More Docked Vesicles and Larger Active Zones at Basket Cell-to-Granule Cell Synapses in a Rat Model of Temporal Lobe Epilepsy

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Temporal lobe epilepsy is a common and challenging clinical problem, and its pathophysiological mechanisms remain unclear. One possibility is insufficient inhibition in the hippocampal formation where seizures tend to initiate. Normally, hippocampal basket cells provide strong and reliable synaptic inhibition at principal cell somata. In a rat model of temporal lobe epilepsy, basket cell-to-granule cell (BC→GC) synaptic transmission is more likely to fail, but the underlying cause is unknown. At some synapses, probability of release correlates with bouton size, active zone area, and number of docked vesicles. The present study tested the hypothesis that impaired GABAergic transmission at BC→GC synapses is attributable to ultrastructural changes. Boutons making axosomatic symmetric synapses in the granule cell layer were reconstructed from serial electron micrographs. BC→GC boutons were predicted to be smaller in volume, have fewer and smaller active zones, and contain fewer vesicles, including fewer docked vesicles. Results revealed the opposite. Compared with controls, epileptic pilocarpine-treated rats displayed boutons with over twice the average volume, active zone area, total vesicles, and docked vesicles and with more vesicles closer to active zones. Larger active zones in epileptic rats are consistent with previous reports of larger amplitude miniature IPSCs and larger BC→GC quantal size. Results of this study indicate that transmission failures at BC→GC synapses in epileptic pilocarpine-treated rats are not attributable to smaller boutons or fewer docked vesicles. Instead, processes following vesicle docking, including priming, Ca²⁺ entry, or Ca²⁺ coupling with exocytosis, might be responsible.

Key words: bouton; dentate gyrus; electron microscopy; GABA; inhibition; ultrastructure

Significance Statement

One in 26 people develops epilepsy, and temporal lobe epilepsy is a common form. Up to one-third of patients are resistant to currently available treatments. This study tested a potential underlying mechanism for previously reported impaired inhibition in epileptic animals at basket cell-to-granule cell (BC→GC) synapses, which normally are reliable and strong. Electron microscopy was used to evaluate 3D ultrastructure of BC→GC synapses in a rat model of temporal lobe epilepsy. The hypothesis was that impaired synaptic transmission is attributable to smaller boutons, smaller synapses, and abnormally low numbers of synaptic vesicles. Results revealed the opposite. These findings suggest that impaired transmission at BC→GC synapses in epileptic rats is attributable to later steps in exocytosis following vesicle docking.

Introduction

One in 26 people develops epilepsy (Hesdorffer et al., 2011), and anti-seizure drugs fail in 23% of cases (Picot et al., 2008) or more (Kwan and Brodie, 2000). Resection of the seizure-generating focus can be a treatment option, and 50%–73% of patients re-

ferred to surgical centers have temporal lobe epilepsy (Téllez-Zenteno and Hernández-Ronquillo, 2012). Depth electrodes sometimes are implanted to localize sites of seizure onset. Results from those evaluations suggest that, in patients with temporal lobe epilepsy, seizures usually start in the hippocampus (Quesney, 1986; Spencer et al., 1987; Sperling and O'Connor, 1989; Spanedda et al., 1997). The dentate gyrus has been suspected to play a role in seizure initiation in part because it is hyperexcitable (Gabriel et al., 2004) and granule cells are less inhibited than in controls (Williamson et al., 1995, 1999) after temporal lobe epilepsy had developed. Animal models of temporal lobe epilepsy display similar abnormalities (Nadler et al., 1978; Cronin et al., 1992). Reduced inhibition of granule cells might be an epileptogenic mechanism.

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In animal models of temporal lobe epilepsy, granule cells display aberrantly low frequencies of miniature IPSCs (Kobayashi and Buckmaster, 2003; Shao and Dudek, 2005; Sun et al., 2007). Most miniature IPSCs recorded in granule cells are generated by somatic GABAergic synapses (Soltesz et al., 1995). Basket cells are the major source of inhibitory synaptic input to granule cell somata (Kraushaar and Jonas, 2000). Reduced miniature IPSC frequency in epileptic subjects could be attributable to lower release probability and/or fewer basket cell-to-granule cell (BC→GC) synapses. However, basket cells are relatively resistant to epileptogenic injuries compared with some other interneurons (Buckmaster and Dudek, 1997; Sun et al., 2007), and numbers of inhibitory synapses with granule cell somata are not significantly reduced in patients with temporal lobe epilepsy (Wittner et al., 2001) or in epileptic pilocarpine-treated rats (Thind et al., 2010). Together, these findings suggest that reduced inhibition of granule cells in temporal lobe epilepsy might be attributable, at least in part, to dysfunction of surviving BC→GC synapses.

Recordings of unitary IPSCs in monosynaptically coupled neurons reveal that BC→GC synaptic transmission is impaired in rats that develop permanent epilepsy after surviving acute status epilepticus induced by systemic treatment with pilocarpine (Zhang and Buckmaster, 2009). Epileptic pilocarpine-treated rats display anatomical and electrophysiological abnormalities in the dentate gyrus similar to patients with temporal lobe epilepsy (Kobayashi and Buckmaster, 2003) and their spontaneous seizures usually initiate in the hippocampal formation (Toyoda et al., 2013). BC→GC synapses in epileptic rats have higher failure rates than in controls, which does not appear to be attributable to abnormally high levels of presynaptic inhibition. Instead, granule cell responses to high-frequency trains of basket cell action potentials reveal smaller readily releasable pools of synaptic vesicles in epileptic rats. In some circuits, including excitatory hippocampal synapses, readily releasable pool size corresponds with the number of docked vesicles (Schikorski and Stevens, 2001). Therefore, we hypothesized that BC→GC synaptic boutons would contain fewer docked vesicles in epileptic pilocarpine-treated rats.

The ultrastructural size principle contends that synaptic efficacy is directly related to synaptic bouton size, which correlates positively with other ultrastructural features, including active zone area and number of synaptic vesicles (Pierce and Lewin, 1994). If this principle, which is based primarily on data from excitatory synapses, also pertains to inhibitory synapses, then BC→GC boutons in animal models of temporal lobe epilepsy might be smaller, have smaller active zones, and have fewer synaptic vesicles than controls. To test these predictions, the present study compared ultrastructural features of BC→GC synaptic boutons in control and epileptic pilocarpine-treated rats. In epileptic pilocarpine- and kainate-treated rats, CA1 pyramidal cells have reduced miniature IPSC frequency that correlates with lower densities of synaptic vesicles in GABAergic axosomatic boutons (Hirsch et al., 1999). To our knowledge, no previous study has evaluated vesicles of BC→GC synapses of the dentate gyrus in temporal lobe epilepsy.

Materials and Methods

All animal procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by an institutional animal care and use committee at Stanford University. Male Sprague Dawley rats (Harlan Laboratories) at 34–37 d of age were treated with pilocarpine. Pilocarpine hydrochloride (380 mg/kg, i.p.) was administered 20 min after atropine methyl bromide

(5 mg/kg, i.p.). Diazepam (10 mg/kg, i.p.) was administered 2 h after the onset of motor seizures and was repeated as needed for the next 10 h to suppress convulsions. To reduce the number of animals used, four rats that had been treated identically with pilocarpine but did not develop status epilepticus were included in the control group. Two more naive control rats were added, so control and epileptic groups would both consist of six animals. Results from both types of controls were combined. Naive and pilocarpine-treated control animals are indicated by different markers in all plots. Previous studies that included larger samples of naive and pilocarpine-treated control rats found no significant differences in electrophysiological and anatomical parameters of interneurons and GABAergic synapses in the dentate gyrus or other brain regions (Kobayashi and Buckmaster, 2003; Kobayashi et al., 2003; Kumar and Buckmaster, 2006), but those studies did not evaluate ultrastructure. All rats that experienced status epilepticus, but none of the controls was observed by chance to have at least one spontaneous motor seizure (grade 3 or greater) (Racine, 1972) at least 14 d after pilocarpine treatment. However, rats were not systematically monitored for spontaneous seizures. Therefore, the total number and timing of seizures experienced were not known and might be a confounding effect on GABAergic synaptic ultrastructure.

Rats were killed by barbiturate overdose (>100 mg/kg pentobarbital, i.p.) when they were 3–9 months old (average 5 and 6 months old in epileptic and control rats, respectively) and perfused through the ascending aorta at 30 ml/min for 1 min with 0.9% sodium chloride and 30 min with 2% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C. Brains postfixed overnight at 4°C. A vibratome was used to make 80- μ m-thick horizontal sections. Sections from −5.1 to −7.1 mm below bregma (Paxinos and Watson, 1998) were trimmed to isolate regions of the dentate gyrus.

Dentate gyrus tissue was postfixed with potassium ferricyanide-reduced 2% OsO₄ in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h, rinsed in the same buffer, and then rinsed in distilled water before being placed in 2% aqueous uranyl acetate overnight on a rotator. After rinses in distilled water, sections were placed in 0.6% lead nitrate in 0.4% L-aspartate for 2 h at 60°C, rinsed in distilled water, dehydrated in a series of ethanols, placed in propylene oxide, and then embedded in Epon and propylene oxide mix (1:1 and 3:1) before being gradually transferred to pure Araldite/Eponate-12 (Ted Pella) and embedded in BEEM capsules at 60°C for 24 h. Block faces were trimmed, and an ultramicrotome (Reichert Ultratuc S, Leica) was used to make ultrathin sections of silver interference color, estimated to be 35 nm thick. Serial sections were collected on coated, nickel single-slot grids. Sections were poststained with Sato's lead stain for 1–3 s.

A transmission electron microscope (JEOL 100CX) at 36,000 $\times$ magnification was used to identify axosomatic symmetric synapses in the granule cell layer that were identified by parallel membranes, cleft material, concentration of presynaptic vesicles, and a thin postsynaptic density (Gray, 1959). Somata were identified as putative granule cells based on their location in the granule cell layer, small size, round shape, and thin cytoplasm. Boutons were chosen for reconstruction by investigators who were blind to experimental groups. Boutons were selected for reconstruction if they fulfilled the following criteria: (1) they made a symmetric synapse with a putative granule cell somata; (2) the synapse was perpendicular to the plane of section; and (3) the bouton could be followed completely across serial sections. Selected boutons were photographed in their entirety across serial sections. Serial electron micrographs were printed at a final magnification of 92,000 $\times$. In some cases, a bouton profile extended beyond the field of view and multiple electron micrographs were made so that montages of prints could be assembled to include the entire bouton profile. A total of 5123 electron micrographs were made. Only 42 sections were missing, which was <1% in both control and epileptic groups.

Synaptic boutons were three-dimensionally reconstructed from serial electron micrographs using Neurolucida software (MBF Bioscience) and an interactive pen display monitor (Cintiq, Wacom). Contours were made to outline synaptic boutons, mitochondria, interfaces (direct appositions of presynaptic and postsynaptic membranes), and active zones (presynaptic border of the synaptic cleft). Different colors designated

Figure 1. Axosomatic symmetric synapses in the granule cell layer of an epileptic pilocarpine-treated rat. Intersection of three granule cells (nuclei indicated by asterisks) with several boutons evident that make axosomatic symmetric synapses (arrowheads). One bouton was reconstructed from serial electron micrographs (arrow).

different mitochondria or different interfaces of individual boutons. Markers were used to designate clear synaptic vesicles, including docked vesicles, which were identified by their direct contact with an active zone. It is possible that vesicles cut at section surfaces might have been counted twice in serial sections, resulting in an overestimation of vesicle number, but relative values of control and experimental groups were not likely to be affected. Reconstructed boutons included both en passant and terminal types. Contours outlining bouton borders extended across narrow points of axon connection. If a mitochondrion projected from a bouton partially into a narrow segment of the axon, the bouton reconstruction included that part of the axon to avoid excluding part of an intact mitochondrion.

To reduce confounding effects of variation from sources other than experimental group differences, the following steps were taken. All rats were handled, treated, and perfused, and 80- μm -thick sections were generated by one investigator. Generation of ultrathin sections and electron micrographs was divided equally between two investigators who each acquired data for five synaptic boutons from every animal. Investigators were blind to experimental groups during data acquisition and analysis.

Measured parameters of 10 boutons from each animal were averaged before calculating control and epileptic group averages. For all statistical comparisons between control and epileptic groups, sample sizes consisted of the number of animals in each group (6). In most cases, a two-tailed *t* test was used to test for differences between control and epileptic groups, which assumes that samples were drawn from normally distributed populations with the same variances (SigmaPlot 12, Systat). The Shapiro–Wilk test was used to measure normality. Equal variance was tested by checking the variability about the group means. If normality or equal variance tests failed, a Mann–Whitney rank sum test was used. Differences were considered significant if $p < 0.05$. The power of a *t* test is the probability that the test will detect a difference between the groups if there really is a difference. Power was calculated using SigmaPlot, which follows the method described by Zar (1984). Results are reported as mean $\pm$ SEM.

Results

In the granule cell layer of control and epileptic pilocarpine-treated rats, numerous boutons were evident that made symmetric axosomatic synapses with round cells containing a thin shell of cytoplasm surrounding a nucleus (Fig. 1). Synaptic clefts, bordered by parallel presynaptic and postsynaptic membranes, were ~ 12 nm wide and contained amorphous, dense material (Fig. 2C,F). Clear vesicles accumulated close to active zones where amorphous material extended into the cytoplasm. Puncta adherentia were sometimes evident between presynaptic and postsynaptic membranes and were distinguished from symmetrical synapses in part by a lack of associated vesicles. Large dense-core vesicles were sometimes evident, but smaller, clear, pleomorphic vesicles predominated. Only clear vesicles were included for analysis.

A total of 120 synaptic boutons that made symmetric axosomatic synapses with granule cells were reconstructed from serial electron micrographs (Fig. 2). Boutons extended across 37 ± 2 (mean $\pm$ SEM) and 45 ± 3 sections in control and epileptic rats, respectively ($p = 0.045$, *t* test, power = 0.54). Reconstructions included enclosed contours of the bouton membrane and mitochondria; markers

depicting clear synaptic vesicles, including those that touched an active zone and were designated as docked; and open contours of active zones and direct appositions of presynaptic and postsynaptic membranes referred to as “interfaces” (Peters and Harri-man, 1990). Ten boutons were reconstructed for each animal (Fig. 3). Previous studies report considerable variability in the size and shape of hippocampal boutons and synapses (Harris and Sultan, 1995; Schikorski and Stevens, 1997), including GABAergic synapses (Biró et al., 2006). Similarly, in the present study, boutons making axosomatic symmetric synapses with granule cells were variable even in individual animals.

To test for scaling relationships between the size of synaptic boutons and other ultrastructural features, bouton volume was plotted against mitochondrial volume, interface area, number and area of active zones, number of clear vesicles, and number of docked vesicles (Fig. 4A–F). All parameters correlated with bouton volume ($p < 0.001$, ANOVA, power = 1.0). In addition, interface area correlated with active zone area (Fig. 4G), active zone area correlated with the number of docked vesicles (Fig. 4H), and the total number of vesicles correlated with the number of docked vesicles (Fig. 4I) (all $p < 0.001$, ANOVA, power = 1.0). These findings revealed scaling relationships between many ultrastructural features of boutons and synapses.

As explained in the Introduction, based on the ultrastructural size principle and previously reported measures of inhibitory synaptic function in granule cells, BC→GC boutons were predicted to be smaller in epileptic animals. However, results revealed the opposite (Fig. 5). Average bouton volume was twice as large in epileptic rats ($0.690 \pm 0.065 \mu\text{m}^3$) compared with controls ($0.333 \pm 0.024 \mu\text{m}^3$, $p < 0.001$, *t* test, power = 1.0) (Fig. 5I).

Figure 2. Reconstructed axosomatic synaptic boutons in the granule cell layer of a control (**A–C**) and epileptic pilocarpine-treated rat (**D–F**). **A, D**, Side views. **B, E**, Top views. Orange represents bouton. Blue and green represent mitochondria (different colors represent different mitochondria). Red represents interface area of presynaptic and postsynaptic membrane apposition. Black represents active zones. Black markers represent clear vesicles. **C, F**, Electron micrographs of one section from reconstructed synapses. Corresponding sections indicated by horizontal arrow in **B** and **E**. **F**, Vertical arrows represent clearly docked vesicles. Filled arrowheads represent borders of active zones. Open arrowheads represent interface areas on the postsynaptic side.

These findings suggested that impaired BC→GC synaptic transmission was not attributable to small, atrophic boutons.

There are reports of ultrastructural damage to mitochondria in the hippocampus of patients with temporal lobe epilepsy (Kunz et al., 2000) and in epileptic pilocarpine-treated rats (Gao et al., 2007), and some evidence suggests that deficient mitochondria can contribute to synaptic dysfunction (Verstreken et al., 2005). To test that possibility, mitochondria in synaptic boutons were evaluated (Fig. 5). Only 1 of 120 sampled boutons lacked a mitochondrion. The average number of mitochondria per bouton was $1.7\times$ higher in epileptic rats (2.25 ± 0.26) compared with controls (1.32 ± 0.10 , $p = 0.009$, Mann–Whitney rank sum test) (Fig. 5J). Similarly, the total mitochondrial volume per bouton

was $1.8\times$ higher in epileptic rats ($0.124 \pm 0.013 \mu\text{m}^3$) compared with controls ($0.070 \pm 0.006 \mu\text{m}^3$, $p = 0.003$, t test, power = 0.93) (Fig. 5K). However, the average volume of individual mitochondria was similar in epileptic rats ($0.057 \pm 0.005 \mu\text{m}^3$) and controls ($0.055 \pm 0.005 \mu\text{m}^3$, Mann–Whitney rank sum test) (Fig. 5L). These values are within the range reported previously for mitochondria in hippocampal synaptic boutons (Eyre et al., 2007). Mitochondrial volume as a percentage of bouton volume in epileptic rats ($18.1 \pm 0.8\%$) was 85% that of controls ($21.2 \pm 1.1\%$, $p = 0.048$, t test, power = 0.53) (Fig. 5M). These findings revealed more numerous mitochondria but not quite proportional total mitochondrial volume in enlarged boutons of epileptic rats.

Figure 3. Reconstructed axosomatic synaptic boutons from the granule cell layer of a control and epileptic pilocarpine-treated rat. Ten boutons were reconstructed for each animal. Orange represents bouton. Blue, green, and magenta represent mitochondria (different colors represent different mitochondria). Red and green represent interface areas of presynaptic and postsynaptic membranes. Black markers represent synaptic vesicles.

Despite larger BC→GC boutons, synapses might be smaller in epileptic rats to explain their reduced transmission rate. Synapses were evaluated by measuring active zone and interface areas (Figs. 6, 7). Interfaces are appositions of the presynaptic bouton and membranes of the postsynaptic cell. All boutons in the sample were selected for reconstruction based on their making at least one synapse with a granule cell soma. Most boutons (79%, 95 of 120) synapsed with only one granule cell, but some synapsed with two (21%, 25 of 120). The average number of interfaces per bouton was identical in epileptic rats (1.22 ± 0.05) and controls (1.22 ± 0.05 , $p = 1.0$, t test, power = 0.50) (Fig. 8A), but the area per interface in epileptic rats ($1.11 \pm 0.10 \mu\text{m}^2$) was $1.4\times$ that of controls ($0.79 \pm 0.08 \mu\text{m}^2$, $p = 0.016$, power = 0.62) (Fig. 8B).

Each interface included one or more active zones. Active zones are synaptic complexes consisting of parallel membranes, amorphous dense material in the cleft, and a dense accumulation of vesicles presynaptically. The proportion of active zone targets was 95.4% granule cell somata (231 of 242), 2.5% dendritic spines (6 of 242), and 2.1% dendritic shafts (5 of 242). Previous studies found that, in contrast to excitatory asymmetric synapses where a large fraction of the interface area is active zone, at symmetric synapses a smaller proportion of interface area is active zone, and active zones can be divided into multiple, irregularly shaped areas (Peters and Harriman, 1990). As explained in the Introduction, some previous studies suggest that active zones are smaller at less reliable synapses, so BC→GC synapses were hypothesized to have smaller active zones in epileptic rats. Results showed quite the opposite (Figs. 6, 7). The number of active

zones per bouton was $1.4\times$ higher in epileptic rats (2.35 ± 0.08) compared with controls (1.73 ± 0.16 , $p = 0.015$, Mann–Whitney rank sum test) (Fig. 8C). Area per active zone was $1.7\times$ larger in epileptic rats ($0.123 \pm 0.016 \mu\text{m}^2$) compared with controls ($0.073 \pm 0.003 \mu\text{m}^2$, $p = 0.002$, Mann–Whitney rank sum test) (Fig. 8D). In control animals, active zones occupied $14.9 \pm 1.4\%$ of the interface area (Fig. 8E), similar to symmetric synapses with pyramidal cell somata in visual cortex (Peters et al., 1990). In epileptic rats, active zone area as a proportion of interface area was $1.3\times$ larger than in controls ($20.0 \pm 1.5\%$, $p = 0.036$, t test, power = 0.59). Total active zone area per bouton was $2.1\times$ larger in epileptic rats ($0.240 \pm 0.029 \mu\text{m}^2$) compared with controls ($0.115 \pm 0.009 \mu\text{m}^2$, $p = 0.002$, Mann–Whitney rank sum test) (Fig. 8F). These findings revealed that active zone area was larger, not smaller, at BC→GC synapses in epileptic rats.

Some evidence suggests that efficacy of synaptic transmission is reduced at boutons with fewer synaptic vesicles (Pierce and Lewin, 1994; Hirsch et al., 1999; Li and Schwarz, 1999), so BC→GC boutons were hypothesized to contain fewer synaptic vesicles in epileptic rats. Results revealed the opposite. The number of vesicles per bouton was $2.4\times$ higher in epileptic rats (2060 ± 190) compared with controls (850 ± 40 , $p < 0.001$, t test, power = 1.0) (Fig. 8G). The number of vesicles per interface was $2.4\times$ higher in epileptic rats (1760 ± 190) compared with controls (740 ± 40 , $p < 0.001$, t test, power = 1.0) (Fig. 8H). The number of vesicles per active zone was $1.9\times$ higher in epileptic rats (1040 ± 110) compared with controls (550 ± 50 , $p = 0.002$, t test, power = 0.96) (Fig. 8I). However, the density of vesicles in

Figure 4. Linear regression revealed correlation of ultrastructural parameters of 120 boutons forming symmetric axosomatic synapses with granule cells in control (red circles) and epileptic pilocarpine-treated rats (black circles). Bouton volume correlated with mitochondrial volume (**A**), interface area (**B**), number of active zones (**C**), active zone area (**D**), number of clear vesicles (**E**), and number of docked vesicles (**F**). **G**, Interface area correlated with active zone area. **H**, Active zone area correlated with number of docked vesicles. **I**, Number of vesicles correlated with number of docked vesicles. In all cases, $p < 0.001$ (ANOVA). R values are indicated.

Figure 5. Axosomatic boutons in the granule cell layer are larger and contain more mitochondria in epileptic pilocarpine-treated rats. Reconstructed boutons (orange) and mitochondria (blue, green, and magenta) in control (**A–D**) and epileptic pilocarpine-treated rats (**E–H**). Side (above) and top (below) views are shown in **A–C** and **E, F**, respectively. **D, H**, (Figure legend continues.)

Figure 6. Surface views of single axosomatic synapses in control (**A–E**) and epileptic pilocarpine-treated rats (**F–J**). Contours of synaptic boutons (orange), interface areas of presynaptic and postsynaptic membrane appositions (red), and active zones (black) are indicated. Yellow circles represent docked vesicles. **E, J**, Electron micrographs of corresponding sections in reconstructions indicated by horizontal arrows in **C** and **I**. Vertical arrows represent docked vesicles in micrographs. Filled arrowheads represent borders of active zones on the postsynaptic side. Open arrowheads represent interface areas on the postsynaptic side. **J**, The left border of the interface is unclear in the section shown but was verified in adjacent sections.

boutons (excluding mitochondrial volume) was not significantly different in epileptic rats (3830 ± 130 per μm^3) and controls (3380 ± 250 per μm^3 , $p = 0.15$, power = 0.30) (Fig. 8J). These findings suggested that epileptic rats were not deficient in synaptic vesicles, which were proportionally more numerous in their enlarged boutons.

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(Figure legend continued.) Electron micrographs correspond to the sections indicated by the horizontal arrow in **C** and **F**, respectively. **D, H**, Vertical arrows represent docked vesicles. Filled arrowheads represent borders of active zones on the postsynaptic side. Open arrowheads represent interface areas on the postsynaptic side. **I–M**, Measured parameters of boutons and mitochondria in control (cont) and epileptic rats (epi). Each marker represents the average of 10 boutons in one animal. Filled circles represent naïve controls. Horizontal lines indicate group averages. * $p < 0.05$. For details of statistical analysis, see text.

Proximity of synaptic vesicles to active zones can affect synaptic release probability (Gracheva et al., 2006; Marra et al., 2012; Park et al., 2012), so BC→GC boutons were hypothesized to contain fewer synaptic vesicles close to active zones in epileptic rats. Distances between all vesicles and active zones were measured and sorted into bins of 50 nm increments. Contrary to the hypothesis, results revealed more vesicles closer to active zones in epileptic rats compared with controls (Fig. 8K). Average values of epileptic rats were greater than those of controls at all increments from the active zone. Differences were significant at all distances ($p < 0.05$, repeated-measures ANOVA, power = 1.0), except the first bin 0–50 nm from the active zone.

Evidence from at least some excitatory hippocampal synapses indicates that docked vesicles are likely to be the morphological correlates of the readily releasable pool (Schikorski and Stevens,

Figure 7. Surface views of double axosomatic synapses made with two different granule cells in control (**A–D**) and epileptic pilocarpine-treated rats (**E–H**). Contours of synaptic boutons (orange), interface areas of presynaptic and postsynaptic membrane apposition (red and green), and active zones (black) are indicated. Yellow circles represent docked vesicles. **B, F**, Side views. Blue and green represent mitochondria (different colors represent different mitochondria). Black circles represent clear vesicles. **D, H**, Electron micrographs of corresponding sections in reconstructions indicated by horizontal arrows in **A, C, E**, and **G**. Filled arrowheads represent borders of active zones on the postsynaptic side. Open arrowheads represent interface areas on the postsynaptic side.

2001; Watanabe et al., 2013). Because results of previous paired recordings suggested that readily releasable pools are smaller at BC→GC synapses in epileptic rats, it was hypothesized that those synapses would contain fewer docked vesicles. Docked vesicles were identified by their contact with an active zone (Figs. 2*F*, 5*D, H*, 6*E, J*). The number of docked vesicles per bouton was 2.5× higher in epileptic rats (23.8 ± 2.9) compared with controls

(9.7 ± 1.2 , $p = 0.001$, t test, power = 0.98) (Fig. 9*A*). The number of docked vesicles per interface was 2.4× higher in epileptic rats (20.3 ± 2.4) compared with controls (8.4 ± 0.9 , $p < 0.001$, t test, power = 0.99) (Fig. 9*B*). Previous studies found an average of 7–10 docked vesicles per active zone at excitatory synapses in the hippocampus (Schikorski and Stevens, 1997; Siksou et al., 2007) and cerebellum (Xu-Friedman et al., 2001). Similar results were

found at BC→GC synapses in the present study. The number of docked vesicles per active zone was $1.9\times$ higher in epileptic rats (11.9 ± 1.6) compared with controls (6.4 ± 0.8 , $p = 0.013$, t test, power = 0.78) (Fig. 9C). However, there was no significant difference in the number of docked vesicles per active zone area (107 ± 9 per μm^2 and 88 ± 7 per μm^2 , $p = 0.14$, t test, power = 0.31) in epileptic and controls rats, respectively (Fig. 9D) or in the proportion of total vesicles in the bouton that were docked ($1.22 \pm 0.11\%$ and $1.50 \pm 0.26\%$, respectively, $p = 0.37$, t test, power = 0.14) (Fig. 9E). Previous studies also found that 1%–2% of vesicles are docked in a variety of different types of synapses (for review, see Rizzoli and Betz, 2005). These findings revealed that, contrary to the hypothesis, epileptic rats were not deficient in docked vesicles at BC→GC synapses. Instead, they had proportionally more docked vesicles for their larger active zone areas.

Discussion

The principal findings of the present study are that, in epileptic pilocarpine-treated rats compared with controls, BC→GC synaptic boutons are larger and the axosomatic synapses they make have more and larger active zones and contain more vesicles, including more docked vesicles. These findings suggest that previously reported impaired transmission at BC→GC synapses in animal models of temporal lobe epilepsy is not attributable to smaller boutons or fewer docked vesicles.

BC→GC boutons and synapses compared with others

Ultrastructural features of boutons and symmetric axosomatic synapses in the granule cell layer of the present study were similar to those reported previously in other regions of the mammalian CNS. Those features included pleomorphic vesicles (Uchi-zono, 1965), active zones consisting of small discrete patches within a larger interface area (Peters et al., 1990), similar bouton volumes and active zone areas (Ledoux et al., 2005), and similar numbers of active zones per bouton (Biró et al., 2006). Like most previous reports, the present study used aldehyde fixation to preserve tissue, which can affect bouton volume and packing density of synaptic vesicles (Rostaing et al., 2006). However, control and experimental groups were treated identically, so comparisons between groups are likely to be valid.

Boutons and synapses evaluated in the present study resembled those reported previously in the granule cell layer that express glutamic acid decarboxylase and therefore are likely to

synthesize GABA (Ribak et al., 1978; Kosaka et al., 1984). Dentate basket cells, which preferentially target granule cell somata (Struble et al., 1978; Ribak and Seress, 1983; Han et al., 1993; Lübke et al., 1998), were the most likely parent cell type of the

Figure 8. Interface (A, B), active zone (C–F), and vesicle parameters (G–I) at BC→GC synapses in control (cont) and epileptic pilocarpine-treated rats (epi). Each marker represents the average of 10 boutons in one animal. Filled circles represent naive controls. Horizontal lines indicate group averages. * $p < 0.05$. For details of statistical analysis, see text. **K.** Number of vesicles at 50 nm increments from an active zone. If a bouton contained multiple active zones, results were averaged. Values indicate mean $\pm$ SEM of control and epileptic pilocarpine-treated groups. Differences in every bin, except the first, were significant ($p < 0.05$, repeated measures two-way ANOVA).

Figure 9. Docked vesicle parameters at BC→GC synapses in control (cont) and epileptic pilocarpine-treated rats (epi). Each marker represents the average of 10 boutons in one animal. Filled circles represent naive controls. Horizontal lines indicate group averages. * $p < 0.05$. For details of statistical analysis, see text.

boutons evaluated in the present study, but the possibility that some boutons belonged to other types of interneurons cannot be excluded. Many, but not all, basket cells in the dentate gyrus express parvalbumin (Kosaka et al., 1987; Ribak et al., 1990), so many of the boutons evaluated were likely to be parvalbumin-positive. In the dentate gyrus, cholecystokinin-expressing interneurons target the inner molecular layer (Hefft and Jonas, 2005), not the principal cell layer as they do in CA1 (Nunzi et al., 1985), so boutons evaluated in the present study were unlikely to be cholecystokinin-positive.

The present study revealed ultrastructural changes at BC→GC synapses different from those reported for BC→CA1 pyramidal cell synapses in epileptic pilocarpine- and kainate-treated rats (Hirsch et al., 1999). In CA1, the frequency and amplitude of miniature IPSCs in pyramidal cells are reduced, but the number and size of GABAergic axosomatic boutons and number of docked vesicles are normal. However, the density of synaptic vesicles is reduced, especially 100–300 nm from active zones. In contrast, BC→GC boutons and synapses were larger and contained more vesicles, including more docked vesicles in epileptic pilocarpine-treated rats. These findings suggest that mechanisms underlying impaired GABAergic synaptic transmission can be regionally specific.

There was a positive correlation between the total number of vesicles and number of docked vesicles per bouton at BC→GC synapses, which is consistent with a mass action model of vesicle docking (Li and Schwarz, 1999; Gitler et al., 2004). In addition, previous studies found scaling relationships between the size of synaptic boutons and other ultrastructural features at excitatory (Harris and Stevens, 1989; Schikorski and Stevens, 1997) and inhibitory hippocampal synapses (Ledoux and Woolley, 2005; Eyre et al., 2007). Results of the present study were consistent and revealed that BC→GC boutons and synapses in epileptic rats were scaled up compared with controls. These findings suggest that there might be homeostatic mechanisms attempting to

increase BC-mediated inhibition in epileptic animals. However, despite hypertrophied boutons and synapses, release probability is reduced at BC→GC synapses (Zhang and Buckmaster, 2009).

Larger boutons and active zones are consistent with larger quantal amplitude

A major finding of the present study was larger BC→GC boutons and synapses in epileptic pilocarpine-treated rats. Periods of high activity cause GABAergic synapses to enlarge in cultured hippocampal neurons (Rannals and Kapur, 2011). Larger boutons and larger active zone areas in the present study are consistent with larger amplitude miniature IPSCs in granule cells (Kobayashi and Buckmaster, 2003; Sun et al., 2007) and larger quantal size at BC→GC synapses in epileptic rats compared with controls (Zhang and Buckmaster, 2009). Similarly, kindling increases miniature IPSC amplitude in granule cells (Otis et al., 1994), increases the size of GABAergic boutons and synapses with granule cells, and increases the number of GABA_A receptors per BC→GC synapse (Nusser et al., 1998). Thus, ultrastructural changes appear to underlie increased quantal amplitude at BC→GC synapses in temporal lobe epilepsy, but that is only part of synaptic efficacy. Release probability is another critical aspect.

Possible mechanisms of impaired release at BC→GC synapses

The motivation of the present study was to identify mechanisms of impaired GABA release at BC→GC synapses in temporal lobe epilepsy. The study tested and rejected the hypothesis that small, atrophic synaptic boutons with deficient docked vesicles were responsible. Consequently, it seems important to discuss alternative possible mechanisms. One of those is fewer synapses per BC→GC pair, which could reduce net release probability. In controls, basket cells make 3–7 release sites per granule cell (Kraushaar and Jonas, 2000). To our knowledge, that parameter has not been measured in temporal lobe epilepsy, but available evidence suggests that fewer release sites are unlikely. Patients with temporal lobe epilepsy (Wittner et al., 2001) and pilocarpine-treated rats (Thind et al., 2010) do not display significant loss of axosomatic inhibitory synapses in the granule cell layer. The probability of finding synaptically coupled BC→GC pairs is not significantly reduced in epileptic pilocarpine-treated rats (Zhang and Buckmaster, 2009). And the present study found identical average numbers of BC→GC synapses per bouton in control and epileptic rats. Together, these findings suggest that impaired transmission at BC→GC synapses is not attributable to fewer synapses, smaller boutons, or fewer docked vesicles.

Another possibility is impaired vesicle priming at BC→GC synapses in temporal lobe epilepsy. After docking, synaptic vesicles must undergo priming before they are ready for Ca^{2+} -triggered fusion (for review, see Südhof, 2013). At many glutamatergic synapses, Munc13 proteins are necessary for vesicle priming (Augustin et al., 1999). Munc13 protein function can be modulated by protein interactions (Betz et al., 2001) and second messengers (Betz et al., 1998), suggesting possibilities for regulating synaptic release probability independently of the number of morphologically docked vesicles. Presumably GABAergic synapses include molecules of comparable function to those of Munc13s, and at least one regulator of vesicle priming at GABAergic synapses has been identified (Kaesler et al., 2009). Therefore, downregulated vesicle priming, possibly via a Munc13-like protein, is another possible mechanism for failure of transmission at BC→GC synapses in temporal lobe epilepsy.

Finally, findings of the present study reveal hypertrophied synaptic boutons and more docked vesicles at synapses previously shown to have reduced release probability, which seems to contradict other published results that release probability is higher at synapses made by larger boutons containing more vesicles, especially more docked vesicles (Murthy et al., 1997, 2001; Mackenzie et al., 1999; Schikorski and Stevens, 2001; Branco et al., 2010; Matz et al., 2010; Holderith et al., 2012). Results from BC→GC synapses in epileptic pilocarpine-treated rats seem to contradict that hypothesis because release probability is reduced (Zhang and Buckmaster, 2009), but boutons and active zones were large and docked vesicles were abundant. There are several possible reasons for the apparent contradiction. First, most of the evidence for correlations between ultrastructural characteristics and release probability were derived from glutamatergic synapses. However, glutamatergic and GABAergic synapses use different vesicle exocytosis mechanisms (Augustin et al., 1999; Schoch et al., 2002; Gitler et al., 2004; Verderio et al., 2004; Bragina et al., 2007; Kerr et al., 2008; Gibson et al., 2009; Kaeser et al., 2009; Benagiano et al., 2011). Therefore, the relationship between synaptic efficacy and bouton ultrastructure, active zone size, and number of docked vesicles might be different in GABAergic synapses. Second, synaptic release probability can be dissociated from ultrastructural features. This occurs naturally; for example, cerebellar climbing fibers and parallel fibers share similar ultrastructural features but display different release probabilities (Xu-Friedman et al., 2001). Release probability can also be experimentally manipulated without affecting docked vesicle numbers (Hess et al., 1993; Augustin et al., 1999; Schoch et al., 2002; Sørensen et al., 2003; Kaeser et al., 2009). Therefore, docked vesicles might not always represent the readily releasable pool.

Results of the present study suggest that previously reported impaired transmission at BC→GC synapses is not attributable to smaller boutons or fewer docked vesicles. Instead, later steps in exocytosis might be deficient in epileptic animals. Tight spatial coupling between a small number of Ca^{2+} channels (Bucurenciu et al., 2010) and Ca^{2+} sensors in the active zone facilitates normally reliable synaptic transmission at BC→GC synapses (Bucurenciu et al., 2008). Therefore, one possibility is that in temporal lobe epilepsy Ca^{2+} entry or Ca^{2+} coupling with exocytosis is impaired at BC→GC synapses. In a neocortical model of post-traumatic epilepsy, inhibitory synaptic transmission is more likely to fail (Faria and Prince, 2010; Ma and Prince, 2012), which correlates with changes in calcium channels (Faria et al., 2012).

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