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Morphological changes among hippocampal dentate granule cells exposed to early kindling-epileptogenesis

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Abstract

Temporal lobe epilepsy is associated with changes in the morphology of hippocampal dentate granule cells. These changes are evident in numerous models that are associated with substantial neuron loss and spontaneous recurrent seizures. By contrast, previous studies have shown that in the kindling model, it is possible to administer a limited number of stimulations sufficient to produce a lifelong enhanced sensitivity to stimulus evoked seizures without associated spontaneous seizures and minimal neuronal loss. Here we examined whether stimulation of the amygdala sufficient to evoke five convulsive seizures (class IV or greater on Racine's scale) produce morphological changes similar to those observed in models of epilepsy associated with substantial cell loss. The morphology of GFP-expressing granule cells from Thy-1 GFP mice was examined either one day or one month after the last evoked seizure. Interestingly, significant reductions in dendritic spine density were evident one day after the last seizure, the magnitude of which had diminished by one month. Further, there was an increase in the thickness of the granule cell layer one day after the last evoked seizure, which was absent a month later. We also observed an increase in the area of the proximal axon, which again returned to control levels a month later. No differences in the number of basal dendrites were detected at either time point. These findings demonstrate that the early stages of kindling epileptogenesis produce transient changes in the granule cell body layer thickness, molecular layer spine density and axon proximal area, but do not produce striking rearrangements of granule cell structure.

Introduction

Changes in the structure and function of hippocampal dentate granule cells are hallmarks of temporal lobe epilepsy (Danzer, 2012). These include changes in spine density (Isokawa, 1998; Swann et al., 2000; Leite et al., 2005; Santos et al., 2011), granule cell layer dispersion (Houser, 1990; Bouilleret et al., 1999; Riban et al., 2002; Heinrich et al., 2006), the appearance of ectopic cells (Scharfman et al., 2000; Dashtipour et al., 2001; Scharfman et al., 2003; Cameron et al., 2011), the appearance of hilar projecting basal dendrites (Spigelman et al., 1998; Ribak et al., 2000; Diaz-Cintra et al., 2009; Murphy et al., 2012; Sanchez et al., 2012), sprouting of granule cell mossy fiber axons (Tauck and Nadler, 1985; Sutula et al., 1989; Shibley and Smith, 2002; Nadler, 2003; McAuliffe et al., 2011) and somatic hypertrophy (Murphy et al., 2012). Abnormalities have been described in numerous

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models of temporal lobe epilepsy. For example, changes in spine density have been observed in systemic pilocarpine (Isokawa, 1998; Santos et al., 2011), systemic kainic acid (Wenzel et al., 2000), intra-hippocampal kainic acid (Suzuki et al., 1997)] and lesion models (Bundman and Gall, 1994) of epilepsy. Notably, these models are associated with both spontaneous seizures and substantial neuron loss (Gall, 1988; Golarai et al., 2001; Riban et al., 2002; Zhang et al., 2002; Wang et al., 2008; Hester and Danzer, 2013).

In the kindling model of epilepsy, electrical stimulations can be applied which initially produce only brief electrographic seizures (≈ 5 s). Repeated stimulation, however, produces longer electrographic seizures, and eventually results in tonic-clonic behavioral seizures (Goddard et al., 1969; Wada et al., 1974; McNamara et al., 1980; Dennison et al., 1995). This increased responsiveness to stimulation lasts lifelong (Moshe and Albala, 1982; Dennison et al., 1995). When only a few tonic-clonic seizures are evoked (≈ 5), the procedure produces minimal neuronal loss (Bengzon et al., 1997; Pretel et al., 1997; Zhang et al., 1998) and does not result in spontaneous recurrent seizures (Bertram and Lothman, 1993; Watanabe et al., 1996; Tuunanen and Pitkanen, 2000; Henshall and Meldrum, 2012). This “early kindling” protocol, in which only a small number of seizures are evoked is believed to model the early phase of epileptogenesis, while evoking repeated seizures may reflect later phases of the epileptogenic process (Bertram, 2007). Extended kindling can also give rise to more pronounced neuronal cell loss and spontaneous seizures (Pinel and Rovner, 1978; Cavazos and Sutula, 1990; Bengzon et al., 1997; Kotloski et al., 2002; Sayin et al., 2003).

Early kindling provides an opportunity to reveal the persistent changes in brain function and structure which might be operative in the earliest stage of epileptogenesis in a model minimally confounded by cell loss (McNamara, 1986; Tuunanen and Pitkanen, 2000). Here, we queried whether some of the more dramatic morphological changes evident in epilepsy models associated with robust cell death would be evident at the earliest stage of kindling epileptogenesis. To explore this possibility, Thy1-green fluorescent protein (GFP) expressing transgenic mice (Feng et al., 2000) received repeated stimulations of the amygdala until 5 successive seizures with clonic and/or tonic motor convulsions of class IV or above (Racine, 1972) were observed. Animals were sacrificed one day after the last evoked seizure, to look for acute effects, and one month after the last seizure, to look for chronic effects.

Methods

Thy1-GFP Expressing Mice

Male and female mice hemizygous for the Thy1-green fluorescent protein transgene (Thy1-GFP, M line) (Feng et al., 2000) were maintained on a C57BL/6 background. Mice from this line express GFP in a subset of hippocampal dentate granule cells ($\sim 11\%$). Labeled granule cells have been found to be morphologically and physiologically indistinguishable from unlabeled cells (Vuksic et al., 2008). Mice were sorted for experiments such that littermates were present in kindled as well as control groups. Analysis of granule cell giant mossy fiber bouton structure in these animals has been previously published (Danzer et al., 2010).

Kindling Model

Two to three-month-old mice were anesthetized with pentobarbital (60 mg/kg, intraperitoneal [i.p.] injection) and stereotactically implanted with a bipolar stimulation-recording electrode in the right amygdala. The following coordinates were used with bregma as reference: 1.0 mm posterior, 2.9 mm lateral and 4.6 mm below dura. A ground electrode was secured to the skull over left frontal cortex. One week post-surgery, a 60Hz, 60 μ A, 1 s

train of 1 ms biphasic rectangular pulses was used to determine the electrographic seizure threshold. Stimulation intensity was increased by 20 μ A increments at 1 min intervals until an electrographic seizure lasting at least 5 s was detected (the electrographic seizure threshold). The experimental animals were subsequently stimulated twice a day at stimulus intensities 100 μ A above the electrographic seizure threshold with a minimum inter-stimulus interval of at least 4 hours. Daily stimulations were administered until five seizures were induced. To be counted as one of the five seizures, the event had to last at least 12 seconds and receive a behavioral severity score of class IV or above according to the Racine scale (Racine, 1972). Surgically implanted control animals (sham) were connected twice daily to the stimulation apparatus, but did not receive any stimulations or undergo seizure threshold determination. Mice were perfused with paraformaldehyde one day (control, N = 7 [4 male, 3 female]; kindled, N = 7 [4 male, 3 female]) or one month (control, N = 8 [7 male, 1 female]; kindled, N = 7 [6 male, 1 female]) after the last evoked seizure or sham protocol.

Tissue preparation

Animals were anesthetized by i.p. injection of 100 mg/kg pentobarbital. They were perfused through the ascending aorta with ice-cold phosphate buffered saline [0.1M PBS] with 1 mM sodium orthovanadate (NaOV) and 1 U/ml heparin for 30 seconds at 10ml/min, immediately followed by 2.5% paraformaldehyde, 4% sucrose and 1mM NaOV in 0.1M PBS at 25°C for 10 minutes. Brains were removed and post-fixed in the same solution for 1 h at 4°C. Brains were cryoprotected in an increasing concentration of sucrose solutions (10%, 20%, 30%) in 1 mM NaOV in PBS, frozen in isopentane pre-cooled to -25°C with dry ice and stored at -80°C until cryosectioning. Coronal sections of 40 μ m thickness corresponding to bregma levels -1.64 to -2.75 (figures 46 – 48, Paxinos and Franklin's mouse atlas, 2001) were cut and mounted on charged slides (Superfrost Plus slides, Fisher Scientific, Waltham, MA). Since only sections from a portion of dorsal hippocampus were examined, the results may not reflect changes occurring in other hippocampal regions.

GFP immunostaining

Sections were thawed in PBS, incubated for 1 h with 0.5% Igepal + 5% normal goat serum in PBS (blocker), and then overnight in rabbit anti-GFP (1:500, Chemicon, Billerica, MA) antibody in blocker at 4°C. Sections were rinsed in blocker and incubated for 2 h in 1:750 Alexa Fluor 488 goat anti-rabbit antibodies (Invitrogen, Eugene, OR). Sections were then rinsed in PBS, dehydrated in alcohols, cleared in xylenes and mounted with Krysalon (EMD chemicals, Gibbstown, NJ).

Microscopy and neuronal reconstruction

The investigator was blind to treatment group during image collection and data analysis. Images of the GFP-labeled dentate granule cells were acquired using a Leica SP5 confocal system, set up on a Leica DMI6000 inverted microscope equipped with a 63X oil immersion objective (numerical aperture 1.4). This system was used to capture three dimensional "image stacks" through the z-depth of the tissue at 0.5 μ m steps with a 1X optical zoom (field size 240 $\times$ 240 μ m). All granule cells selected for analysis were brightly labeled with GFP and had their somata fully contained within the tissue section. To reduce variability, cells selected for this study were restricted to the upper blade of the dentate gyrus within dorsal hippocampus. On average 20 cells (range 18 – 23) were selected from each animal with an average of 2 sections per animal. All cells in an image stack that met selection criteria were used for analyses.

Confocal z-series image stacks were imported into Neurolucida software for analysis (Version 5.65, Microbrightfield Inc., Williston, VT). For each cell, maximum soma profile area and the number of primary apical and basal dendrites was determined. Basal dendrites

were counted as those dendrites that originated from the hilar side of the soma (i.e. arising from a region below the equator of the soma). Basal dendrites that projected into the hilus were defined as “hilar basal dendrites” and basal dendrites that turned back into the dentate molecular layer were termed “recurrent basal dendrites”. Dendrites arising from above the equator of the soma were scored as apical dendrites. Portions of the proximal axon contained within the granule cell body layer (GCL) were also examined to determine their mean cross sectional area. This was done by first encoding the volume of the first 8.0 – 13.0 μm of the axon (lengths varied slightly due to artificial axon truncation at the tissue surface, although groups were not significantly different [at 1 day after the last kindling evoked seizure: control, $11.1 \pm 0.22 \mu\text{m}$, kindled, $10.9 \pm 0.15 \mu\text{m}$; $p=0.26$, t-test]) and then dividing by the length measured. The density of axonal varicosities along the portion of axon contained within the granule cell body layer was also determined. Axonal varicosities were defined as expansions of the axon with a diameter greater than 0.5 μm . Proximal axon varicosity number was normalized by dividing by the length of axon measured. Image stacks were also used to determine the width of the granule cell body layer. Two methods were used to determine layer width: 1) distance from the hilar border to the molecular layer border at the midpoint of the upper blade; 2) area of the entire granule cell body layer in the brain section divided by the length of the entire cell body layer. Further, to correct for potential bias introduced by the angle of sectioning, the thickness of the granule cell layer was normalized relative to the thickness of the CA3 and CA1 pyramidal cell layers in same sections, using the following equations: GCL thickness/pyramidal cell layer thickness $\times 100$. Image stacks were also used to determine the relative depth of each neuron examined within the GCL (cell distance from GCL-hilar border/GCL width).

For analyses of dendritic spine density, images of dendritic segments located within the inner and the outer molecular layers were captured using a 0.2 μm step at 6X optical zoom (field size $40 \times 40 \mu\text{m}$). Inner molecular layer dendritic segments were imaged by placing the lower edge of the imaging field on the granule cell body layer - molecular layer border and by scanning the initial projection into the molecular layer. Outer molecular segments were scanned by placing the upper edge of the field at the hippocampal fissure and imaging the terminal segments of the dendritic tree. Only dendritic segments fully contained within the tissue section were imaged. Images were imported into Neurolucida software for analysis. Dendritic spines were defined as protrusions from the dendritic shaft with a length greater than 0.25 μm . Spine density was determined by dividing the number of spines counted by the length of dendrite examined. Although confocal microscopy does facilitate visualizing spines located above and below the dendritic shaft, it is likely that smaller spines are still obscured in these regions, so spine measurements likely underestimate actual values. In addition, images/analyses were not adjusted for shrinkage of the tissue, so comparisons between the present findings and work using different methodologies should be made with these caveats in mind.

Statistical Analysis

All statistical analyses were performed using Sigma Plot (version 12.0). For each parameter, data from each animal was averaged to generate individual animal means. Animal means were used for determination of significance instead of cell numbers, as cells from the same animal are not completely independent and may result in artificially low p-values. Significance was determined using two-tailed Students t-tests for data that met assumptions of normality and equal variance. The Mann-Whitney rank sum test was used for non-normal data. Values presented are means $\pm$ standard error of the mean (SEM) or medians (range), as appropriate. Pearson product moment correlation was used for correlations. Since both male and female animals were included in the study, data were examined for effects of sex. No significant effects of sex were found for any parameter (data not shown). Removing the

female animals from the one month groups and analyzing only males did not change statistical outcomes either (data not shown). For these reasons, male and female animals were binned and analyzed together.

Figure Preparation

Maximum projections from confocal z-series stacks were prepared using Leica's LAS AF Confocal software (1.3.1 build 525). Background artifact reduction was accomplished using an erosion filter run for one iteration with a three pixel radius (Leica software). Contrast, brightness, montage adjustments and figure preparation were done using Adobe Photoshop CS5 (version 12.0). Identical filtering and adjustments to brightness and contrast were done for images used for comparison.

Results

Part I: Morphological analyses one day after the last kindling-evoked seizure

Gross hippocampal morphology is unchanged in kindled mice—Granule cells in the Thy1-GFP line, brightly labeled with GFP, clearly revealed the axonal and dendritic structure of each labeled neuron (Figure 1). Each kindled animal experienced 5 evoked seizures, with cumulative electrographic seizure duration of 527 ± 101 seconds/mouse. The mean number of stimulations required to evoke 5 class IV or above behavioral seizures was 22.0 ± 3.4 stimulations/mouse. The number and distribution of GFP-expressing granule cells in kindled and controls animals was statistically similar between groups, as demonstrated in previous work with the mice used for the present study (Danzon et al., 2010). The morphological architecture of the hilus, the granule cell layer and the molecular layer was also comparable between control and kindled mice. No evidence of gross neuronal loss or overt neuronal injury (e.g. dendritic beading or retraction) was observed.

Granule cell body layer thickness is increased one day after the last kindling-evoked seizure—Status-epilepticus models of epilepsy can produce dramatic granule cell dispersion and somatic hypertrophy (Houser, 1990; Murphy et al., 2012). Here, we observed a modest increase in the width of the granule cell body layer in kindled mice compared to controls. At the midpoint of the upper blade, control animals had an average granule cell body layer width of $46.9 \pm 2.7 \mu\text{m}$ ($N=7$ mice, 2 sections/mouse) compared to $63.0 \pm 6.5 \mu\text{m}$ ($N=7$ mice, 2 sections/mouse) in kindled animals ($p=0.02$, t-test, Figure 2). Similar results were obtained by dividing the entire area of the granule cell body layer by the entire length of the granule cell body layer (control, $48.9 \pm 2.4 \mu\text{m}$, $N=7$ mice, 2 sections/mouse; kindled, $61.1 \pm 4.5 \mu\text{m}$, $N=7$ mice, 2 sections/mouse; $p=0.03$, t-test, Figure 2). This latter finding suggests that the increase in width affects most of the layer. This difference in the width of the granule cell body layer also remains significant when normalized to the width of CA1 and CA3 (Table 1), indicating that the angle of sectioning did not interfere with our results. Although non-significant, a tendency for granule cell somata to be larger in kindled mice likely contributed to the increase in layer width. The average soma area in cells from kindled animals was $95.3 \pm 8.8 \mu\text{m}^2$ ($N=7$ mice, 140 cells from 2 sections/mouse) compared to $78.9 \pm 6.3 \mu\text{m}^2$ ($N=7$ mice, 133 cells from 2 sections/mouse) in control animals ($p=0.15$, t-test, Figure 2). There was no significant change, however, in the relative location of GFP-expressing granule cell somata within the granule cell layer (cell distance from GCL-hilar border/GCL width) (Table 1) suggesting that the observed expansion of the granule cell body layer is relatively uniform. We did not observe a difference in the mean width of either CA3 or CA1 at this time point (Table 1).

Apical and basal dendrites—Changes in the number of apical dendrites (Murphy et al., 2011) and the *de novo* appearance of basal dendrites projecting into the dentate hilus

(Spigelman et al., 1998) have been described in multiple models of epilepsy. Whether and to what extent these changes are a feature of the early phases of kindling epileptogenesis is, however, unclear. To explore these questions, apical and basal dendrite number was assessed among granule cells from control and kindled mice one day after the last kindling-evoked seizure. No differences were found in the number of apical or basal dendrites in the kindled mice relative to controls (Table 1). All granule cells in the study had at least one apical dendrite, with a few cells having as many as four. Hilar basal dendrites, on the other hand, were only rarely found on granule cells from control animals, and this did not change significantly in kindled mice (Table 1). No recurrent basal dendrites were observed.

Reduced dendritic spine density in kindled mice—Spine density along dendritic segments located within the inner and outer molecular layers was significantly decreased in kindled animals relative to controls one day after the last evoked seizure. Within the inner molecular layer, spine density was reduced 30% from 1.81 ± 0.13 spines/ μm (N=7 mice, 133 cells from 2 sections/mouse) in controls to 1.26 ± 0.09 spines/ μm (N=7 mice, 140 cells from 2 sections/mouse) in kindled mice ($p=0.005$, t-test, Figure 3A). Similar reductions were observed in the outer molecular layer, where spine density dropped by 21% from 3.57 ± 0.18 spines/ μm (N=7 mice, 133 cells from 2 sections/mouse) in controls to 2.81 ± 0.16 spines/ μm (N=7 mice, 140 cells from 2 sections/mouse) in kindled mice ($p=0.01$, t-test, Figure 3A). Finally, we conducted a correlation analysis between mean inner and outer molecular layer spine density for each animal, and found that the values were positively correlated with each other across the entire group of mice (controls and kindled combined, $R=0.609$, $p=0.0207$; Figure 3B). This finding indicates that animals with a decrease in inner molecular layer spine density are likely to exhibit a concomitant reduction in the outer molecular layer spine density.

Increased volume of the proximal axon one day after the last kindling-evoked seizure—To determine whether axonal swelling occurs in the kindling model, we measured the mean cross sectional area of the proximal axon of GFP-expressing granule cells. One day after the last seizure, cross sectional area of the proximal axon was increased in kindled animals relative to controls. Proximal axon segments from kindled animals had an average cross sectional area of $0.75 \pm 0.08 \mu\text{m}^2$ (N=7 mice, 113 cells from 2 sections/mouse) compared to $0.54 \pm 0.03 \mu\text{m}^2$ (N=7 mice, 90 cells from 2 sections/mouse) in control animals ($p=0.04$, t-test, Figure 4). By contrast, no difference was found between groups in the density of axonal varicosities along the portion of the axon located within the granule cell body layer (Table 1).

Part II: Morphological analyses one month after last kindling-evoked seizure

Animals examined one month after the last of 5 kindling-evoked seizures experienced a cumulative electrographic seizure duration of 397 ± 69 seconds. The mean number of stimulations required to evoke 5 class IV or above behavioral seizures was 17.0 ± 2.2 stimulations/mouse. Similar to animals examined one day after the last seizure, the pattern of GFP expressing cells and the gross structure of the dentate gyrus were qualitatively similar between control and kindled animals (Figure 5). Kindled animals did not show any gross neuronal loss and the morphological architecture of the hilus, hippocampal pyramidal cell layers and molecular layers were qualitatively similar between groups.

Granule cell body layer width normalizes one month after the last kindling-evoked seizure—The increase in granule cell layer width observed one day after the last kindling-evoked seizure was no longer detectable one month later. Kindled animals had an average granule cell body layer width of $49.9 \pm 4.2 \mu\text{m}$ (N=7 mice, 2 sections/mouse) compared to $49.5 \pm 3.9 \mu\text{m}$ (N=8 mice, 2 sections/mouse) in control animals ($p=0.94$, t-test,

Figure 6). Similarly, no difference was observed in the area divided by length of the granule cell body layer, either for the raw (kindled, $56.1 \pm 4.3 \mu\text{m}$, $N=7$ mice, 2 sections/mouse; control, $49.8 \pm 4.9 \mu\text{m}$, $N=8$ mice, 2 sections/mouse; $p=0.35$, t-test) or normalized values (Table 2). The trend towards increased soma area observed at one day after the last kindling-evoked seizure was also absent at this time point. The average soma area from kindled animals was $63.8 \mu\text{m}^2$ (range: $57.8 - 116.8 \mu\text{m}^2$, $N=7$ mice, 184 cells from 2 sections/mouse) compared to $65.9 \mu\text{m}^2$ (range: $57.9 - 102.4 \mu\text{m}^2$, $N=8$ mice, 122 cells from 2 sections/mouse) in control animals ($p=0.83$, t-test, Figure 6). Similar to the results obtained one day after the last kindling-evoked seizure, relative somata position within the granule cell body layer remained unchanged between control and kindled animals (Table 2). Likewise, we did not observe any difference in the thickness of CA1 or CA3 (Table 2).

Apical and basal dendrites—Control and kindled animals had comparable numbers of apical and basal dendrites one month after the last kindling-evoked seizure (Table 2). Basal dendrites were rare, with only $2.4 \pm 1.2\%$ of granule cells (3 of 122 cells from 2 sections/mouse, $N=8$ mice) from control animals and $2.7 \pm 3.4\%$ of granule cells (5 of 184 cells from 2 sections/mouse, $N=7$ mice) from kindled animals possessing a basal dendrite. Only hilar basal dendrites were observed.

Kindled animals exhibit normal spine density one month after the last kindling-evoked seizure—In comparison to the results at one day, at one month after the last kindling-evoked seizure smaller reductions of dendritic spine density were evident in the inner (23% reduction in kindled animals) and outer molecular layers (13% reduction in kindled animals). In the inner molecular layer, kindled animals had 1.39 ± 0.16 spines/ μm ($N=7$ mice, 184 cells from 2 sections/mouse), compared to 1.79 ± 0.30 spines/ μm ($N=8$ mice, 122 cells from 2 sections/mouse) in control animals. These differences were not statistically significant ($p=0.279$, t-test, Figure 7). Similarly, in the outer molecular layer, the kindled animals had 3.52 ± 0.19 spines/ μm ($N=7$ mice, 184 cells from 2 sections/mouse), compared to 4.06 ± 0.36 spines/ μm ($N=8$ mice, 122 cells from 2 sections/mouse) in control animals; also non-significant ($p=0.21$, t-test, Figure 7). Further, no correlation was observed between spine densities in the inner and outer molecular layers of kindled and control animals at this time point ($p=0.39$, $r=0.235$).

Proximal axon cross sectional area is indistinguishable from control one month after the last kindling-evoked seizure—The cross sectional area of the proximal axon did not show a significant difference between kindled and control animals one month after the last kindling-evoked seizure. Proximal axon segments from kindled animals had an average cross sectional area of $0.55 \pm 0.03 \mu\text{m}^2$ ($N=7$ mice, 148 cells from 2 sections/mouse) compared to $0.61 \pm 0.05 \mu\text{m}^2$ ($N=8$ mice, 122 cells from 2 sections/mouse) in control animals ($p=0.36$, t-test, Figure 8). Similarly, the density of axonal varicosities along the portion of the axon located within the granule cell body layer did not change significantly between control and kindled groups (Table 2).

Discussion

In the present study, hippocampal granule cells were examined either one day or one month after early kindling, in which five generalized, tonic-clonic seizures were evoked. We observed an increase in the thickness of the granule cell body layer, a decrease in the number of apical dendrite spines in the inner and outer molecular layers and an increase in the area of the proximal axon one day after the last kindling-evoked seizure. Apart from a persistent non-significant reduction in spine density, these changes had remitted in animals examined one month later. Morphological features seen in other models of temporal lobe

epilepsy, such as ectopic granule cells or granule cells with hilar-projecting basal dendrites were not observed in kindled animals examined at either time point.

Significance of increased granule cell body layer thickness

The increase in granule cell body layer thickness observed one day after the last kindling-evoked seizure could reflect a variety of processes, such as increased neurogenesis, somatic hypertrophy or reactive astrogliosis. All of these phenomena have previously been demonstrated in different models of epilepsy (Houser, 1990; Ribak and Dashtipour, 2002; Jessberger et al., 2005; Shapiro et al., 2007; Kraev et al., 2009; Murphy et al., 2012). Increased neurogenesis following seizure activity could increase granule cell layer thickness (Ribak and Dashtipour, 2002; Parent et al., 1998). The transient nature of the increase, however, favors other explanations, although it is possible that new (or old) cells might undergo apoptosis, thus restoring granule cell width to control values at one month. Reactive gliosis has been reported in the kindling model of epilepsy (Kraev et al., 2009; Jafari et al., 2012) and increased number and thickness of astroglial fibers might also contribute to the changes observed in the present study. Finally, the trend towards increased somata area described here likely contributes to the observed increase in granule cell layer thickness. Acute increases in cell volume can occur following osmotic changes caused by neuronal activity (Andrew et al., 1989; Ostby et al., 2009). In addition, work by Kato and colleagues (Kato et al., 2001) described dendritic hypertrophy a few hours after the last kindled seizure. Electron microscopy studies of these animals revealed an increase in stable polymerized microtubules in these dendrites, raising the possibility that cytoskeletal changes might contribute to increased granule cell layer width.

Development of hilar-projecting aberrant basal dendrites has been described in numerous models of temporal lobe epilepsy (Spigelman et al., 1998; Ribak et al., 2000; Murphy et al., 2012) and is hypothesized to promote epileptogenesis by creating recurrent excitatory circuits. In the present study, no increase in the incidence of basal dendrites was observed either one day or one month after the last kindling-evoked seizure. This finding is consistent with findings by Wood and colleagues (Wood et al., 2011), who used a retroviral approach to label adult-generated granule cells after rapid-kindling, but contrasts with work by Pekcec and colleagues (Pekcec and Potschka, 2007; Pekcec et al., 2011) where an increase in basal processes following 15 kindling-evoked seizures was observed among granule cells labeled with doublecortin. Differences in the kindling protocols used could account for the divergent results, with the number of evoked Class IV or greater seizures (15 vs. 5 in the present study) and the kindling protocol used (rapid vs. standard kindling) being likely candidates. Alternatively, the labeling strategy and time points examined could also contribute to the observed differences. Doublecortin is transiently expressed by developing granule cells ($\approx$ 1–2 weeks old) (Brown et al., 2003), while Thy1-driven GFP is only expressed in mature granule cells ($>$ 5 weeks) (Walter et al., 2007; Vuksic et al., 2008; Murphy and Danzer, 2011; Santos et al., 2011). The retroviral strategy used by Wood and colleagues targeted developing granule cells generated after rapid kindling; however, labeled cells were examined $\approx$ 2 months later, similar to the present study. The three studies, therefore, examined cells of different ages at different time points after kindling. Significantly, therefore, work by Walter and coworkers (Walter et al., 2007), using the same Thy1-GFP expressing mouse line examined here, found that 20% of Thy1-GFP expressing granule cells exhibited basal dendrites one month after pilocarpine induced status epilepticus; indicating that the neuronal population examined in the present study can develop basal dendrites under the right conditions. Whether more robust seizures, associated cell death or other differences between the pilocarpine status epilepticus and kindling models of epilepsy account for the contrasting morphological responses remains to be determined.

Significance of spine density changes

Dendritic spines are post-synaptic specializations which house the synaptic machinery that allows a neuron to respond to excitatory pre-synaptic input. Since most of the excitatory inputs received by principal neurons are targeted to dendritic spines, spine counts provide an indirect means to assess the density of excitatory synapses on a neuron. Spines are highly plastic and can exhibit rapid changes in structure and number in response to a variety of physiological and pathological changes (Gray, 1959; Drakew et al., 1996; Fiala et al., 2002; Kasai et al., 2010; Gonzalez Burgos et al., 2012; Kulkarni and Firestein, 2012).

One plausible mechanism contributing to the lifelong enhanced propagation of seizure activity from the stimulated amygdala to hippocampus and extra-limbic sites in kindled animals is increased numbers of excitatory synapses formed between principal neurons. This proposal predicts an increase in the density of spines on the apical dendrites of the dentate granule cells. Paradoxically, significant decreases in spine density were observed in both the inner (30% reduction) and outer (21% reduction) molecular layers one day after the last evoked seizure. The magnitude of these reductions had diminished by one month after the last evoked seizure; statistically significant decreases were no longer detected. The reductions in spine density observed here suggest that associational/commissural excitatory input to the inner molecular layer and lateral entorhinal cortex excitatory input to the outer molecular layer are reduced. The direction of the change, namely a decrease, together with its time course support the idea that these reductions underlie a homeostatic response aimed at reducing excitability following repeated seizures. Alternatively, since spines are an indirect measure of synapse number, it is also possible that spines are lost, but excitatory synapses are preserved. Conversion of excitatory spine synapses to direct synaptic innervation of dendritic shafts could also account for the present results, and would alter the electrotonic properties of the affected synapses by eliminating the thin spine necks. Distinguishing between these possibilities will require more direct measures of synapse number, such as electron microscopy techniques.

Finally, studies of spines and excitatory synapses in the kindling model have revealed both decreases and increases (Geinisman et al., 1992; Teskey et al., 2001). We suspect these complex changes can be accounted for in part by the population of synapses studied, with different neuronal populations potentially exhibiting distinct responses. Additional factors likely to be important include the stimulation paradigm used (Zhao et al., 2012) and time points examined (Teskey et al., 2001; Teskey et al., 2006). The time point examined also appears to be important for status epilepticus models of epilepsy, in which reductions are observed at early time points (Olney et al., 1983; Pyapali and Turner, 1994; Isokawa, 1998) while partial recovery can occur at later time points (Isokawa, 1998).

Significance of changes in the volume of the proximal axon

The axon initial segment is located within the proximal axon. The axon initial segment is the site at which the action potential originates, and is therefore critical to neuronal function (Palay et al., 1968). Marked swelling and dilation of the proximal axon has previously been seen in tissues from human epileptic patients (Chronister et al., 1995), and recent studies of the initial segment reveal changes in response to neural activity (Grubb and Burrone, 2010; Grubb et al., 2011) and seizures (Wimmer et al., 2010). Whether the proximal axon swelling observed here reflects changes in the composition of the initial segment, or is merely an extension of the trend towards swelling in the somatic compartment is not known. Even transient swelling, however, could alter action potential initiation if the density of ion channels relative to internal axon volume was substantially changed.

Conclusions

Our findings demonstrate that early kindling produces acute, transient changes in the thickness of the granule cell layer, molecular layer spine density and the area of the proximal granule cell axon. Early kindling did not, however, produce the striking rearrangements of granule cell structure seen in other models of epilepsy, at least for the cell populations examined. Whether the absence of robust restructuring in these animals accounts for the absence of spontaneous seizures remains to be determined.

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Figure 1. Confocal maximum projections of GFP-expressing hippocampal dentate granule cells from Thy1-GFP mice. The endogenous GFP signal was enhanced by immunostaining for GFP. Sections from either control (A) or kindled (B) animals are qualitatively similar. Scale bar, 250 μ m.

Figure 2.

One day after the last kindling-evoked seizure, there was a significant increase in granule cell layer thickness in kindled animals (B) in comparison to control (A) animals. There was also a trend towards increased somata area in kindled animals. Quantification of granule cell layer thickness and somata area are shown graphically in (C) and (D). * t-test, $p < 0.05$. Scale bar for (A) and (B), 20μm. Note: mossy cells axons, also labeled with GFP in the Thy1-GFP line, are present in the molecular layer of both the control and kindled animals (A & B, examples marked by blue arrowheads).

Figure 3.

Images shown in (A) are confocal maximum projections showing segments of GFP-expressing granule cell apical dendrites in the inner (IML) and outer (OML) molecular layers. One day after the last kindling-evoked seizure, spine density in both regions was significantly decreased in the kindled mice compared to control animals. Scale bar, 5 μm . The difference is depicted graphically below. * t-test, $p < 0.05$. (B) Correlation between inner and outer molecular layer spine density for each animal in the study (kindled, red dots; control, green squares).

Figure 4.

Proximal axon cross sectional area was quantified using Neurolucida software by calculating the volume of the first $\approx 10 \mu\text{m}$ of axon and dividing by the length measured. A confocal maximum projection of a sample neuron is shown in A, with the neurolucida tracing of the cell shown in B. One day after the last kindling-evoked seizure, the cross sectional area of the proximal axon was significantly increased in kindled mice (D) relative to controls (C). Arrows in each image delineate the portion of the proximal axon that was quantified. Bar graphs (E) show means $\pm$ SEM for each group. * t-test, $p < 0.05$. A= Cross sectional area of the axon, V= Volume of the axon, L=Measured length of the axon segment. All scale bars, $5 \mu\text{m}$.

Figure 5. Confocal maximal projections showing GFP immunostaining in the hippocampal dentate gyrus of control and kindled mice one month after the last kindling-evoked seizure. The gross morphology of the dentate was similar between groups. Scale bar, 250 μ m.

Figure 6.

Confocal maximum projections showing GFP-expressing granule cells within the granule cell body layer. The thickness of the cell body layer was similar between control (A) and kindled (B) animals one month after the last kindling-evoked seizure. Granule cell somata area was also similar between groups. Quantification of granule cell layer thickness and somata area are shown in the bar graphs in (C) and (D). Scale bar, 10 μm . Note: mossy cells axons, also labeled with GFP in the Thy1-GFP line, are present in the molecular layer of both the control and kindled animals (A & B, examples marked by blue arrowheads).

Figure 7.

Images are confocal maximum projections showing segments of GFP-expressing granule cell apical dendrites in the inner (IML) and outer (OML) molecular layers. One month after the last kindling-evoked seizure, spine density in both regions was statistically similar between control and kindled mice. Bar graphs show means $\pm$ SEM for each group. Scale bar, 5 μ m.

Figure 8.

One month after the last kindling-evoked seizure, the cross sectional area of granule cell proximal axons was statistically indistinguishable from control values. Arrows delineate the portion of the proximal axon that was quantified. Bar graphs show means $\pm$ SEM for each group. Scale bar, 5 μm .

Table 1

Additional parameters compared between control and kindled mice one day after the last evoked seizure.

Parameter	Control Mean (N=7 mice)	Kindled Mean (N=7 mice)	p-value (t-test)
Number of Apical Dendrites/ Cell	1 [1 – 4] (Median[Range]) (133 cells from 2 sections/mouse)	1 [1 – 3] (Median[Range]) (140 cells from 2 sections/mouse)	0.21
% of Cells with Basal Dendrites	1.5 ± 0.9% (Mean ± SEM) (133 cells from 2 sections/mouse)	3.6 ± 1.4% (Mean ± SEM) (140 cells from 2 sections/mouse)	0.25
Axon Length within the granule layer (µm)	26.2 ± 2.4 (Mean ± SEM) (90 cells from 2 sections/mouse)	30.3 ± 1.3 (Mean ± SEM) (113 cells from 2 sections/mouse)	0.17
Distance from Hilus (µm)	21.6 ± 3.2 (Mean ± SEM) (133 cells from 2 sections/mouse)	27.0 ± 1.9 (Mean ± SEM) (140 cells from 2 sections/mouse)	0.17
Axonal Varicosities/µm	0.006 ± 0.002 (Mean ± SEM) (90 cells from 2 sections/mouse)	0.013 ± 0.004 (Mean ± SEM) (113 cells from 2 sections/mouse)	0.21
Thickness of the CA1 (µm)	44.4 ± 2.7 (Mean ± SEM) (2 sections)	49.8 ± 1.9 (Mean ± SEM) (2 sections)	0.22
Thickness of the CA3 (µm)	66.2 ± 3.6 (Mean ± SEM) (2 sections)	65.0 ± 2.1 (Mean ± SEM) (2 sections)	0.82
Thickness of the granule cell body layer normalized to thickness of CA1 (µm)	1.0 ± 0.08 (Mean ± SEM) (2 sections)	1.3 ± 0.06 (Mean ± SEM) (2 sections)	0.03
Thickness of the granule cell body layer normalized to thickness of CA3 (µm)	0.7 ± 0.06 (Mean ± SEM) (2 sections)	1.0 ± 0.09 (Mean ± SEM) (2 sections)	0.009
Cell distance from GCL-hilar border/GCL width	61.0 ± 4.3% (Mean ± SEM) (133 cells from 2 sections/mouse)	54.1 ± 3.7% (Mean ± SEM) (140 cells from 2 sections/mouse)	0.25

Table 2

Additional parameters compared between control and kindled mice one month after the last evoked seizure.

Parameter	Control Mean (N=8 mice)	Kindled Mean (N=7 mice)	p-value (t-test)
No. of Apical Dendrites/ cell	1 [1 – 4] (Median[Range]) (122 cells from 2 sections/mouse)	1 [1 – 3] (Median[Range]) (184 cells from 2 sections/ mouse)	0.13
% of Cells with Basal Dendrites	2.4 ± 1.2% (Mean ± SEM) (122 cells from 2 sections/mouse)	2.7 ± 3.4% (Mean ± SEM) (184 cells from 2 sections/ mouse)	0.39
Axon Length within the granule layer (µm)	32.83 ± 1.76 (Mean ± SEM) (122 cells from 2 sections/mouse)	32.1 ± 1.83 (Mean ± SEM) (148 cells from 2 sections/ mouse)	0.82
Distance from Hilus (µm)	28.56 ± 3.28 (Mean ± SEM) (122 cells from 2 sections/mouse)	31.48 ± 3.28 (Mean ± SEM) (184 cells from 2 sections/ mouse)	0.54
Axonal Varicosities/µm	0.017 ± 0.007 (Mean ± SEM) (122 cells from 2 sections/mouse)	0.008 ± 0.002 (Mean ± SEM) (148 cells from 2 sections/ mouse)	0.25
Thickness of the CA1 (µm)	35.9 ± 1.1 (Mean ± SEM) (2 sections/mouse)	40.4 ± 2.5 (Mean ± SEM) (2 sections/mouse)	0.16
Thickness of the CA3 (µm)	60.4 ± 5.0 (Mean ± SEM) (2 sections/mouse)	59.0 ± 2.6 (Mean ± SEM) (2 sections/mouse)	0.80
Thickness of the granule cell body layer normalized to thickness of CA1 (µm)	1.3 ± 0.11 (Mean ± SEM) (2 sections/mouse)	1.2 ± 0.13 (Mean ± SEM) (2 sections/mouse)	0.52
Thickness of the granule cell body layer normalized to thickness of CA3 (µm)	0.85 ± 0.11 (Mean ± SEM) (2 sections/mouse)	0.85 ± 0.07 (Mean ± SEM) (2 sections/mouse)	0.97
Cell distance from GCL-hilar border/GCL width	58.91 [35.94–65.54] Median [Range] (122 cells from 2 sections/mouse)	55.43[42.97–62.26] Median [Range] (184 cells from 2 sections/ mouse)	0.41