# Recurrent limbic seizures do not cause hippocampal neuronal loss: A prolonged laboratory study

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## Abstract

**Purpose:** It remains controversial whether neuronal damage and synaptic reorganization found in some forms of epilepsy are the result of an initial injury and potentially contributory to the epileptic condition or are the cumulative affect of repeated seizures. A number of reports of human and animal pathology suggest that at least some neuronal loss precedes the onset of seizures, but there is debate over whether there is further damage over time from intermittent seizures. In support of this latter hypothesis are MRI studies in people that show reduced hippocampal volumes and cortical thickness with longer durations of the disease. In this study we addressed the question of neuronal loss from intermittent seizures using kindled rats (no initial injury) and rats with limbic epilepsy (initial injury).

**Methods:** Supragranular mossy fiber sprouting, hippocampal neuronal densities, and subfield area measurements were determined in rats with chronic limbic epilepsy (CLE) that developed following an episode of limbic status epilepticus ($n = 25$), in kindled rats ($n = 15$), and in age matched controls ($n = 20$). To determine whether age or seizure frequency played a role in the changes, CLE and kindled rats were further classified by seizure frequency (low/high) and the duration of the seizure disorder (young/old).

**Results:** Overall there was no evidence for progressive neuronal loss from recurrent seizures. Compared with control and kindled rats, CLE animals showed increased mossy fiber sprouting, decreased neuronal numbers in multiple regions and regional atrophy. In CLE, but not kindled rats: 1) Higher seizure frequency was associated with greater mossy fiber sprouting and granule cell dispersion; and 2) greater age with seizures was associated with decreased hilar densities, and

increased hilar areas. There was no evidence for progressive neuronal loss, even with more than 1000 seizures.

**Conclusion:** These findings suggest that the neuronal loss associated with limbic epilepsy precedes the onset of the seizures and is not a consequence of recurrent seizures. However, intermittent seizures do cause other structural changes in the brain, the functional consequences of which are unclear.

### Keywords

Epilepsy pathology; Axon plasticity; Mossy fibers; Epileptogenesis; Pathogenesis; Temporal lobe epilepsy

## 1.    Introduction

Mesial temporal lobe epilepsy (MTLE) with hippocampal sclerosis (HS) is a common epilepsy syndrome (Babb and Brown, 1987; Blumcke et al., 1999; Wieser, 2004). Whether HS is the cause or the consequence of seizures has been debated for years (Mathern et al., 1997a, 1997b). One proposal holds that HS occurs early in brain development or as a result of an early injury (initial precipitating injury: IPI) such as prolonged febrile seizures. The neuronal loss is thus acute and non-progressive (Margerison and Corsellis, 1966). A second hypothesis suggests that HS is the cumulative consequence of repeated seizures and that individual, self-limited seizures are harmful (Sutula et al., 1994; Kalviainen et al., 1998). MRI studies in people with TLE have reported volume loss over several years of the disease, and the authors have concluded that the findings are supportive of the progressive seizure induced neuronal loss (Kalviainen and Salmenpera, 2002; Briellmann et al., 2002; Fuerst et al., 2003; Bernhardt et al., 2009; Caciagli et al., 2017, Tasch et al., 1999.). In examining animal models of limbic seizures a few investigators have suggested that recurrent kindled seizures can lead to neuronal loss and hence be a possible source of HS (Cavazos and Sutula, 1990; Cavazos et al., 1994). However studies from other investigators of the pathology in animals and people have not found strong evidence for progressive neuronal loss. These studies have concluded that the majority of the neuronal loss precedes the development of epilepsy. (Bertram et al., 1990; Bertram 3rd and Lothman, 1993; Davies et al., 1996; Gorter et al., 2003; Thom et al., 2005; Sen et al., 2005; Garbelli et al., 2017).

Adult rats that have status epilepticus (SE) greater than 3 to 4 hours frequently have acute, severe hippocampal neuron loss and mossy fiber synaptic reorganization similar to human HS (Mathern et al., 1998a, 1998b). Furthermore, post-SE rats with neuronal loss usually have a latent period before the development of spontaneous seizures (Nissinen et al., 2000; Mathern et al., 1998a, 1998b; Lothman et al., 1990), an observation that suggests that the critical processes leading to seizures take place during the recovery from the neuronal injury. One study found that neuronal cell death in CLE rats was due to the initial SE and not by repeated spontaneous seizures and that the majority of cells that die, die within the first week following SE (Gorter et al., 2003). The issue of whether recurrent intermittent seizures can cause progressive damage has reappeared with the recent observation that a number of people with Alzheimer's disease may have frequent clinically unrecognized seizures involving the temporal lobes (Lam et al., 2017; Vossel et al., 2017). The question has been

raised whether in these people control of the seizures might alter the progression of the disease. Because of these questions it is important to determine whether recurrent seizures do cause neuronal loss and if the loss is cumulative over time.

The present study was designed to address this question by examining hippocampal pathology (volume changes, mossy fiber sprouting and neuronal loss) in post-SE (IPI) and kindled rats (intermittent seizures only) over many months. We had previously shown that hippocampal neuron loss and supragranular mossy fiber sprouting is greater in rats with spontaneous epilepsy compared with kindled and controls (Mathern et al., 1997a, 1997b). In this study we hypothesized that increased seizure frequency would be associated with greater neuronal loss and that cumulative seizure numbers would add to the loss.

## 2. Materials and methods

In this study, all of the whole animal work from surgery to preparing the brains for the histological studies was performed at the University of Virginia. Each blocked brain was given a code before shipment to UCLA in batches that included animals from each group. The histological analysis was performed at UCLA, and that team was blinded to which group each brain belonged. The code was only broken after the data collection was finished before the statistical analysis was performed.

### 2.1. Experimental Design and Comparison Groups

All experimental procedures were performed using approved institutional animal research protocols, and three groups of rats were prepared. Listed ages are time after first stimulation in months. All rats were approximately three months of age when stimulation started.

**Chronic limbic epilepsy (CLE; *n* = 25).**—These rats developed spontaneous limbic seizures following an episode of limbic status epilepticus induced by electrical hippocampal stimulation. The animals survived between 4 and 16 months following SE.

**Kindled (*n* = 15).**—These rats were kindled through a hippocampal electrode. They were included in this study to determine the cumulative effect of seizures over time in the absence of an initial event that caused neuronal loss (i.e. SE in this study).

**Controls. (*n* = 20)**—This group consisted of rats without chronic seizures. It was composed of three subgroups: 1) rats implanted with electrodes but not stimulated (*n* = 7). 2) CHS rats that did not go into SE and did not have chronic seizures (n = 7). This subgroup was included to determine the effect of stimulation alone on hippocampal structure. 3) CHS rats that experienced acute SE but did not show chronic epilepsy (*n* = 6). This subgroup was included to determine if the experience of SE that did not lead to chronic epilepsy had an effect on hippocampal anatomy. These latter two groups were initially evaluated independently, but were included in the overall control group when it was determined that there were no differences among these 3 subgroups. These control groups were chosen to evaluate the effects of age, stimulation and status epilepticus without the possible effect of intermittent seizures.

A primary goal of this study was to determine whether there was a relationship between the changes in hippocampal pathology and the severity of the seizure disorder (defined by seizure frequency). A separate goal was to determine the effect of cumulative numbers of seizures as determined, in part by the age of the animal. For seizure frequency, kindled and CLE rats were separated into low (5 or less stimulated or spontaneous seizures per week) and high frequency categories (greater than 5 events per week. The low frequency rats had less than 100 total seizures and the high frequency animals had more than 100. To examine the potential effect of the duration of the seizure disorder on hippocampal structure, kindled and CLE rats were also separated into young (less than 6 months from first kindled or SE event) or old categories (more than 6 months). There were several reasons for these seemingly arbitrary definitions of grouping by seizure numbers and age. There was a clear separation between the low and high frequency, with the greatest number seizures in the low frequency group was 98 and the lowest number of seizures in the high frequency group was 130. The six month age separation had a similar clear split, and our previous studies had largely used animals that were less than 6 months of age.

## 2.2. Animal Surgery

At the University of Virginia, male Sprague-Dawley rats (225–275 g) were anesthetized (ketamine/xylazine) and bipolar twisted insulated stainless steel electrodes placed symmetrically in both posterior ventral hippocampi using stereotactic coordinates (from bregma AP −5.3 mm; ML ±4.9 mm; DV −5.0 mm, incisor bar −3.3 mm). Ground and reference electrodes were placed over the cerebellum, and all electrodes were connected to a strip connector (Amphenol), and the electrode assembly was secured to the skull with stainless steel jeweler's screws and dental acrylic.

## 2.3. Continuous Hippocampal Stimulation to Induce SE

At least one week after electrode placement, 38 rats were electrically stimulated in one hippocampus continuously for 90 min according to a standard protocol (10 s trains of biphasic 50 Hz, 1 ms pulse width, 400 μA peak-to-peak square waves every 11 s) (Lothman et al., 1990). After 90 min, CHS was discontinued and CHS-SE rats continued in self-sustained limbic status epilepticus (SSLSE) for the next 6 to 12 h. The duration of SE was monitored electrographically and behaviorally, and no drugs were given during SSLSE.

## 2.4. Seizure Documentation

At a minimum of 2 months post-CHS, rats were monitored from the same electrode array 24 h per day over multiple weeks to record the number and frequency of spontaneous seizures. Previous studies of the CHS rat model indicate that there is a standard evolution pattern in the development of CLE (Bertram and Cornett, 1994). There is an initial post-CHS seizure-free latent period with a mean of 14 days. Following the latent period spontaneous seizures begin, and the early seizures are often brief, showing limbic EEG-onsets with behavioral arrest and staring without secondary generalization (Bertram, 1997). Over time, the seizures increase in number and severity such that by 12 to 16 weeks post CHS the seizure frequency per rat becomes stable for the life of the animal, and ictal EEG onsets involve multiple limbic sites simultaneously (Bertram and Cornett, 1994; Bertram, 1997).

During the chronic EEG recordings rats were housed in special acrylic cages that allowed full freedom of movement, and they were connected via electrical swivels and cables to an analog Grass Model 8 EEG machine interfaced with a commercial seizure detection package (Monitor, Stellate Systems). Although our experience has shown that this system detects more than 95% of the spontaneous seizures from the intracerebral electrodes, when multiple animals are recorded simultaneously, there are many false positive detections. For this reason, the full 24 h of EEG was reviewed manually in fast forward mode daily. Six CHS rats that had status epilepticus had no recorded spontaneous seizures over a minimum of 4 weeks of continuous EEG recording, and they were studied separately, initially as the status epilepticus without CLE group that was subsequently included in the overall control group. Previous studies indicate that if these rats had not demonstrated spontaneous seizures by the end of 4 weeks of monitoring (12 weeks after CHS) they were unlikely to develop chronic epilepsy (studied for 8 months) (30,31). The remaining 25 CHS rats showed spontaneous long-term seizures and were studied up to 16 months post-SE (CLE). For the longer survivors, monitoring was performed intermittently (every one to two months) for 2–4 weeks each time to determine that the seizure frequency remained stable. Overall cumulative seizure numbers were extrapolated from a mean seizure frequency (seizures/week) multiplied by the number of weeks of survival after CHS, less 4 weeks for the latent period, during which the seizure frequency is very low.

## 2.5. Kindling Protocol

Fifteen additional electrode implanted rats were electrically kindled to stage V seizures as previously described (Mathern et al., 1997a, 1997b). Kindled rats do not have an SE episode. At least one week after surgery, after-discharge thresholds were determined using 10 s, 50 Hz, biphasic 1 ms pulse width constant current trains. The hippocampi with the lower thresholds were subsequently stimulated throughout the study, and all stimulations were at after-discharge threshold. Kindled rats were stimulated between 1 and 24 times per week, and all animals developed stage V seizures. The stimulation frequency was adjusted for a low and high frequency group. Stimuli were given no more than once per hour and eight per day. Kindled rats were maintained on this schedule from 4 to 12 months. After-discharge duration and behavioral accompaniment were recorded for each animal, and there was a minimum of one week between the last kindled stimulated seizure and perfusion. None of the kindled rats were witnessed to have spontaneous (i.e. non-stimulated) seizures even after 200 or more stimulations.

## 2.6. Tissue Processing

At sacrifice the animals were deeply anesthetized and perfused via an aortic cannula with; buffered normal saline for 1 min, 0.1% sodium sulphide in Millonig's buffer at pH 7.3 for 5 min, and 4% buffered paraformaldehyde for 5 to 10 min (Katzir et al., 2000). The brains were removed, blocked, kept in fixative, and shipped via express mail from the University of Virginia to UCLA. Rat brains were sent in batches, and prior to shipment each brain was given an identity number that blinded the UCLA team. Each shipment contained brains from different experimental groups. Upon arrival the specimens were cryoprotected overnight in 10% sucrose in 0.12 M phosphate buffer (pH 7.3), quick frozen, and sectioned in a standard horizontal plane from ventral to dorsal on a cryostat (−15°C). Serial sections beginning at

the dorsal - ventral level of the posterior commissure were collected for neoTimm's histochemistry (2 sections at 30 μm) and Nissl stain (10 and 30 μm). Serial sections were collected two more times for a total of 3 sequential sites that sampled bilaterally mid-ventral hippocampi (i.e., total of six sample sites per animal, 3 right and 3 left). Electrode positions were confirmed based on Nissl sections.

## 2.7. Neo-Timm's histochemistry

This procedure was the same as previously published (Mathern et al., 1997a, 1997b; Katzir et al., 2000). Briefly, two sets of adjacent 30 μm sections, one developed lightly and the other darkly, were processed to confirm that the staining was specific for mossy fibers. Cryostat sections were mounted on chromium-alum gelatin-coated slides and air-dried. The slides were immersed in a 'physical developer' maintained at $26\overset{\circ}{C}$ in the darkroom. The developer consisted of 180 ml of a 50% gum arabic solution, 30 ml of an aqueous solution of 7.65 g citric acid and 7.05 g sodium citrate, 90 ml of an aqueous solution of 5.3 g hydroquinone and 1.5 ml of a 17% silver nitrate solution. Development time for light sections was 40 min and for dark sections 50 min. The slides were washed in distilled water for 5 min, running tap water for 10 min, air dried, dehydrated through alcohol to xylene and coverslipped.

## 2.8. Tissue Analysis for Molecular Layer neoTimm's Staining

The optical density (darkness) of molecular layer neoTimm's staining was measured by a computer image analysis system as an average of the gray value (GV) between white (0) to black (255) (Mathern et al., 1998a, Mathern et al., 1998b). The dark neoTimm's sections were imaged using a video monochrome charge-coupled device camera (CCD; Hamamatsu) attached to a Zeiss microscope, and captured, averaged, and digitized using a frame grabber (Data Translation Quick Capture; average of 16 serially collected video frames) on a Macintosh computer (Model 8100/110). Luminance was uniformly maintained and checked after every 10 measurements using optical density standards (Kodak). The image was analyzed using NIH Image; v. 1.56; public domain. The operator imaged the fascia dentata molecular layer from the hippocampal fissure to the stratum granulosum and outlined the inner molecular layer (IML), and the middle and outer molecular layers (collectively referred to as the OML). The computer determined the average GV of the pixels within the encircled region. Measurements were performed on the right and left hippocampi at the 3 horizontal sections (i.e. 6 samples sites) and averaged into a single IML and OML GVs per animal. The GV differences between the IML and OML were calculated, which provided an accurate measure of IML neoTimm's staining based on previous human and animal studies.

## 2.9. Neuron Counts

Adjacent sections were Nissl stained with cresylecht violet (CV) for histopathological review (30 μm thick) and cell densitometry (10 μm) (Mathern et al., 1998a, Mathern et al., 1998b). We used Nissl stain to assess neuronal cell loss because it clearly identifies viable neurons in the rodent hippocampus. Neuron counts were performed on both the right and left hippocampus in all three horizontal sections and averaged into single values for each animal. Counts were obtained visually with manual counting at a magnification of 400× using grid

morphometric techniques, and the hippocampal subfields counted were based on Lorente de Nó's classification (Lorente De No, 1934). The subfields were the granule cells and hilar neurons of the fascia dentata, CA3c, CA3b, CA1 stratum pyramidale, and subicular neurons. An ocular grid consisting of 10 by 10 boxes was placed over the hippocampal region of interest. For the stratum pyramidale, the 20 boxes in sequential 2 by 2 box segments ($104 \times 520$ μm area) that followed the laminar profile of neurons were selected, and all nuclei belonging to pyramids within this region counted except for those nuclei touching the superior and right edges of the grid. For the smaller granule cells, a linear 1 by 5 box ($52 \times 260$ μm area) was used, the measure was repeated, and the results averaged per section and then per animal.

For hilar counts a slightly different technique was applied. Previous investigators (Bertram 3rd and Lothman, 1993; Adams et al., 1998; Mathern et al., 1997a, 1997b) have shown that seizures induce an enlargement of the cross-sectional area of the hilus that results in a secondary reduction in neuronal density because of the increased area of the hilus. For this reason it is important to count all neurons in the hilus rather than an estimate of density. Using the image of the hilus, defined as the region between the dentate granule cell blades excluding the line of CA3c pyramidal neurons, was outlined and the area measured. Within the outlined area **all** hilar neurons (i.e. non-pyramidal cells) were counted. Total hilar neuronal counts were combined with area to determine neuronal density (number of neurons per 1000μm$^2$). This approach has been standard in our laboratories for a number of years (Bertram et al., 1990; Bertram 3rd and Lothman, 1993; Mathern et al., 1997a, 1997b).

To determine if there was atrophy or hypertrophy of hippocampal structures, the hippocampal subfield areas were obtained from the digitized images. The fascia dentata molecular layer, stratum granulosum, hilus, CA3c, CA3b stratum pyramidale and radiatum, CA1 stratum pyramidale and radiatum, and subiculum were outlined from the same 10 μm horizontal sections used for neuron counts. The average area (μm$^2$) was determined for each section, and this process was repeated for each of the three sections per side for each rat and the results averaged into a single value per rat.

### 2.10. Data analysis

After tissue analysis, the code was broken and animals assigned to the following categories for statistical comparison: 1) Controls (initially electrode control, stimulation without status epilepticus, and status epilepticus without CLE, all combined into one group when no differences were found among the three subgroups), 2) Kindled, and 3) CLE rats. Additional comparisons were made between kindled and CLE rats with: 1) Low or High seizure frequencies, and 2) Young or Old for durations of epilepsy using the criteria described above. Data were analyzed using a statistical program (StatView, SAS Institute, Cary, NC). Relationships between a particular data type (neuronal counts or regional area) and a particular category (e.g. age or seizure frequency) was examined with an analysis of covariance (ANCOVA, Table 2). Testing across groups was performed with an ANOVA with post hoc Student-Newman-Keuls. Pairwise testing was with an unpaired *t*-test.

## 3.   Results

### 3.1.   The Control Groups

There were no statistically significant differences in hippocampal pathology between the 3 control rat groups. They were combined into a single control category to compare with kindled and CLE rats. Electrode Control rats ($n = 7$), stimulation without SE rats and (n = 7), and SE without CLE rats ($n = 6$) showed no significant differences in hippocampal neuron densities, hippocampal subfield areas or IML-OML GV differences. These results indicate that neither stimulation alone nor the seizure activity of SE itself that did not lead to CLE had a significant impact on hippocampal pathology or mossy fiber sprouting.

## 4.   Comparison between control, kindled, and CLE rats

### 4.1.   Age and Seizure Frequency

The mean (±SEM) age at sacrifice was not statistically different between controls, kindled, and CLE rats (Table 1). Overall the average age at sacrifice was $6.0 \pm 0.59$ months (ANOVA; $P = 0.59$). Similarly there was no significant difference between kindled and CLE animals with regard to mean frequency of seizures or in total numbers of estimated seizures. These data indicate that the rat groups were statistically equivalent for age at sacrifice, and the kindled and CLE rats were comparable for frequency of spontaneous or stimulated seizures. For the kindled animals the total number of seizures ranged from 20 to 82 for the low frequency animals and 136 to 912 for the high frequency rats. For the CLE animals, the ranges were 6 to 98 for the low frequency animals and 130 to 12,096 for the high frequency animals.

Three CLE rats had seizure frequencies that were much greater (83, 160, and 216 seizures per week, 4316, 7680 and 12,096 total seizures respectively) than the kindled group (14.6 average frequency per week). *Re*-analysis omitting these 3 rats did not modify the demographic results comparing controls, kindled, and CLE rats (modified CLE category; age $6.4 \pm 1.0$, $P = 0.49$; Seizures/week $8.7 \pm 2.6$, $P = 0.064$; Total Estimated Seizures $184 \pm 73$, $P = 0.95$). For some comparisons, these three CLE rats with extreme seizure frequency will be excluded or reported separately to assure that for some analyses that the groups are as comparable as possible with regard to seizure frequency and total seizures experienced.

### 4.2.   Hippocampal Neuronal Densities and Subfield Area Measurements

Qualitatively, the Nissl sections showed no apparent differences in hippocampal neuronal densities or in subfield areas for controls (Fig. 1A) and kindled rats (Fig. 1B). By comparison, CLE rats showed apparent neuronal loss and decreased subfield areas throughout the fascia dentata and Ammon's horn (Fig. 1C). In addition, CLE rats often showed dentate granule cell dispersion into the molecular layer. Dispersion could be either focal (Fig. 1C arrow) or diffuse along the length of the granule cell layer (Figs. 2B & D). Kindled animals, including those with hundreds of stimulated seizures over many months, did not show granule cell dispersion (Fig. 1B and 2C). CLE rats also frequently had a decrease in thickness of Ammon's horn stratum radiatum (SR) as compared with the other rat groups (Figs. 1C, asterisks). There were also clear differences among the groups with

regard to mossy fiber sprouting. No obvious differences were seen between the kindled and control groups, whereas there was obvious and dense staining in the inner molecular layer of the CLE rats (Fig. 1 D,E and F).

These qualitative observations were supported by the quantitative data (Fig. 3). As noted in previous work, kindling was associated with an increase in area in many of the regions. However, in combining the areas with the densities, we found that neuronal loss was only seen in the CLE rats. The reductions were significant in all regions except the subiculum. *Re*-analysis removing the 3 very high frequency CLE rats (modified category) did not alter the results.

### 4.3. Neo-Timm's IML staining

Compared with controls and kindled animals, CLE rats demonstrated increased neoTimm's staining in the fascia dentate inner molecular layer (IML). Five kindled rats (33%) with a total of 21, 82, 136, 178, and 912 stimulated stage V seizures visually showed a slight amount of IML neoTimm's puncta, but it was often scant and barely detectable at low magnification (Figs. 1E & 4B). Ten kindled rats without visible mossy fiber sprouting had 7 to 384 total stimulated seizures, and there were no statistically significant differences between rats with or without mossy fiber sprouting for weekly seizure frequency (*t*-test; $p = 0.54$), total seizures (t-test; $p = 0.39$), and age at sacrifice (t-test; $p = 0.81$). By contrast, most CLE rats showed darkly stained IML puncta (Figs. 1F, 4C, and D). Aberrant supragranular mossy fiber sprouting was asymmetric in 14 and symmetric in the rest of the CLE rats (Fig. 5). Mean (±SEM) seizure frequencies per week were not different in CLE rats with asymmetric (27.6 ± 11.9) compared with symmetric IML neoTimm's staining (28.5 ± 23.6; $p = 0.97$).

The IML-OML gray value differences corroborated the qualitative observations (Fig. 6; left). IML-OML gray value differences were increased in CLE rats compared with the kindled and controls (ANOVA; $p < 0.0001$). Furthermore, IML-OML GVs were not different in CLE rats with asymmetric (mean ± SEM; 50.5 ± 7.5) compared with symmetric mossy fiber sprouting (43.5 ± 11.4; $p = 0.60$).

## 5.  Effect of seizure frequency and epilepsy duration on anatomy

Comparison of kindled and CLE rats with different seizure frequencies showed hippocampal pathological changes in high frequency CLE rats. Visual inspection showed that IML neoTimm's staining was often greater and granule cell dispersion was increased in high frequency CLE rats compared with low frequency CLE rats and low and high frequency kindled rats (Fig. 4). Quantitative measures showed IML-OML Timm's GV differences were increased in high frequency CLE rats compared with low frequency CLE rats (Fig. 6; middle). Duration of the epilepsy also contributed to increased mossy fiber staining when comparing the younger to older CLE rats. In the kindled rats no differences were found based on either duration of kindling or seizure frequency. We did not have a formal quantitative measure for granule cell dispersion, which was qualitatively obvious. Some support to dispersion can be derived from the increased area of stratum granulosum as well as decreased density of the granule cells (Fig. 3).

The hilus was enlarged with fewer neurons per unit area in older CLE rats compared with younger CLE and older and younger kindled animals, and these findings were statistically different in quantitative assessments. Total counts of neurons in the hilus and CA1 showed that there was no association between how long the animals had had epilepsy and neuronal numbers, nor was there an association between the numbers of seizures (Figs. 7 and 8). However, there is a clear association of hilar area (increasing) with increasing total numbers of seizures (Fig. 9). This observation suggests that the common finding of reduced density of neurons in the hilus over time is likely the result of the same number of neurons spread over a larger area as was shown in Fig. 3. It is also of note that a similar relationship exists for mossy fiber sprouting (Figs. 6 and 9), an observation that raises the possibility that ongoing increased sprouting contributes to the expanding hilar area. On average, 2-dimensional hilar areas in **CLE** rats more than doubled in size between 3 and 15 months of age (0.31 to 0.74 $mm^2$). Statistical analysis for control rats between age and hippocampal anatomic variables found no statistically significant results (ANOVA; $P > 0.061$) indicating that age by itself was not a confounding factor that might explain the findings in CLE rats. 2-dimensional hilar areas in kindled rats remained unchanged between 3 and 15 months of age (0.51 to 0.48 $mm^2$) (Table 2).

## 6. Discussion

In this study we sought to answer two questions. The first was whether there is any evidence for progressive neuronal loss in the hippocampus as a consequence of repeated intermittent limbic seizures. The second question was whether there was any evidence of hippocampal change that could be related to the severity of the disorder or the duration of the disorder. With regard to the first question, we found no evidence in this study to support the hypothesis that repeated limbic seizures cause neuronal loss in the hippocampus. This conclusion is based on the observation that increasing numbers of seizures do not result in progressive neuronal loss in either the kindling or the CLE model. With regard to the second question, there are changes associated with higher seizure frequencies in CLE animals. These changes include increased hilar area and an increase in IML Timm's staining (mossy fiber sprouting). Together these findings suggest that there are seizure associated changes, but the overwhelming majority of neuronal loss precedes the onset of spontaneous seizures, indicating that hippocampal damage is not likely the consequence of the recurrent focal seizures. These conclusions are based, in part, on the observations that CLE animals had widespread neuronal loss, whereas the kindled animals, even with many seizures, had none. Overall these findings extend the observations of a number of previous studies, but conflict with the results of others. What is the basis for these differences? It is possible that the studies really are looking at the same phenomenon, but interpreting it differently.

The issue over the of the differences among the studies hinges on the interpretation of decreased neuronal density. The general interpretation of decreased density is that there are fewer neurons. But, as we have shown in this and previous studies, decreased density can be seen even when there is no neuronal loss. Density, as stereologists have pointed out, is only one measure in estimating (and the emphasis is on estimating) a total population. Density is a measure of number per cross sectional area or unit volume. The only way that the total population can be estimated is by using the density together with the area or volume. Most

biologic tissue presents a problem in this regard in that it can atrophy or hypertrophy, a process that can bring a given element (neurons for example) closer together or spread them further apart. An hypertrophied region could therefore have a greatly decreased density of neurons even though the population was unchanged. In contrast, after significant neuronal loss, shrinkage of the tissue can bring the remaining elements together, resulting in an essentially normal density, which could be misinterpreted as meaning no loss, unless the change in tissue volume was taken into account. When looking at neuronal density alone, all studies are in agreement: it does decrease with time and seizure number. However, when corrected for changes in area, the estimated total number of neurons is unchanged. In past studies we have shown that the entire hippocampus increases in size with kindling (Bertram et al., 1990; Bertram 3rd and Lothman, 1993). There is no evidence for progressive atrophy or neuronal loss with continued seizures. In support of the hypothesis that seizures can increase tissue volume, Adams et al. (1998) demonstrated a significant upregulation of activated glial cells during kindling that was associated with an expansion of volume, and that his glial activation subsided over three months following the last kindled seizure. It is a well established observation that seizures activate glial cells which in turn will increase the volume of the neuropil (Steward et al., 1991; Adams et al., 1998) although the role of this activation in seizures and epilepsy is not understood.

The role of mossy fiber sprouting in the development of epilepsy and the initiation and support of seizures is not clear, although it is a form of synaptic reorganization. In the CLE model it appears primarily after neuronal loss (much less prominent sprouting with kindling) and increases with time and seizure number. The mechanisms behind sprouting are not well understood, but it may occur in part because there are "synaptic openings" from synapses originating from neurons that were lost after status epilepticus. Continued seizures may encourage increased staining, but whether the sprouting is supporting the generation and spread of seizures or whether it is a reaction that is an attempt to oppose the seizures is entirely unknown. Many studies have examined the potential physiological consequences of sprouting in animals to help understand the potential role it might play in human temporal lobe epilepsy. The concept that supragranular mossy fiber sprouting may form recurrent excitatory axon circuits leading to increased neuronal hyperexcitability and seizures was proposed by Tauck and Nadler (1985) with supporting data from others (Cavazos and Sutula, 1990; Leite et al., 1996; Mathern et al., 1993). The evidence from animals and people with HS suggests that the sprouting is associated with hippocampal neuronal hyperexcitability and spontaneous seizures, but the role of these findings is unclear. Some have suggested that the changes in the granule cell layer may be related to the neoneurogenesis of granule cells from local stem cells (Jessberger and Parent, 2015; Danzer, 2019). It is a well described phenomenon but the role of this activity in the development of epilepsy is not known. Further, other recent studies have shown that neoneurogensis falls off with age in people (Sorrells et al., 2018), so how much this phenomenon plays in the role of the changes found in the older CLE rats is also unclear.

With the recent reports of frequent, clinically unrecognized seizures in patients with Alzheimer's disease, the question has been raised whether these seizures contribute to the progression of the disease (Lam et al., 2017; Vossel et al., 2017). The results from this study suggest that the seizures do not, but it is important to add a few caveats to that conclusion.

The neurons involved with seizure activity in Alzheimer's Disease may have a very different response to seizures than the neurons in kindled animals or in the rats with epilepsy. It would thus be possible that an "Alzheimer's" neuron that is already compromised by the disease might be more prone to injury from a seizure than would otherwise be the case. The best way to resolve this issue is to evaluate this group of patients for subclinical seizures, and to treat those people with seizures and determine whether seizure suppression could alter the course of the disease. However, the data from this paper suggest that even relatively frequent seizures do not promote neuronal loss and the potential negative cognitive and other neurological effects of the medications in such a trial would make interpretation of the results problematic. For this reason, although we cannot completely exclude the possibility that unrecognized seizures contribute to the structural substrate of Alzheimer's disease, these results suggest that they are not a major contributor.

There have been a number of quantitative MRI studies in people with epilepsy that have reported volume loss in the hippocampus over time, and this finding has been interpreted as progression of hippocampal sclerosis and volume loss with greater duration of the disease (Bernhardt et al., 2009; Caciagli et al., 2017) although some reviews have left it unclear whether one is seeing the effect of disease, or the effects of time (Caciagli et al., 2017). There are several potential contributing factors that these studies did not consider. One of the most common comorbidities of epilepsy is depression, and there are a number of MRI studies that consistently show that volume loss in the hippocampus is associated with depression (Mervaala et al., 2000; Sheline et al., 1996; Sheline et al., 1999). Further, the potential effect of drugs on hippocampal volume was not taken into consideration. Although the literature is limited on this topic, there are a number of papers that link valproate with a potentially reversible volume loss in the hippocampus and cortex (Guerrini et al., 1998; Fleisher et al., 2011; Pardoe et al., 2013). There is less information for other epilepsy medications. These two observations suggest that the reported loss of hippocampal volume found in MRIs could be more the result of comorbidities and drug exposures than progressive neuronal loss as a result of the seizures.

Many more recent studies examining surgical and autopsy specimens have also suggested against progression or the development of hippocampal atrophy as a consequence of repeated seizures (Thom et al., 2005; Sen et al., 2005; Garbelli et al., 2017). These studies have either not found a strong relation between duration of the disease or the severity of the epilepsy and the hippocampal pathology or, in cases in which the seizures originated outside the hippocampus but which involved the region secondarily, there was no evidence for neuronal loss, even after years of recurrent seizures. The many animal studies that reported loss used density alone as the primary marker for neuronal loss, and the weaknesses in this approach have been discussed.

In summary, this study provides further experimental evidence supporting the concept that injury-induced epileptogenic foci are generated by an initial precipitating event, like status epilepticus, that damages neurons and then evolves secondarily to synaptic reorganization and other processes, and is not the consequence of repeated brief seizures. The study also provides support for the hypothesis that seizures, overtime, do cause changes in the structure of the brain in epileptic animals, as evidenced by the hypertrophy in the neuropil and the

increase in mossy fiber staining. The work of others also indicates that recurrent seizures affect glial cells which remain activated for a period of time after the seizures stop. The role of these changes in supporting or opposing seizure activity remains unclear, and clearly require further study.

## Acknowledgments

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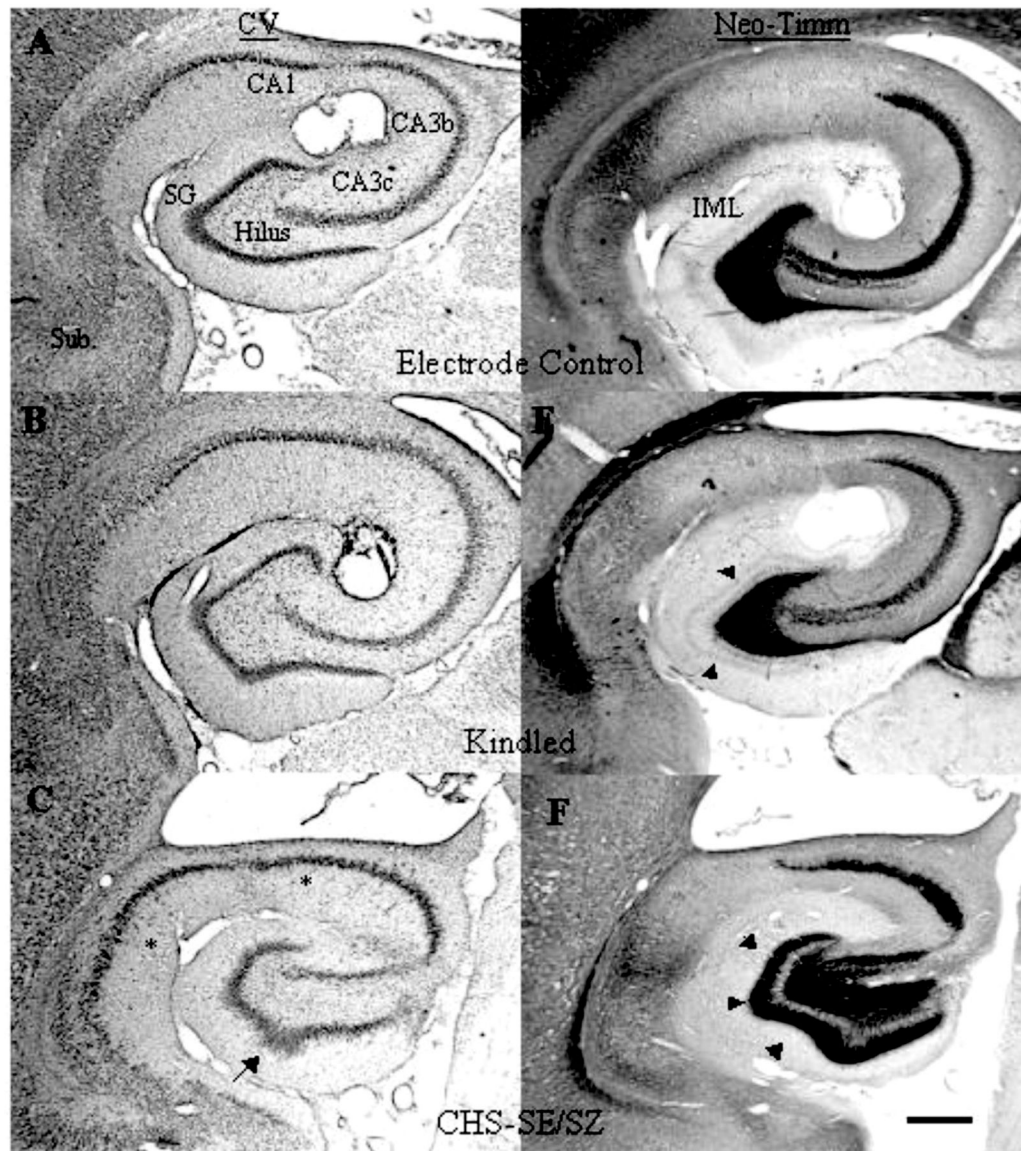
**Fig. 1.**
Micrographs showing hippocampi from Electrode Control (Panels A & D), kindled (Panels B & E), and **CLE** rats (Panels C & F) for Nissl stain (CV; left column) and neo-Timm's histochemistry (right column). All animals were 3 to 4 months old when tissue processed. The kindled rat had 16 stimulated stage 5 seizures per week for a total of 80 events. The **CLE** rat averaged 2.5 spontaneous seizures per week for a total of 107 seizures. **Panel A**: Electrode Control with the labeled hippocampal subfields used for neuron counts. Notice the electrode tract between the upper SG blade and CA1 stratum pyramidale. **Panel B**: Kindled rat hippocampus showed no visible neuron loss compared with the Electrode Control. **Panel C**: The **CLE** rat showed diffuse hippocampal neuron loss, signs of focal granule cell dispersion (arrow), and loss of stratum radiatum thickness (asterisks). **Panels D**: The neoTimm's stain shows a normal pattern in the Electrode Control rat with no staining in the inner molecular layer (IML). **Panel E**: The kindled rat showed minimal IML neoTimm's

staining (arrowheads). **Panel F**: The **CLE** rat showed significant IML neoTimm's staining (arrowheads). All panels of equal magnification; calibration bar equals 500 μm.
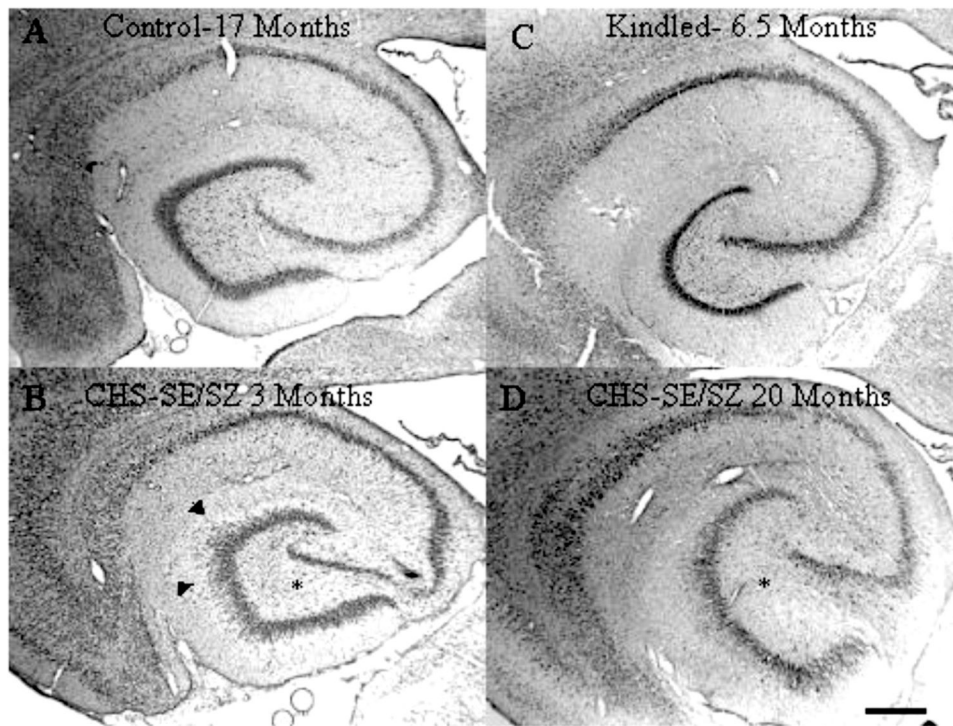
**Fig. 2.**
Nissl sections illustrating hippocampal anatomic changes in older rats. **Panel A**: A 17 month old Electrode control rat for comparison with the other panels. **Panel B**: This **CLE** rat had 1.5 spontaneous seizures per week for a total of 15 seizures. There is hippocampal neuron loss, hilar atrophy (asterisk), and diffuse granule cell dispersion (arrowheads). **Panel C**: Kindled rat with 24 stimulated events per week for a total of 384 seizures over 6.5 months. Visually, there are no qualitative anatomic differences compared with the older control rat (Panel A). **Panel D**: This older **CLE** rat averaged 0.5 seizures per week for a total of 40 recorded seizures over 14 months. The hilar area is larger than the other examples (asterisk)and, as in Panel B, there is diffuse dispersion of the granule cells. All panels of equal magnification; calibration bar equals 500 μm.
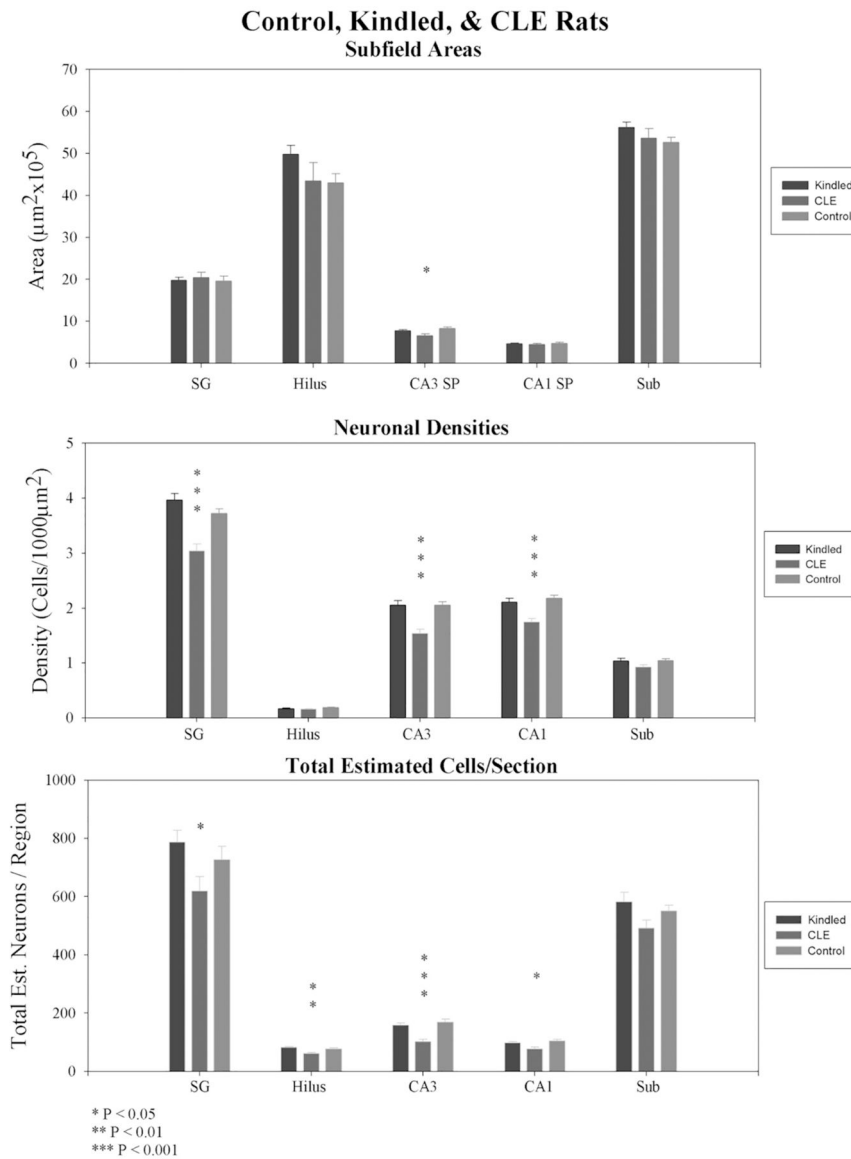
**Fig. 3.**

Bar graphs showing hippocampal subfield areas (top row) neuron densities (middle row) and total estimated neurons per region for controls (n = 20), kindled (n = 15), and CLE (n = 25) rats. Significant post-hoc differences are indicated by asterisks. **Area measures**: CLE rats showed decreased subfield areas compared with Kindled and control rats for CA3 SP (post-hoc; $p = 0.047$). **Neuron counts**: CLE rats showed decreased densities compared with Kindled and control rats for SG, ($p < 0.0001$), CA3 ($p < 0.0001$), and CA1 ($p < 0.001$). **Total neuron numbers**: CLE rats showed decreased total neuron numbers compared with Kindled and control rats for SG ($p = 0.045$), hilus ($p = 0.004$), CA3 ($p < 0.001$), and CA1 ($p = 0.021$).
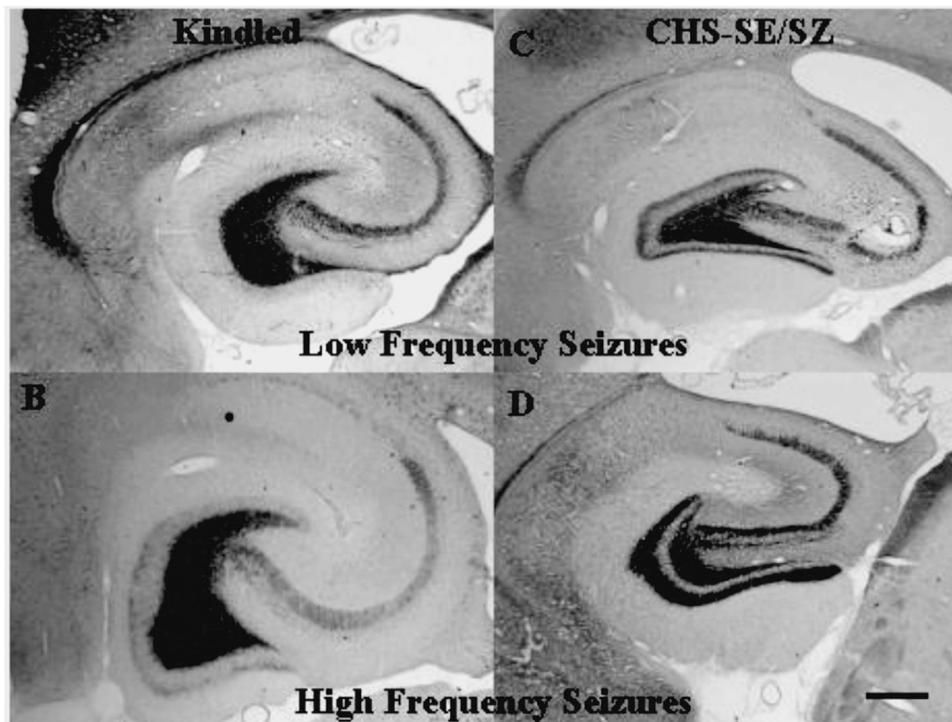
**Fig. 4.**
Neo-Timm's staining in kindled (left column) and **CLE** rats (right column) with low (top row) and high seizure frequencies (bottom row). **Panel A**: Kindled rat with 3 stage 5 seizures per week over 7 weeks for a total of 21 seizures, and sacrificed 3 months after electrode implantation. There is no visible IML neoTimm's stain. **Panel B**: Kindled rat with 24 kindled seizures per week for a total of 912 events, and sacrificed 9.5 months after implantation. There is some neoTimm's IML puncta, which was the most stain observed in all of our kindled rats. **Panel C**: **CLE** rat averaged 1.5 spontaneous seizures per week for a total of 15 recorded events, and sacrificed at 4 months. Sprouting was unilateral and greater than the kindled rat with a higher weekly seizure frequency (Panel B). **Panel D**: Another **CLE** rat averaged 32 seizures per week for a total of 157 seizures, and was killed at 3 months. There was bilateral aberrant IML mossy fiber sprouting, which was greater than the low frequency **CLE** rat (Panel C). All panels of equal magnification; calibration bar equals 500 μm.
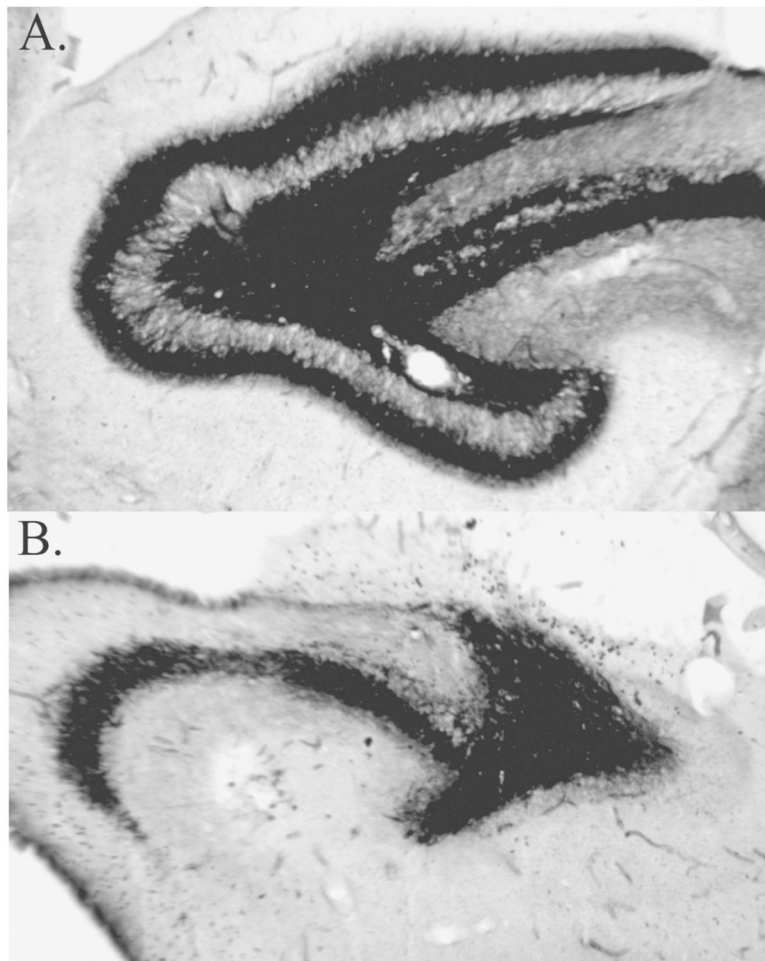
**Fig. 5.**
Micrographs of asymmetric Timm's staining in a CLE rat 4 months following SE with 29 total recorded seizures. Note that the absent supragranular staining is in the more atrophic hippocampus. Both micrographs at same magnification.
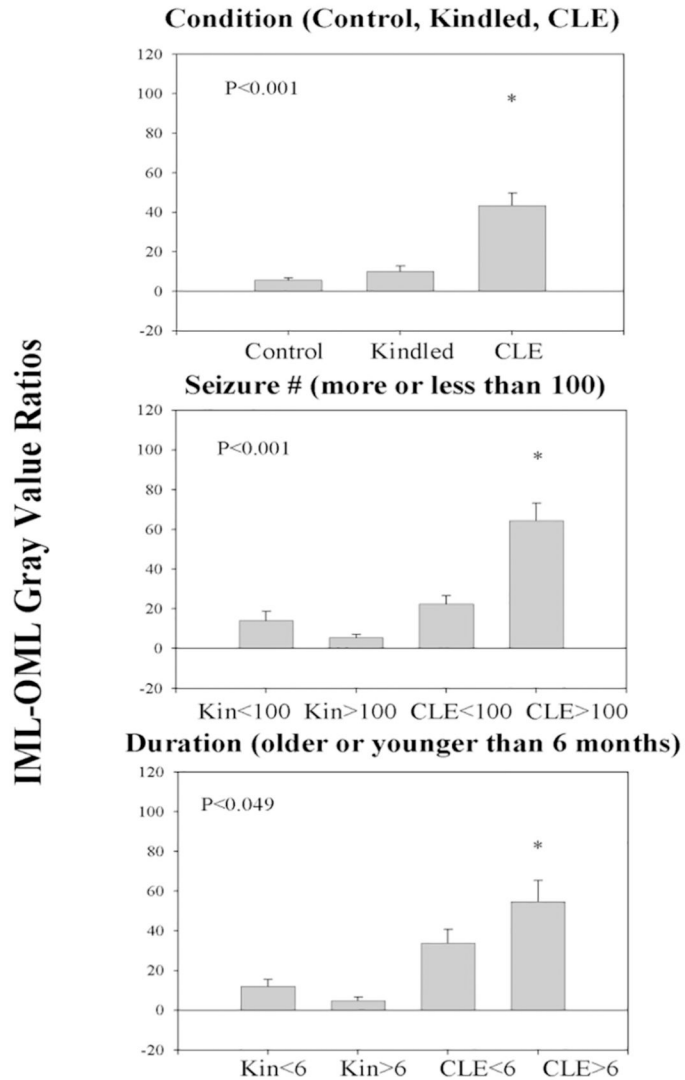
## Mossy Fiber Sprouting



**Fig. 6.**
Mossy fiber sprouting. Quantitative comparison among the three primary groups and for kindled and CLE rats based on numbers of seizures and duration of seizure disorder as determined by inner molecular layer-outer molecular layer gray value ratios.. Top: only the CLE rats had a significant increase in mossy fiber staining. Middle: only the CLE rats with high frequency seizures had a significant increase in staining compared rats with lower frequency seizures (Kindled rats: <100 seizures $n = 8$; >100 seizures $n = 7$; CLE rats <100 seizures $n = 13$; >100 seizures $n = 12$). Bottom: the duration of the epilepsy played some role, as the CLE animals with longer durations of the disorder also had significantly greater staining compared to CLE animals with shorter duration epilepsy (Kindled rats: <6 months n = 8; >6 months n = 7; CLE rats <6 months $n = 14$; >6 months $n = 11$).
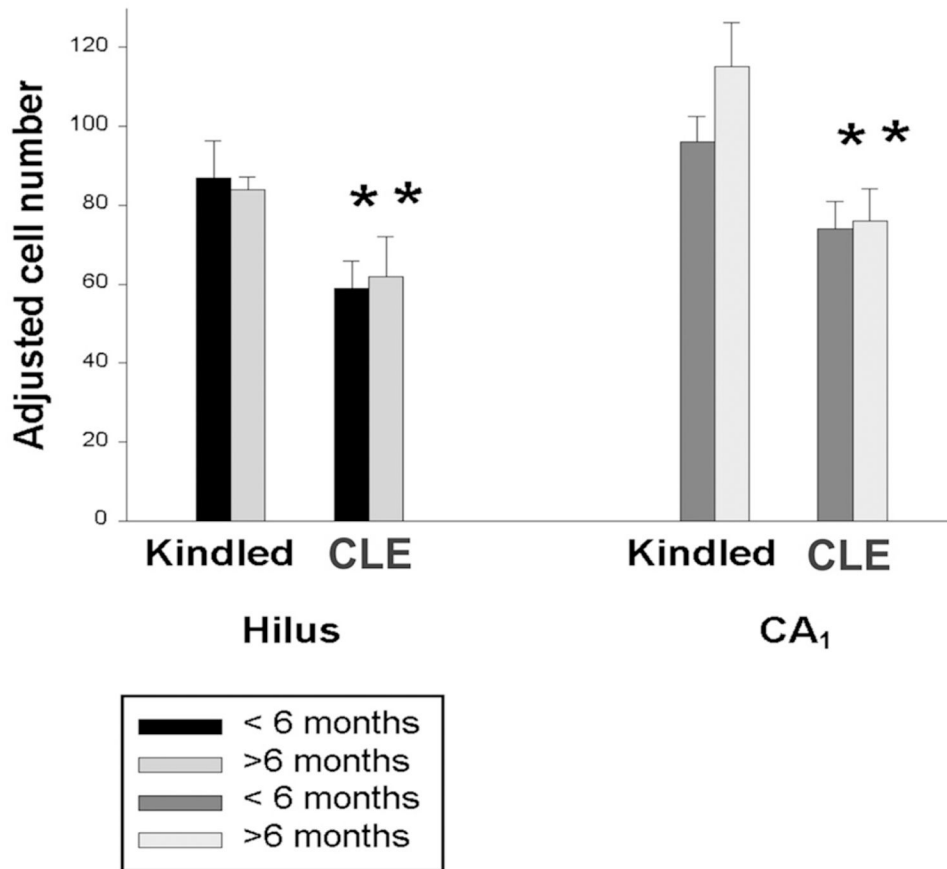
**Fig. 7.**
Bar graphs showing differences with longer seizure durations for adjusted neuronal numbers in the hilus and CA1 pyramidal cell layer for kindled and TLE rats. **Left Graph**: Hilar neuronal numbers were decreased in TLE rats, but there were no differences between the younger and older rats. **Right Graph:** Similarly, the adjusted numbers in the CA1 pyramidal layer were lower in the TLE rats compared to kindled animals, but duration of disease did not affect these numbers. ($p < 0.05$ hilus) (Kindled rats: <6 months n = 8; >6 months n = 7; CLE rats <6 months n = 14; >6 months n = 11).
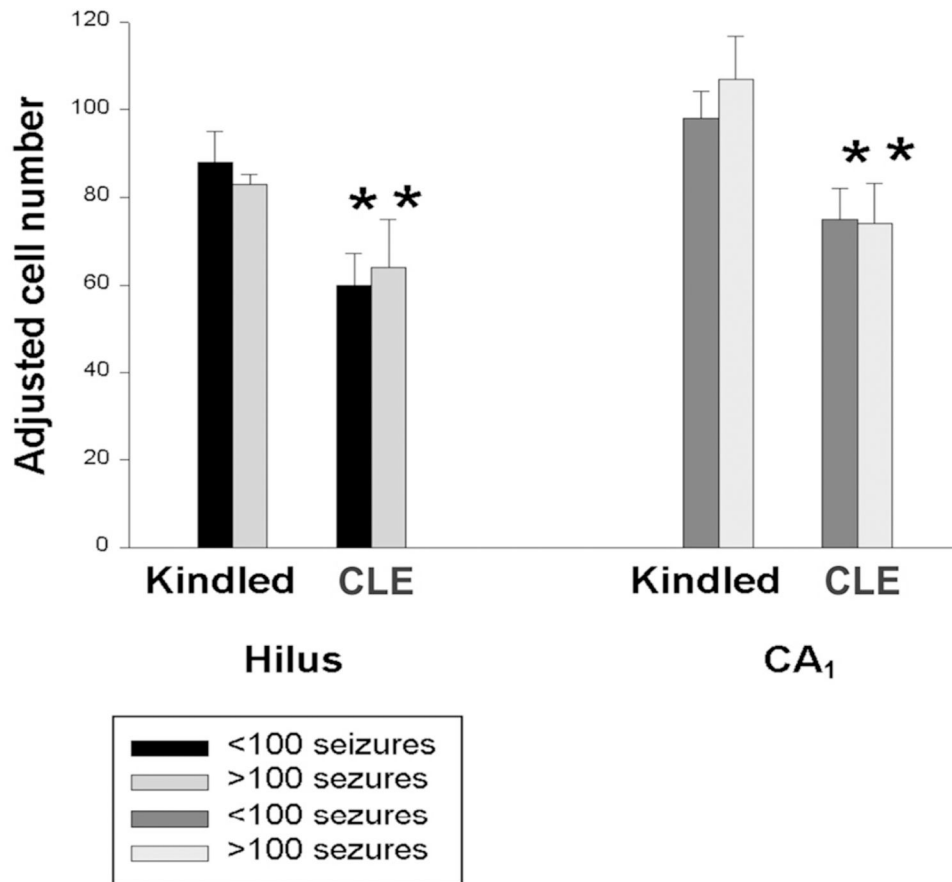
**Fig. 8.**
Bar graphs showing differences with greater seizure number for adjusted neuronal numbers in the hilus and CA1 pyramidal cell layer for kindled and TLE rats. **Left Graph**: Hilar neuronal numbers were decreased in TLE rats, but there were no differences between the rats based on total seizure number. **Right Graph:** Similarly, the adjusted numbers in the CA1 pyramidal layer were lower in the TLE rats compared to kindled animals, but number of seizures did not affect these numbers. ($p < 0.05$) (Kindled rats: <100 seizures n = 8; 0.100 seizures n = 7; CLE rats <100 seizures n = 13; >100 seizures n = 12).
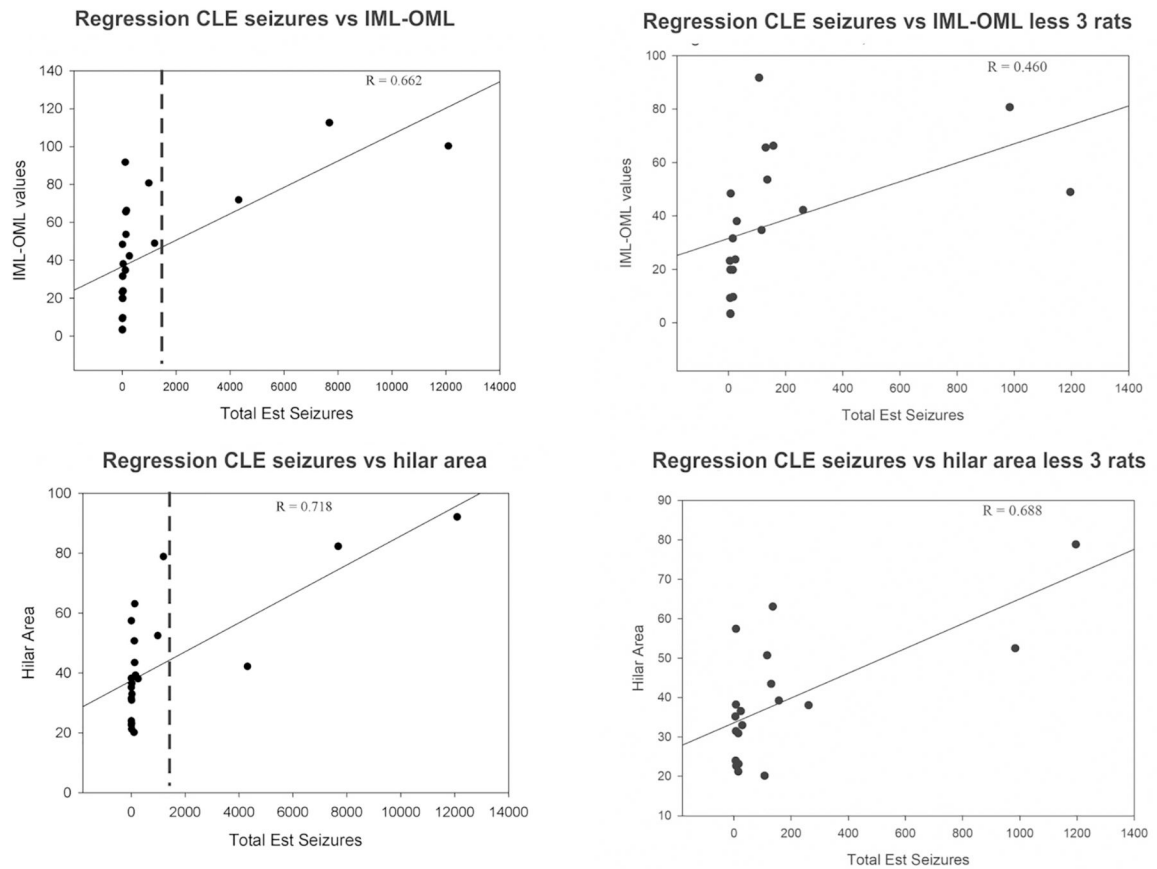
**Fig. 9.**

Relationship in the CLE rats between total number of seizures and IML-OML mossy fiber staining (top) and hilar area. In both cases there is a relation between both measures and the number of seizures. On the left the graphs show the data for all of the CLE animals and on the right with the rats with over 4000 seizures removed. Note that the vertical axis is different for all rats compared to the rats with fewer seizures. The vertical dotted lines in the graphs on the left define the limits of the seizure number that are displayed on the right (right graphs remove the 3 rats with the greatest number of seizures.

**Table 1**

Comparison between Controls, Kindled, and CLE rat categories for age at sacrifice (Age; mean ± SEM), seizures per week (Sz/Wk), and total estimated seizures (Total Est Szs).

| Category | N | Age (Months) | Sz/Wk | Total Est Szs |
|---|---|---|---|---|
| Controls | 20 (Range) | 6.0 ± 1.1 (3–17) | 0 | 0 |
| Kindled | 15 (Range) | 4.4 ± 0.6 (3–10) | 14.6 ± 2.5 (1–24) | 189 ± 61.0 (20–912) |
| CLE | 25 (Range) | 7.1 ± 1.0 (3–20) | 26.0 ± 10.6 (0.25–216) | 1165 ± 593 (6–12,096) |
| *p*-values | | p = 0.59 | *p* = 0.41 | *p* = 0.20 |

p-values tabulated with ANOVA (Age) or *t*-tests (Sz/Wk; Total Est. Szs). Total Est Szs = age in weeks times Sz/Wk.

**Table 2**

Analysis of covariance (ANCOVA) comparing seizure duration and frequency to hippocampal pathology for kindled and CLE rats as separate groups. Data presented as F-values/*P*-values, significant results indicated in **bold** type and an *. A significant value indicates that there is a relation between that particular data point (e.g. neuron counts) and category (e.g. age of seizures/week).

| Pathologic Factor | Kindled Age | Kindled Sz/Week | CLE Age | CLE Sz/Week |
|---|---|---|---|---|
| Neo-Timm's | | | | |
|   IML-OML GVs | | | | |
| | 0.863/0.373 | 0.899/0.363 | 0.571/0.458 | **33.2/0.0001*** |
| *Neuron Counts* | | | | |
|   SG Density | | | | |
| | 0.001/0.974 | 0.522/0.485 | 0.075/0.787 | 0.003/0.957 |
| Hilar Density | | | | |
| | 0.026/0.875 | 0.006/0.942 | **7.34/0.014*** | 4.20/0.067 |
| CA3c Density | | | | |
| | 0.285/0.604 | 0.008/0.929 | 1.52/0.233 | 0.118/0.735 |
| CA3b Density | | | | |
| | 0.374/0.553 | 0.228/0.643 | 0.188/0.669 | 0.002/0.964 |
| CA1 Density | | | | |
| | 0.835/0.380 | 0.013/0.911 | 0.627/0.437 | 0.026/0.873 |
| Sub Density | | | | |
| | 0.037/0.851 | 1.65/0.225 | 0.011/0.918 | 0.037/0.849 |
| *Hippo. Areas* | | | | |
|   SG Area | | | | |
| | 0.115/0.740 | 0.290/0.600 | 0.063/0.804 | **6.14/0.022*** |
| Hilar Area | | | | |
| | 0.377/0.552 | 0.167/0.690 | **5.70/0.028*** | 1.28/0.273 |
| CA3c SP Area | | | | |
| | 0.056/0.817 | 0.282/0.606 | 0.678/0.420 | 0.615/0.442 |
| CA3b SP Area | | | | |
| | 1.59/0.233 | 3.22/0.100 | 0.478/0.497 | 0.012/0.912 |
| CA1 SP Area | | | | |
| | 4.02/0.070 | 3.21/0.101 | **11.3/0.003*** | 3.44/0.078 |
| Sub Area | | | | |
| | 4.27/0.063 | **7.56/0.019*** | **5.38/0.031*** | 0.382/0.543 |
| Mol. Layer Area | | | | |
| | 2.85/0.129 | 2.85/0.119 | 0.095/0.761 | 1.85/0.188 |
| CA3 SR Area | | | | |
| | 0.834/0.381 | 0.966/0.347 | 1.28/0.272 | 0.705/0.411 |
| CA1 SR Area | | | | |
| | 5.02/0.056 | 1.35/0.269 | 4.34/0.051 | 0.411/0.529 |