 ± 3 s, and it was reduced to 52% of baseline during TTX infusion (31 ± 11 s, $p = 0.04$). The percentage of seizures that were convulsive was 46 ± 12% during baseline and 17 ± 15% during TTX infusion ($p = 0.22$). These findings showed that unilateral infusion of 10 μM TTX in the ventral hippocampal formation reversibly blocked seizures completely or reduced their frequency. The treatment also reduced seizure duration.

Lower doses were tested to determine the minimal amount of TTX that would reduce seizure frequency. For rats treated with 5 μM TTX in the ventral hippocampal formation unilaterally ($n = 2$), seizure frequency during baseline was 1.21 ± 0.16 seizures/h, and it was reduced to 21% of baseline during 12 d of TTX infusion (0.25 ± 0.17 seizures/h, $p = 0.003$) (Fig. 2). Seizure duration during baseline was 45 ± 5 s, and it was reduced to 38% of baseline during TTX infusion (17 ± 2 s, $p = 0.03$). The percentage of seizures that were convulsive was 65 ± 7% during baseline and 45 ± 42% during TTX infusion ($p = 0.34$). These findings revealed that unilateral infusion of 5 μM TTX also reduced seizure frequency, so a lower dose was tested.

For rats treated with 1 μM TTX ($n = 9$), seizure frequency during baseline was 0.76 ± 0.13 seizures/h, and it was reduced to 71% of baseline during 13.3 ± 2.3 d of TTX infusion (0.54 ± 0.11 seizures/h, $p = 0.004$) (Fig. 2). Seizure duration was 56 ± 5 s during baseline and 48 ± 7 s during TTX infusion ($p = 0.07$). The percentage of seizures that were convulsive was 70 ± 10% during baseline, and it was reduced to 52 ± 12% during TTX infusion ($p = 0.01$). These findings revealed that unilateral infusion with 1 μM TTX also reduced seizure frequency, so an even lower dose was tested.

For rats treated for 9.7 ± 2.4 d with 0.1 μM TTX ($n = 3$), seizure frequency was 1.02 ± 0.08 seizures/h during baseline and

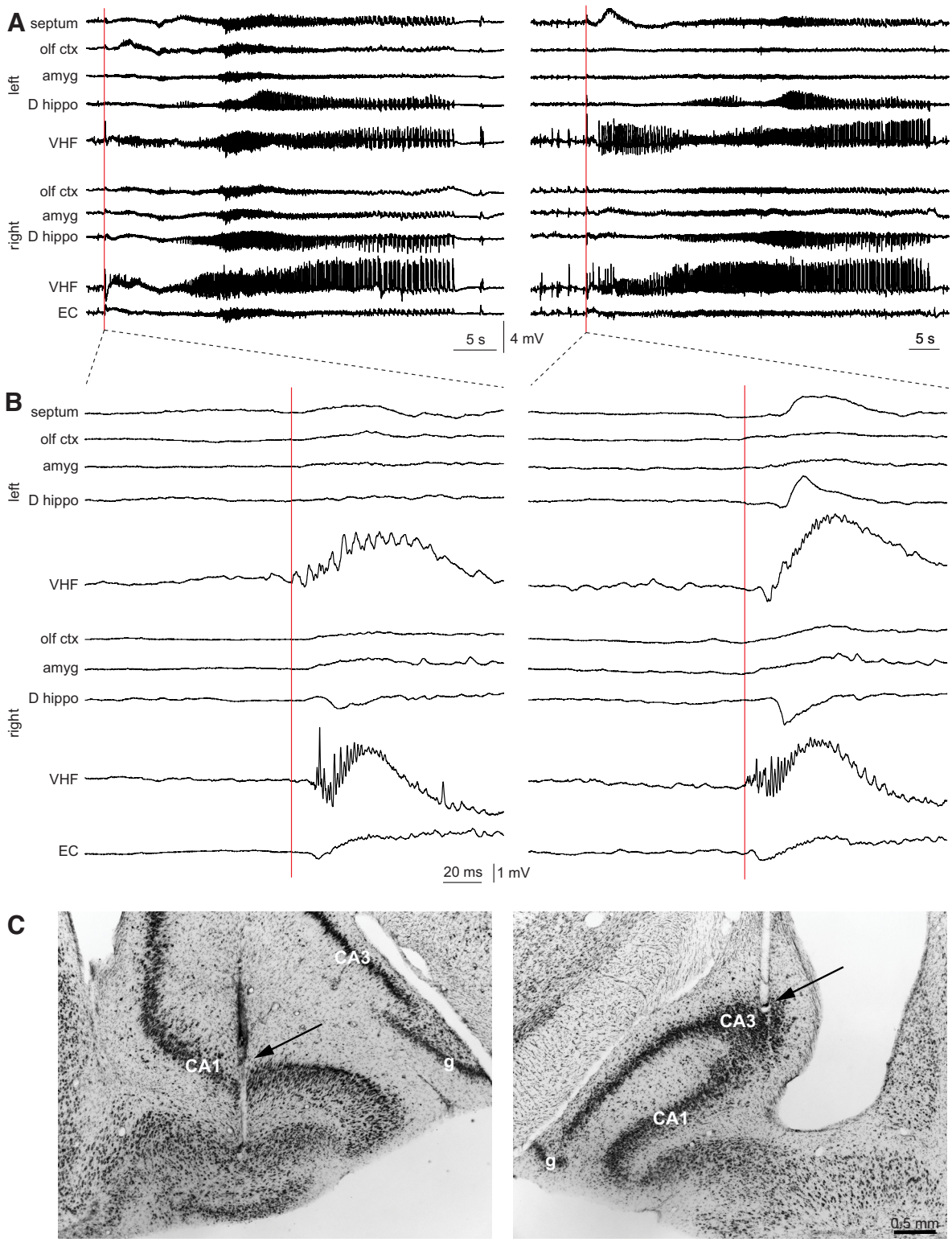

Figure 3. The earliest recorded seizure activity could occur in either hemisphere for different seizures in the same rat (data from Rat 6 in Fig. 4). ***A***, Entire seizures. Red line indicates onsets. Olf ctx, Olfactory cortex; amyg, amygdala; D hippo, dorsal hippocampus; VHF, ventral hippocampal formation; EC, entorhinal cortex. ***B***, The earliest recorded seizure activity was in the left ventral hippocampal formation for the seizure shown in the left column, and the right ventral hippocampal formation for the seizure shown in the right column. ***C***, Electrode locations of earliest recorded seizure activity in the ventral hippocampal formation of the left and right sides, shown in ***B***. In both cases, arrows indicate tips of the shorter of the two bipolar electrode tracks.

0.92 ± 0.08 seizures/h during TTX infusion ($p = 0.22$) (Fig. 2). Seizure duration was 48 ± 12 s during baseline and 51 ± 10 s during TTX infusion ($p = 0.28$). The percentage of seizures that were convulsive was $81 \pm 7\%$ during baseline and $99 \pm 0.2\%$ during TTX infusion ($p = 0.06$). Together, these findings revealed stronger reductions in seizure frequency and seizure duration at higher doses. The minimal dose of TTX that significantly reduced seizure frequency when infused unilaterally in the ventral hippocampal formation was 1 μM.

Table 1. Seizure onset data from Rat 6 in Figure 4[a]

	Electrodes	Onsets	*z* score
Left			
Septum	1	0	−1.77
Olfactory cortex	2	0	−2.58
Amygdala	2	0	−2.58
Dorsal hippocampus	2	0	−2.58
Ventral hippocampal formation	3	31	7.92
Entorhinal cortex	0	—	—
Right			
Septum	0	—	—
Olfactory cortex	2	0	−2.58
Amygdala	3	0	−3.24
Dorsal hippocampus	2	3	−1.29
Ventral hippocampal formation	1	29	15.38
Entorhinal cortex	1	0	−1.77
Other	4	0	−3.46
Total	23	63	

[a]*z* scores >1.96 indicate more onsets than expected by chance based on the number of recording electrodes ($p < 0.05$).

LFP analysis of seizure onset

The strong reduction in seizure frequency at high doses suggested that seizure onset sites in the ventral hippocampal formation had been directly inactivated by TTX infusion. To test that possibility, sites of earliest recorded seizure activity during the initial baseline period were identified. An example from Rat 6 is shown in Figure 3. Rat 6 had 23 useful electrodes (Table 1). Recorded target regions included the left septum, right entorhinal cortex, and bilaterally in the olfactory cortex, amygdala, dorsal hippocampus, and ventral hippocampal formation. Of the 63 seizures analyzed, 49% were earliest in the left ventral hippocampal formation, 46% in the right ventral hippocampal formation, and 5% in the right dorsal hippocampus. The left and right ventral hippocampal formation had significantly more earliest recorded seizures than expected by chance. Yet, infusion of 10 μM TTX only into the left ventral hippocampal formation reduced seizure frequency to 8% of baseline in this rat. In other rats with significant sites of earliest recorded seizure activity bilaterally (Rats 1 and 2; Fig. 4), infusion of 10 μM TTX unilaterally into only one ventral hippocampal formation blocked seizures completely. (Fig. 1 illustrates data from Rat 1.) Together, these findings suggested that unilateral infusion of 10 μM TTX into just one ventral hippocampal formation reduced seizure frequency, even when both ventral hippocampal formations were generating seizures.

Group data from all unilaterally infused rats revealed that 72 ± 5% of sites of earliest recorded seizure activity during the baseline period were in the ventral hippocampal formation; 14 ± 3% in the dorsal hippocampus; 4%-5% in the olfactory cortex, entorhinal cortex, or amygdala, each; and <1% in the septum (Fig. 5*A*). The ventral hippocampal formation was a statistically significant site of earliest recorded seizure activity in 19 of 22 rats, bilaterally in 7 of 19 rats (Fig. 4). Other statistically significant sites of earliest recorded seizure activity included the amygdala (1 rat), olfactory cortex (1), entorhinal cortex (2), and dorsal hippocampus (5). These findings confirmed previous reports that, in pilocarpine-treated rats, the ventral hippocampal formation is the most common site of earliest recorded seizure activity (Toyoda et al., 2013), frequently bilaterally (Wyeth et al., 2020).

Rats displayed convulsive and nonconvulsive seizures. Do the different types of seizures have different onset sites? To test this, 2 × 2 contingency tables were constructed. The first criterion of classification was whether seizures were convulsive or nonconvulsive. The second criterion of classification was whether seizure activity was recorded earliest in the brain region with the highest *z* score versus any other region. Twelve rats had sufficient numbers of both convulsive and nonconvulsive seizures to analyze. χ^2 or Fisher exact tests were used. In only 1 rat was the proportion of convulsive and nonconvulsive seizures first recorded in the region with the highest *z* score different from the proportion recorded in other regions. The lack of contingency of earliest recorded seizure activity on whether a seizure was convulsive or not in 11 of 12 rats analyzed suggested that it was reasonable to combine both convulsive and nonconvulsive seizures for further analysis.

The discordance, in some cases, between seizure-reducing effects of unilateral TTX infusion and sites of earliest recorded seizure activity raised questions. Could it be that earliest recorded seizure activity was simply attributable to fortuitous electrode tip location in a cell layer where LFP amplitudes were large? In the 22 rats treated unilaterally with TTX, there were 568 useful electrodes. Of those, 89 (16%) were located in a dense cell layer. The dorsal hippocampus and ventral hippocampal formation had the highest percentages of electrodes in a cell layer (35 of 102, 34% and 37 of 163, 23%, respectively). The percentage of electrodes in a cell layer was greater in the dorsal hippocampus and ventral hippocampal formation combined (72 of 265, 27%), compared with all other brain regions combined (17 of 302, 6%, $p \leq 0.001$, χ^2 test).

To evaluate LFPs more directly, they were analyzed during a 1 min period of slow wave sleep during each day in all rats. The maximum extent of the spontaneous LFP amplitude during that period was measured. Then, the average during the baseline period was calculated for each electrode in all rats (Fig. 5*B*). LFP amplitudes were largest in the dorsal hippocampus (5.5 ± 0.3 mV) and ventral hippocampal formation (4.7 ± 0.2 mV). They were not significantly different from one another, but both were larger than all other brain regions whose mean amplitudes were 1.9-2.3 mV ($p < 0.05$, Kruskal–Wallis one way ANOVA on ranks with Dunn's method). Together, these findings revealed that, in the dorsal hippocampus and ventral hippocampal formation, electrode tips were more likely to be located in a cell layer, and LFP amplitudes were larger compared with other brain regions. However, if results of earliest recorded seizure activity depended solely on the likelihood of the electrode tip being located in a cell layer and having large LFP amplitudes, then the dorsal hippocampus should have had as many seizure onsets as the ventral hippocampal formation, but that was not the case (Figs. 4, 5*A*).

To further test the possibility that results were attributable to recording conditions and not actual seizure onsets, in each rat the electrode that recorded the most seizure onsets was evaluated. The electrode that most often recorded the earliest seizure activity was in the olfactory cortex in 1 rat, the dorsal hippocampus in 2 rats, and the ventral hippocampal formation in 19 rats. Of the 22 electrodes that most often recorded the earliest seizure activity for each rat, 5 (23%) were in a cell layer. This finding revealed that electrodes that most often recorded the earliest seizure activity usually were not in a cell layer.

For electrodes that most often recorded the earliest seizure activity, LFP amplitude during slow wave sleep (7.6 ± 0.8 mV, median = 5.6 mV) was less than the maximum value of all electrodes in each rat (10.6 ± 2.8 mV, median = 11.7 mV, $p \leq 0.001$,

Wilcoxon signed rank test). Therefore, while LFP amplitudes tended to be large, they were on average significantly less than the maximum. In summary, these results did not support the hypothesis that earliest recorded seizure activity was simply attributable to fortuitous electrode tip location in a cell layer where LFP amplitudes were large. Nevertheless, the LFP recording method of identifying seizure onset sites is limited, which was part of the rationale for deactivating tissue with TTX to discover epileptogenic zones (see Introduction).

The analysis of electrode locations provided an opportunity to address whether specific subfields of the hippocampus were more or less likely to generate earliest recorded seizure activity. In the 22 rats treated unilaterally with TTX, 265 electrodes were in the dorsal hippocampus or ventral hippocampal formation. Of those, 24% (63) were in CA1, 17% (46) in CA3, 33% (88) in the dentate gyrus, and 26% (68) in the subicular complex. The fraction of electrodes that displayed earliest recorded seizure activity at least once was not significantly different between subfields: 37% (23 of 63 electrodes) in CA1, 48% (22 of 46) in CA3, 66% (58 of 88) in the dentate gyrus, and 57% (39 of 68) in the subicular complex ($p = 0.23$, χ^2 test). In 21 rats, the electrode that recorded earliest seizure activity the most was in the dorsal hippocampus or ventral hippocampal formation: 4 in CA1, 0 in CA3, 8 in the dentate gyrus, and 9 in the subicular complex. Based on the total number of recording electrodes in those subfields, there were no significant differences: 6% in CA1, 0% in CA3, 9% in the dentate gyrus, and 13% in the subicular complex ($p = 0.11$, χ^2 test). Together, these findings did not provide compelling evidence for differences in probability of seizure onset sites between hippocampal subfields, but sample sizes are limited.

rat		septum	olfactory cortex	amygdala	dorsal hippocampus	ventral hippocampal formation	entorhinal cortex
1 - 10 μM	left	0	4	0	2	6	1
	right	0	2	1	2	4	1
2 - 10 μM	left	0	3	1	1	5	1
	right	1	1	0	3	4	0
3 - 10 μM	left	2	0	0	2	4	0
	right	2	2	2	3	1	1
4 - 10 μM	left	0	1	2	2	4	0
	right	0	0	1	1	5	0
5 - 10 μM	left	4	1	2	2	4	2
	right	0	1	0	0	5	1
6 - 10 μM	left	1	2	2	2	3	0
	right	0	2	3	2	1	1
7 - 10 μM	left	0	2	1	2	4	2
	right	0	0	1	0	2	3
8 - 10 μM	left	1	2	0	0	3	2
	right	1	0	3	1	5	0
9 - 5 μM	left	1	2	2	3	1	1
	right	0	2	0	3	4	2
10 - 5 μM	left	0	1	1	4	5	1
	right	0	0	2	3	6	0
11 - 1 μM	left	0	2	2	3	5	1
	right	0	2	2	2	5	0
12 - 1 μM	left	4	3	4	3	2	1
	right	0	2	3	2	3	0
13 - 1 μM	left	0	2	1	2	4	2
	right	0	1	3	2	3	0
14 - 1 μM	left	0	3	0	4	3	1
	right	0	1	3	3	5	0
15 - 1 μM	left	2	4	1	2	3	2
	right	0	0	4	2	4	1
16 - 1 μM	left	1	1	1	1	1	5
	right	0	1	2	3	1	3
17 - 1 μM	left	2	2	0	4	7	0
	right	1	1	0	2	5	0
18 - 1 μM	left	0	3	1	3	3	2
	right	1	1	3	3	6	0
19 - 1 μM	left	0	2	2	2	3	3
	right	1	0	5	3	4	0
20 - 0.1 μM	left	2	1	1	4	6	1
	right	0	3	1	4	4	1
21 - 0.1 μM	left	4	2	2	2	2	4
	right	0	2	2	3	3	1
22 - 0.1 μM	left	1	3	2	4	4	1
	right	1	2	2	4	3	1

Figure 4. Sites of earliest recorded seizure activity during the baseline period before TTX was infused unilaterally in the ventral hippocampal formation. Values indicate number of recording electrodes. Brain regions with significantly more sites of earliest recorded seizure activity than chance are highlighted in yellow or red (highest *z* score for that individual). The ventral hippocampus was infused with TTX on the side of the brain highlighted in green.

Electrode locations were identified in Nissl-stained sections that also provided an opportunity to evaluate neuron loss. The pattern and extent of hippocampal cell loss were similar to that reported previously for pilocarpine-treated rats (Mello et al., 1993; Fujita et al., 2014). Qualitatively, neuron loss was sometimes evident in the CA1 and CA3 fields and especially in the hilus. Neuron loss did not appear to be obviously more severe in the ventral hippocampal formation or dorsal hippocampus. Although not quantified, the extent of cell loss in rats appeared to be less than that in tissue resected to treat patients with temporal lobe epilepsy and classified as hippocampal sclerosis Types 1-3 (Blümcke et al., 2013).

TTX effect on LFP

LFP analysis revealed that unilateral infusion of 1-10 μM TTX at 0.25 μl/h in the ventral hippocampal formation reduced seizure frequency, even when sites of earliest recorded seizure activity were in the ventral hippocampal formation contralateral to TTX infusion (Rats 4 and 17), bilateral in the ventral hippocampal formation (Rats 1, 2, 3, 6, 9, 12, and 14), or in other brain regions (Rats 3, 5, 11, 13, 15, and 16). How could focal infusion of TTX reduce seizure frequency if onset sites were remote? It has been proposed that inhibition of any one single epileptogenic node would block seizures in an extended and functionally interdependent hyperexcitable network (Spencer, 2002). Alternatively, concentrations might have been so high that TTX diffused to and directly inhibited nontargeted regions. To help distinguish between these possibilities, effects of unilateral TTX infusion on neural activity were measured. Maximal amplitudes of spontaneous LFPs during slow wave sleep were measured before, during, and after TTX infusion. For example, the right ventral hippocampal formation was infused with 10 μM TTX in Rat 1. The amplitude of the LFP in the ipsilateral ventral hippocampal formation was greatly reduced (Fig. 6*A*). The effect was strongest in the TTX-infused ventral hippocampal formation, but nontargeted regions also were affected. In this case and others, inhibition of the LFP gradually returned to pre-TTX baseline levels after TTX infusion ceased.

To evaluate the spatial extent of focal TTX infusion, sites of cannulas and electrodes were three-dimensionally reconstructed from Nissl-stained coronal sections (Fig. 6*B1*). Distances between electrodes and TTX infusion sites were measured. As

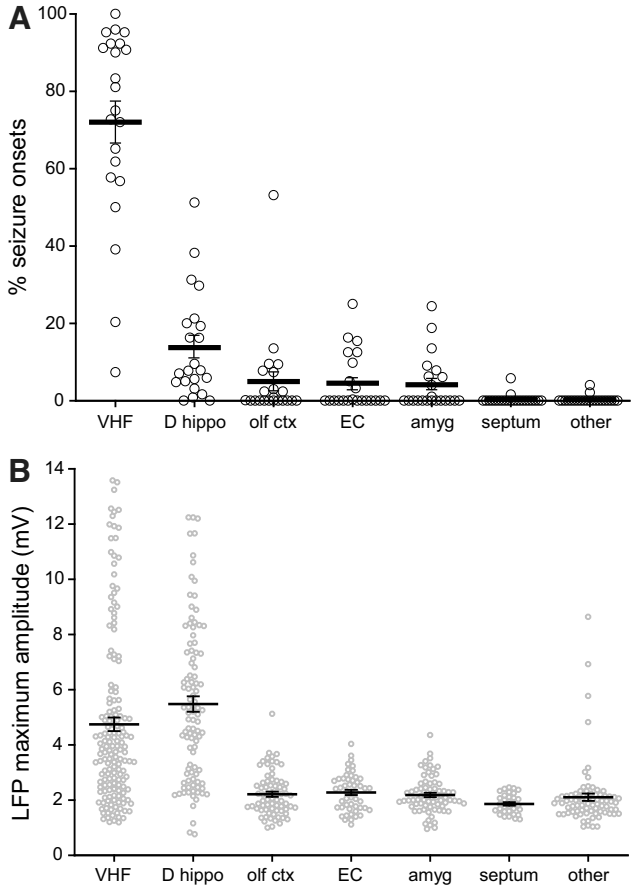

Figure 5. Sites of earliest recorded seizure activity and LFP amplitudes. ***A***, Percent of sites of earliest recorded seizure activity during the initial baseline period. For example, in Rat 1, 95% of the sites of earliest recorded seizure activity were in the ventral hippocampal formation, 1% in the dorsal hippocampus, 1% in the olfactory cortex, 3% in the entorhinal cortex, and 0% in all other brain regions. Values are from all rats that were unilaterally infused with TTX. Horizontal lines indicate averages. Error bars indicate SEM. VHF, Ventral hippocampal formation; D hippo, dorsal hippocampus; olf ctx, olfactory cortex; EC, entorhinal cortex; amyg, amygdala. ***B***, Maximal amplitudes of spontaneous LFPs during a period of slow wave sleep for all electrodes in rats unilaterally infused with TTX. On average, LFPs were largest in the dorsal hippocampus and ventral hippocampal formation, but the earliest seizure activity was 5× more likely to be recorded in the ventral hippocampal formation than in the dorsal hippocampus.

expected, average distances from the TTX cannula to electrodes in the TTX-infused ventral hippocampal formation were shortest, and distances to electrodes in the ipsilateral cerebral hemisphere were shorter than distances to contralateral electrodes (Fig. 6*B2*). To quantify the effect of focally infused 10 μM TTX, average LFP amplitude during infusion was divided by average LFP amplitude during the first baseline period for each electrode. Based only on recording electrode distance from the infusion site (not specifying brain region), group data revealed that inhibitory effects of 10 μM TTX were strongest proximally, decreased with distance, and plateaued at <75% baseline level at 5 mm distance and beyond (Fig. 6*B3*). Next, LFP amplitude data were grouped by brain region. The ventral hippocampal formation is an example. Figure 4 shows that 20 of 22 TTX-infused ventral hippocampi contained multiple recording electrodes (3.8 ± 0.3, range 1-7). The maximum distance between electrodes in a TTX-infused ventral hippocampal formation was 2.6 ± 0.2 mm (range 1.3-4.5 mm). The ventral hippocampal formation, therefore, was sampled with multiple, dispersed electrodes in most cases. Values of all electrodes were averaged for each brain region on each side. Grouping LFP amplitude data by brain region revealed that inhibitory effects were strongest in the ipsilateral ventral hippocampal formation but extended to other regions (Fig. 6*C1*). Together, these findings showed that focal infusion of 10 μM TTX had substantial inhibitory effects beyond the infused ventral hippocampal formation.

The effect of lower doses of TTX on LFP amplitude in nontargeted brain regions was calculated as follows: percent inhibition = 100 × (1 – LFP amplitude (during TTX infusion/during pre-infusion baseline)). The percent inhibition in the TTX-infused ventral hippocampal formation was greatest with 10 μM (58 ± 7%) and least with 0.1 μM (7 ± 10%) (Fig. 6*C2*). Averaging all regions together, except the TTX-infused ventral hippocampal formation, percent inhibition was greatest with 10 μM (28 ± 2%) and virtually zero at 0.1 μM (−1 ± 2%) (Fig. 6*C3*). These findings revealed that 0.1 μM TTX had negligible effects on LFP amplitudes in brain regions beyond the infused ventral hippocampal formation.

Bilateral TTX infusion

The low dose of TTX (0.1 μM) had no significant effect on seizure frequency, duration, or severity when infused unilaterally (Fig. 2). We tested whether that dose would be more effective if infused bilaterally. Surprisingly, bilateral 0.1 μM TTX in the ventral hippocampal formation for 11.3 ± 1.1 d blocked seizures (Fig. 7). For all rats in this group (n = 5), seizure frequency during the pre-TTX baseline period was 0.81 ± 0.32 seizures/h (Fig. 8). Seizure frequency was reduced to 15% of baseline during TTX infusion (0.12 ± 0.04 seizures/h, p = 0.04). Seizure duration was 54 ± 4 s during baseline and 42 ± 11 s during TTX infusion (p = 0.22). The percentage of seizures that were convulsive was 53 ± 19% during baseline and 53 ± 20% during TTX infusion (p = 0.49). These findings showed that low-dose bilateral infusion of TTX in the ventral hippocampal formation reversibly and strongly reduced seizure frequency.

An even lower dose was tested to determine the minimal amount of bilaterally infused TTX that would reduce seizure frequency. For rats treated for 11.7 ± 1.2 d with 0.01 μM TTX in the ventral hippocampal formation bilaterally (n = 3), seizure frequency was 1.42 ± 0.58 seizures/h during the entire baseline period and 1.18 ± 0.63 seizures/h during TTX infusion (p = 0.25) (Fig. 8). Seizure duration during baseline was 68 ± 15 s and 70 ± 17 s during TTX infusion (p = 0.23). The percentage of seizures that were convulsive was 44 ± 5% during baseline and 60 ± 9% during TTX infusion (p = 0.12). Together, these findings suggested that 0.01 μM was not effective, and the minimal dose of TTX that significantly reduced seizure frequency when infused bilaterally into the ventral hippocampal formation was 0.1 μM.

Why was 0.1 μM TTX effective at reducing seizure frequency when infused into the ventral hippocampal formation bilaterally but not unilaterally? One possibility is that TTX was not unilaterally infused at the site of seizure onset. On the contrary, the 3 rats treated unilaterally with 0.1 μM TTX (Rats 20, 21, and 22) were infused in the same ventral hippocampal formation with the highest z score for earliest recorded seizure activity (Fig. 4). Why then didn't 0.1 μM TTX block seizures when infused unilaterally in the region of seizure onset? In all 3 rats, TTX reduced but did not completely eliminate the probability of earliest recorded seizure activity in the infused ventral hippocampal formation. Average z score for earliest recorded seizure activity in the TTX-infused ventral hippocampal formation decreased from 5.33 ± 2.18 during vehicle infusion to 31% of baseline during TTX infusion (1.66 ± 1.90, p = 0.003). Although the probability

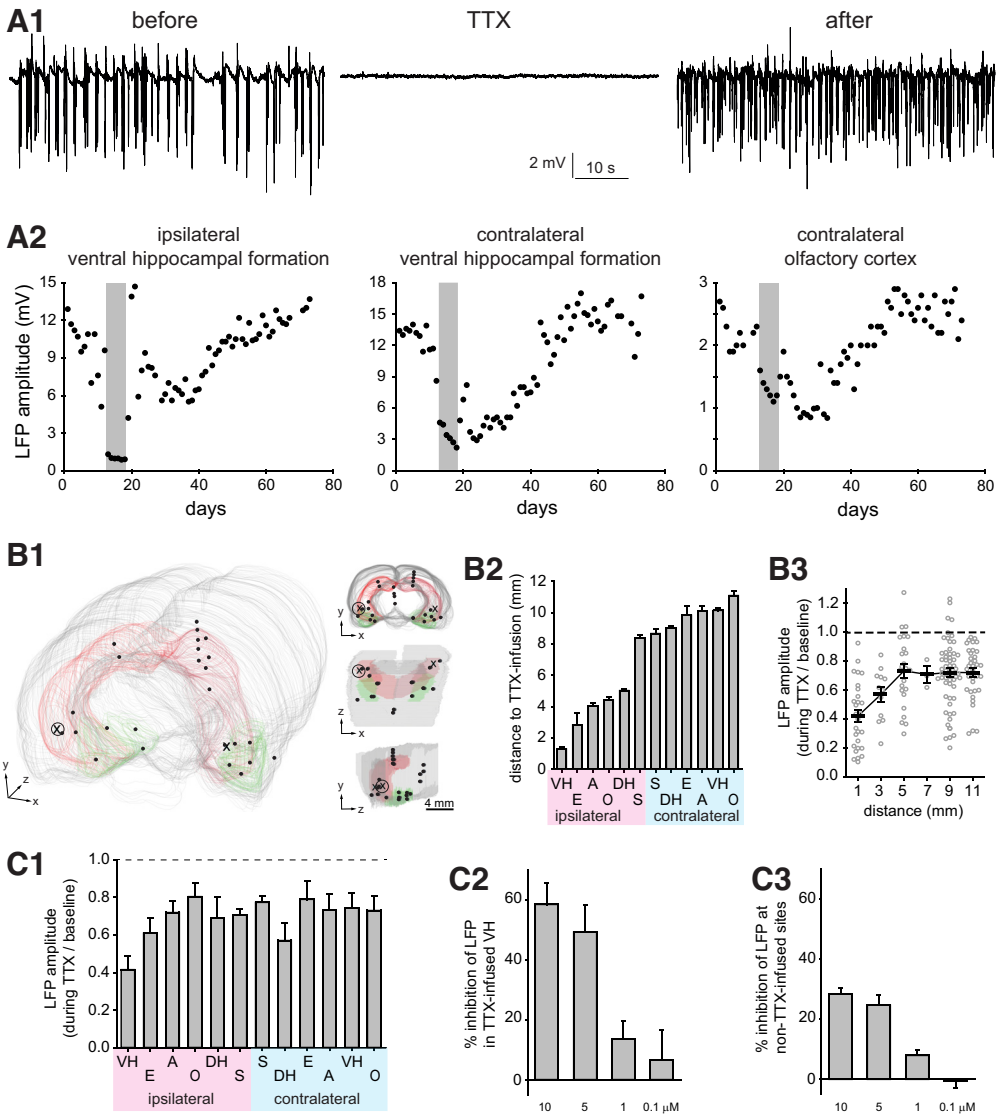

Figure 6. Unilateral 10 μM but not 0.1 μM TTX reduced LFP amplitude outside the infused ventral hippocampal formation. ***A1***, Spontaneous LFPs during slow wave sleep in the ipsilateral ventral hippocampal formation before, during, and after focal infusion of 10 μM TTX (data from Rat 1 in Fig. 4.) ***A2***, Maximum amplitude of LFP during slow wave sleep before, during (gray bar), and after focal infusion of 10 μM TTX. ***B1***, Sites of focal TTX infusion and recording electrodes (data from Rat 3 in Fig. 4). x indicates cannula locations. x in a circle indicates TTX infusion site. Filled circles represent recording electrode sites. Red outline indicates hippocampus. Green outline indicates amygdala. ***B2***, Recording electrode distances to focal TTX infusion site by brain region. Values are mean ± SEM; $n = 8$ rats. VH, Ventral hippocampal formation; E, entorhinal cortex; A, amygdala; O, olfactory cortex; DH, dorsal hippocampus; S, subiculum. ***B3***, Effect of 10 μM TTX infusion on LFP amplitude by distance. Values indicate amplitude during TTX infusion divided by amplitude during baseline for each electrode in all 8 rats infused unilaterally with 10 μM TTX. ***C1***, Effect of 10 μM TTX infusion on LFP amplitude by brain region. ***C2***, Dose effect of TTX infusion on LFP amplitude in the infused ventral hippocampal formation. ***C3***, Dose effect of TTX on LFP amplitude in all brain regions, except the infused ventral hippocampal formation.

of recording earliest seizure activity decreased in the TTX-infused ventral hippocampus (Fig. 9), seizure frequency was not significantly reduced in these rats (Fig. 2). Evaluating sites of earliest recorded seizure activity during the period of TTX infusion revealed that they become more likely in other brain regions, especially the contralateral ventral hippocampal formation. For example, in Rat 20, the TTX-infused ventral hippocampal formation remained the only significant site of earliest recorded seizure activity, but earliest recorded seizure activity in the contralateral ventral hippocampal formation increased from 14% during vehicle infusion to 33% during TTX infusion. In Rat 21, the TTX-infused ventral hippocampal formation ceased being a significant site of earliest recorded seizure activity. The contralateral dorsal hippocampus remained a site of earliest recorded seizure activity. In addition, the contralateral ventral hippocampal formation became the site with the highest z score during TTX infusion. In Rat 22, the TTX-infused ventral hippocampal formation was no longer a significant site of earliest recorded seizure activity, nor was the contralateral entorhinal cortex. Instead, the contralateral ventral hippocampal formation became the only significant site of earliest recorded seizure activity during TTX infusion. After TTX infusion ceased, z scores for earliest recorded seizure activity in the infused ventral hippocampal formation returned toward pre-TTX baseline levels in rats 21 and 22 but not in rat 20. Together, these findings showed that, when focally inhibiting a ventral hippocampus where most earliest seizure activity was recorded, earliest recorded seizure activity became more frequent primarily in the contralateral ventral hippocampal formation, while overall seizure frequency remained stable.

To test whether treatment outside of the ventral hippocampal formation would block seizures, other regions were infused bilaterally with 0.1 μM TTX. In human patients with temporal lobe

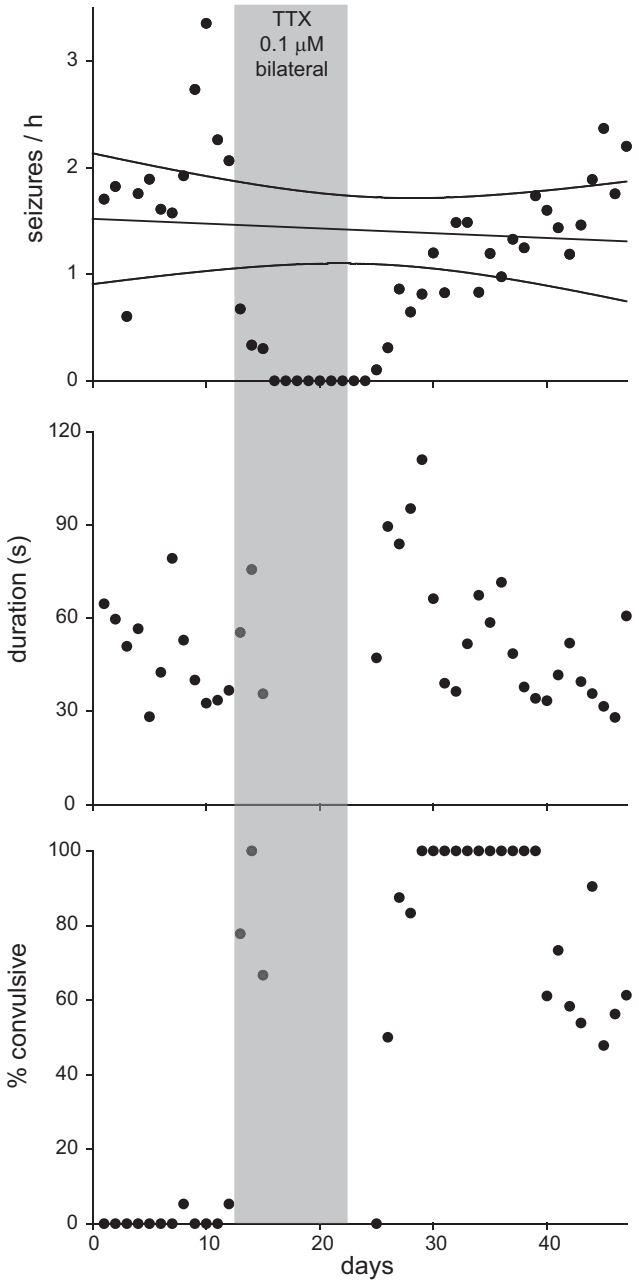

Figure 7. Bilateral infusion of 0.1 μM TTX at 0.25 μl/h into the ventral hippocampal formation reversibly blocked seizures in a pilocarpine-treated rat. Average daily seizure frequency, average daily seizure duration, and average daily percentage of seizures that were convulsive. Gray bar represents period of TTX infusion. The plot of average daily seizure frequency includes a regression line and 99% CIs for baseline values, including those before TTX infusion and after, starting when seizure frequency began to recover from zero.

epilepsy, the amygdala is second only to the hippocampal formation in seizure initiation (Quesney, 1986; So et al., 1989a; Sperling and O'Connor, 1990; Spanedda et al., 1997; Wennberg et al., 2002). Rats were infused for 12.3 ± 1.9 d at 0.25 μl/h with 0.1 μM TTX bilaterally in the amygdala ($n = 3$). Nissl staining revealed proper placement of infusion cannulas (Fig. 10*A*). Like most other rats in this study, sites of earliest seizure activity were in the ventral hippocampal formation, not the amygdala. Seizure frequency was 0.69 ± 0.35 seizures/h during baseline and 0.55 ± 0.25 seizures/h during TTX infusion ($p = 0.16$) (Fig. 8). Seizure duration was 72 ± 18 s during baseline, and it was reduced to 78% of baseline during TTX infusion (56 ± 12 s, $p = 0.047$). The percentage of seizures that were convulsive was 81 ± 10% during baseline and 93 ± 6% during TTX infusion ($p = 0.10$). These findings revealed that the amygdala was not as epileptogenic as the ventral hippocampal formation in pilocarpine-treated rats.

The dorsal hippocampus was evaluated because, after the ventral hippocampal formation, it is the next most common site of earliest recorded seizure activity in pilocarpine-treated rats (Figs. 4, 5*A*) (Toyoda et al., 2013; Wyeth et al., 2020). Nissl staining revealed proper placement of infusion cannulas (Fig. 10*B*). For rats treated bilaterally for 13.0 ± 0.7 d with 0.1 μM TTX in the dorsal hippocampus ($n = 4$), seizure frequency was 1.32 ± 0.39 seizures/h during baseline, and it was reduced to 1.10 ± 0.36 seizures/h during TTX infusion ($p = 0.01$) (Fig. 8). All 4 rats had significant sites of earliest recorded seizure activity in the ventral hippocampal formation, and 2 of the 4 rats had a significant site of earliest recorded seizure activity in a dorsal hippocampus. Those with a dorsal hippocampus early seizure site did not have greater seizure reductions than those without. Seizure duration was 58 ± 5 s during baseline and 50 ± 6 s during TTX infusion ($p = 0.11$). The percentage of seizures that were convulsive was 69 ± 9% during baseline and 52 ± 14% during TTX infusion ($p = 0.09$). These findings suggested that the dorsal hippocampus could be an epileptogenic zone in pilocarpine-treated rats, but less than the ventral hippocampal formation.

In rats infused bilaterally in the dorsal hippocampus ($n = 4$), the dorsal hippocampus accounted for 15 ± 5% of the sites of earliest recorded seizure activity during the baseline period. TTX infusion in the dorsal hippocampus reduced seizure frequency 17% to 83% of baseline. Thus, there was similarity in the average percentage of sites of earliest recorded seizure activity during the baseline period and the average percent reduction in seizures during bilateral inhibition. These findings raised the question whether the same was true for the ventral hippocampal formation. The percent reduction in seizure frequency by bilateral infusion of the ventral hippocampal formation ($n = 5$) with 0.1 μM TTX was 85% (Fig. 8), which was close to the sum (84%) of sites of earliest recorded seizure activity in the ventral hippocampal formation (55%) and dorsal hippocampus (29%) in this group. These results suggested that TTX infusion in the ventral hippocampal formation might also have reduced seizure onsets in the dorsal hippocampus, but not vice versa.

Results of TTX infusion on seizure frequency are summarized in Figure 11. Unilateral infusion of the ventral hippocampal formation with TTX at 10, 5, and 1 μM significantly reduced seizure frequency. However, those concentrations also reduced LFP amplitudes in noninfused brain regions (Fig. 6*C3*). At 0.1 μM TTX, a concentration that did not affect average LFP amplitudes in noninfused brain regions, unilateral infusion had no significant effect on seizure frequency. Bilateral infusion of 0.1 μM TTX in the amygdala did not significantly reduce seizure frequency. In contrast, bilateral infusion of 0.1 μM TTX in the ventral and dorsal hippocampus significantly reduced seizure frequency. The effect was >4 times stronger in the ventral hippocampal formation. Together, these findings suggested that the bilateral hippocampus, especially the ventral hippocampal formation, is the primary epileptogenic zone in pilocarpine-treated rats.

Discussion

In pilocarpine-treated rats, the electrode location recording earliest seizure activity is variable but mostly in the ventral hippocampal formation. Seizures are blocked by continual infusion of high-dose TTX into the ventral hippocampal formation

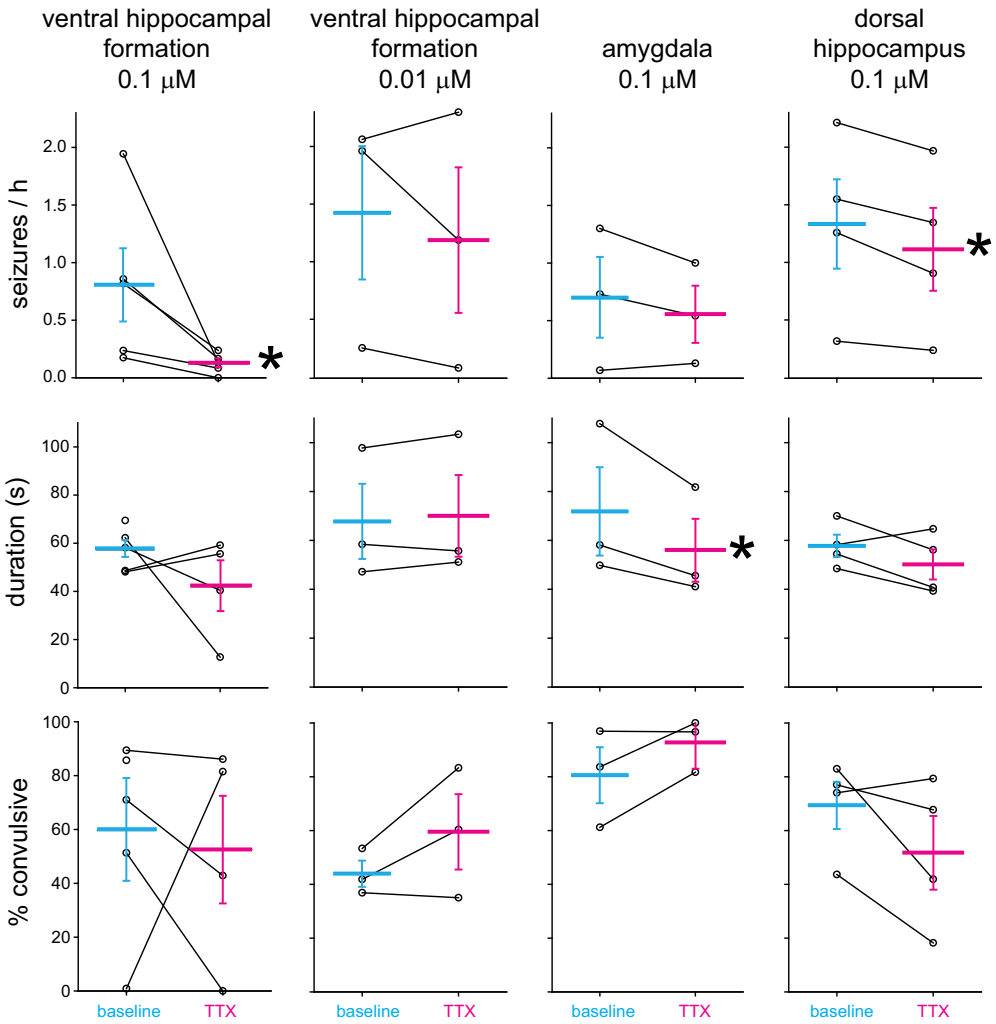

Figure 8. Bilateral infusion of 0.1 μM TTX reduced seizure frequency when targeted to the ventral hippocampal formation, with less effect in the dorsal hippocampus and no significant effect in the amygdala. Infused brain regions and TTX concentrations indicated. Horizontal lines indicate averages. Error bars indicate SEM. *$p < 0.05$, significant difference (paired t test).

unilaterally or by low-dose TTX bilaterally. Low-dose bilateral infusion in the ventral hippocampal formation is more effective than in the amygdala or dorsal hippocampus.

Ventral hippocampal formation is a primary, bilateral, independent epileptogenic zone

LFP recording reveals the ventral hippocampal formation as the most common site of earliest recorded seizure activity in pilocarpine-treated rats (Toyoda et al., 2013; Wyeth et al., 2020). The finding that focal, bilateral inhibition of neural activity in the ventral hippocampal formation blocks seizures almost completely in pilocarpine-treated rats confirms that it is the most common onset site. The same treatment in other brain regions has much smaller effects. The smaller or negative effects outside the ventral hippocampal formation additionally serve as controls for damage by cannulas and for spread of TTX to nontargeted regions.

Bilateral ablation of the ventral hippocampal formation can reverse amygdala-kindling permanence (Yoshida, 1984). Inhibitory neuron transplantation to the bilateral hippocampus, including the ventral hippocampal formation, reduces seizure frequency in pilocarpine-treated mice (Hunt et al., 2013). Therefore, results of recording earliest seizure activity and experimental manipulations converge to support the conclusion that the ventral hippocampal formation is the primary epileptogenic zone in this and perhaps some other animal models of temporal lobe epilepsy. Resection or ablation of the homologous anterior part of the hippocampus is a common surgical therapy for patients (Kaiboriboon et al., 2015). Thus, the epileptogenic role of the homologous part of the hippocampal formation is a common characteristic of human patients with temporal lobe epilepsy and pilocarpine-treated rats.

Bilateral infusion of low-dose TTX into the ventral hippocampal formation strongly suppressed seizures and had no obvious side effects, although behavioral testing was not performed. These findings support the possibility of convection-enhanced drug delivery as a therapeutic option for some patients with temporal lobe epilepsy (Rogawski, 2009).

Bilateral infusion of low-dose TTX into the ventral hippocampal formation strongly suppressed seizures. Yet, during baseline recording, earliest recorded seizure activity sometimes occurred outside the ventral hippocampal formation. The reason for the apparent discrepancy between the broader range of sites of earliest recorded seizure activity and the more focused site most sensitive for blocking seizures is unclear. It might be attributable to challenges of identifying seizure onset sites with LFP recording. Perhaps sparse spatial

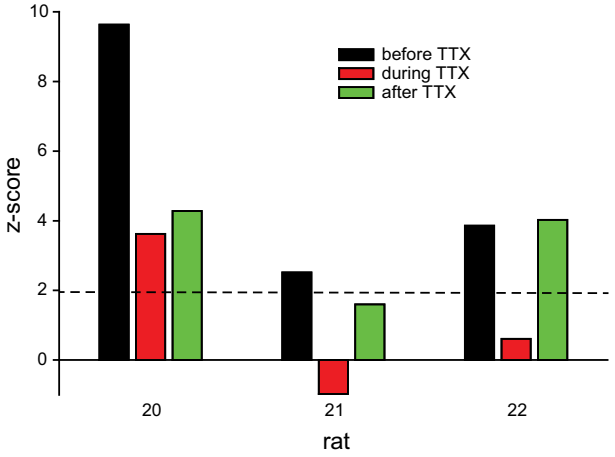

Figure 9. TTX reduced the probability that the infused hippocampal formation was the site of earliest recorded seizure activity. Data from rats treated unilaterally with 0.1 μM TTX in the ventral hippocampal formation with the highest probability for earliest recorded seizure activity indicated by *z* score (details on Rats 20, 21, and 22 are provided in Fig. 4). Dashed line at 1.96, above which *z* scores are statistically significant ($p < 0.05$). In each rat, 0.1 μM TTX reduced the likelihood of seizures being first recorded in the infused ventral hippocampal formation. However, in these unilaterally infused rats, seizure frequency was unchanged as seizure onset became more likely at other sites, especially the contralateral ventral hippocampal formation.

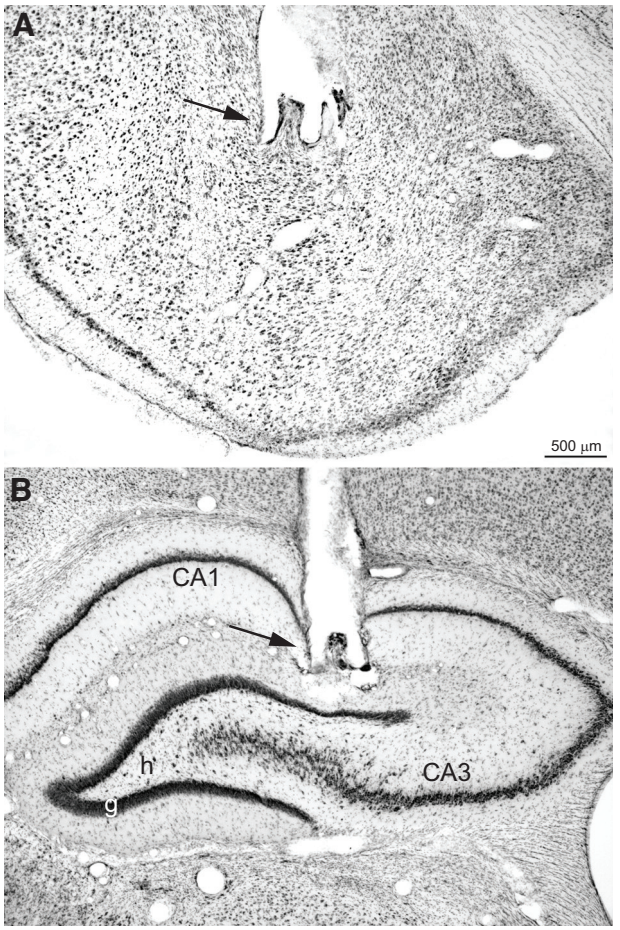

Figure 10. Cannula sites (arrows) of rats infused bilaterally in the amygdala (***A***) or dorsal hippocampus (***B***) with 0.1 μM TTX. h, Hilus; g, granule cell layer. All cannula sites were confirmed histologically.

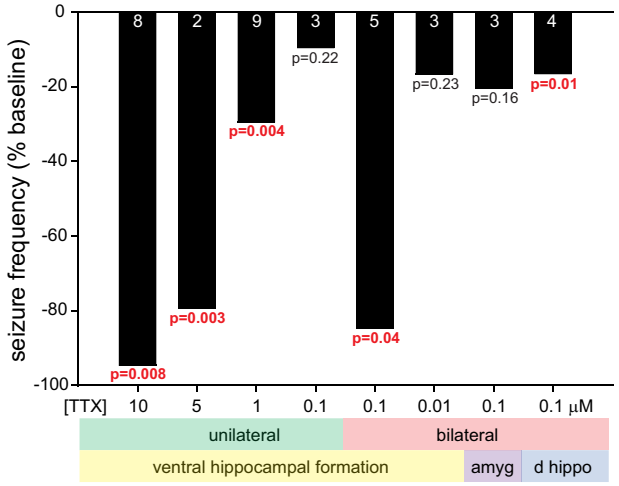

Figure 11. Average effects of TTX infusion relative to baseline seizure frequency. Summary of results from Figures 2 and 8. Number of subjects indicated in bars.

sampling and fast seizure spread result in "noise" in the earliest recorded seizure activity. Alternatively, onset sites might be genuinely variable across brain regions, and yet the ventral hippocampal formation is the primary critical hub for controlling seizures. If so, it would suggest a more complicated concept of focal seizure onsets.

In pilocarpine-treated rats, both ventral hippocampal formations are epileptogenic zones, independently generating seizures. Earliest recorded seizure activity commonly varies between the right and left ventral hippocampal formation. Focal inactivation of only one ventral hippocampal formation with low-dose TTX does not reduce seizure frequency. Similarly, in some people with temporal lobe epilepsy, interictal spikes and seizure onsets can be bilateral; and in those cases, unilateral surgical treatment typically fails to control seizures (So et al., 1989a,b). When one ventral hippocampal formation is inhibited in rats, seizure onsets tend to shift to the contralateral ventral hippocampal formation. Available evidence from scalp recording and semiology suggest that, after unilateral surgical resection, persistent seizures can begin ipsilaterally or contralaterally in patients with bilateral temporal lobe epilepsy (So et al., 1989b), similar to pilocarpine-treated rats.

Cases of temporal lobe epilepsy in people can be bilateral or unilateral. For example, 75% of patients with hippocampal sclerosis have unilateral lesions (Briellmann et al., 1999). In contrast, in rodents that develop temporal lobe epilepsy after status epilepticus caused by systemic treatment with convulsants, hippocampal neuron loss is consistently bilateral (Buckmaster and Dudek, 1997). The cause of the difference is unclear. The initial precipitating injury is not a likely explanation. Rats develop bilateral temporal lobe epilepsy after status epilepticus caused by systemic treatment with an excitotoxin. However, similar systemic exposure to an excitotoxin usually results in unilateral temporal lobe epilepsy in sea lions (Buckmaster et al., 2014). Sea lions, like humans, have much larger brains than rats. Larger brain size and less commissural connectivity are possible causes for the greater likelihood of unilateral temporal lobe epilepsy in humans versus rats.

Dorsal hippocampus is a secondary epileptogenic zone

The dorsal and ventral hippocampus in rodents have different patterns of connectivity, gene expression, and function (for

review, see Moser and Moser, 1998; Fanselow and Dong, 2010). They also activate different networks when seizing (Duffy et al., 2020). Confirming previous studies with pilocarpine-treated rats (Toyoda et al., 2013; Wyeth et al., 2020), the present study found that, after the ventral hippocampal formation, the dorsal hippocampus is the next most common site of earliest recorded seizure activity, accounting for ~15%. A novel finding is that seizure frequency deceases ~15% after bilateral focal inhibition of the dorsal hippocampus. A simple explanation for these results is that 15% of the seizures start in the dorsal hippocampus; and when it is inhibited, seizures that would have started there are instead blocked. However, underlying ictogenic mechanisms might be more complicated.

In pilocarpine-treated rats, ictogenesis in the dorsal hippocampus might depend on the ventral hippocampal formation. Bilateral infusion of low-dose TTX into the ventral hippocampal formation reduces seizure frequency to an extent equal to the sum of seizure onsets in ventral hippocampal formation plus dorsal hippocampus. This finding suggests that activity generated in the ventral hippocampal formation might propagate to the dorsal hippocampus and trigger a seizure there. In patients with temporal lobe epilepsy, interictal spikes are most common in the anterior temporal lobe (Emerson et al., 1995), and they propagate posteriorly (Umeoka et al., 2012). Anterior hippocampus in humans is homologous with ventral hippocampus in rats. Transection of the human hippocampus between anterior and posterior parts can block propagation of interictal spikes and greatly reduce or eliminate seizures in patients with unilateral temporal lobe epilepsy (Shimizu et al., 2006; Umeoka et al., 2012; Patil and Andrews, 2013; Koubeissi et al., 2016). In addition, some patients in whom depth recording reveals bilateral hippocampal seizure onsets can still become seizure-free after unilateral resection of just one anterior temporal lobe, if rigorous case selection criteria are used (Hirsch et al., 1991). Together, these findings are consistent with the possibility that a primary epileptogenic region can trigger seizure onsets in a secondary region, and that seizures can be blocked effectively by resecting or inactivating only the primary region. Pilocarpine-treated rats might be a useful animal model to further investigate this phenomenon.

Limitations

Pilocarpine-treated rats do not represent all aspects of all patients with temporal lobe epilepsy. Rats were extensively implanted with 32 LFP recording electrodes. Nevertheless, constraints of LFP recording result in sparse spatial sampling for seizure onset detection, raising the question of whether a seizure started in an unrecorded area. Rats were extensively monitored for seizures, but not 24/7. TTX provided long-lasting reversible inhibition of infused brain regions. However, it could inhibit action potentials in fibers of passage and have unintended side effects. Sparse spatial sampling with LFP recording electrodes prevented complete characterization of the extent of inhibited neural activity by TTX infusion. Focal inhibition of one brain region could have indirect downstream effects by reducing afferent activity of other regions. Nevertheless, TTX infusion demonstrated that the bilateral ventral hippocampal formation is a primary zone for generating seizures. Finally, despite evaluating 37 rats and >18,000 seizures, sample sizes are limited.

Ictogenesis hypotheses

It has been proposed that ictogenesis in temporal lobe epilepsy involves a network that includes the hippocampi, amygdalae, entorhinal cortices, lateral temporal neocortices, and components of the medial thalamus and frontal lobes, and that treatment directed to any single region of the network should be just as effective as treatments directed at a specific focus (Spencer, 2002). Results of the present study from a rat model of temporal lobe epilepsy do not support this hypothesis. Low-dose focal, bilateral infusion of TTX in the ventral hippocampal formation was much more effective at blocking seizures than in the dorsal hippocampus or amygdala.

Another ictogenesis hypothesis of temporal lobe epilepsy contends that hyperactivity coalesces among distributed small pathologic aggregates of neurons to initiate a clinically observable seizure (Bragin et al., 2000). Support for this hypothesis includes recordings in human patients of microseizures (Schevon et al., 2008; Weiss et al., 2016) that are more frequent and dense in epileptogenic zones (Stead et al., 2010). Data from the present study are consistent with this hypothesis. Findings in pilocarpine-treated rats suggest the possibility that small, ictogenic aggregates of neurons distributed in olfactory cortex, amygdala, entorhinal cortex, and dorsal hippocampus might account for the broader distribution of seizure onsets, but with the highest density in the ventral hippocampal formation bilaterally. Furthermore, activity in the ventral hippocampal formation sometimes might be necessary for ictogenesis in remote regions. This hypothesis requires further testing.

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