Temporal profile of hilar basal dendrite formation on dentate granule cells after status epilepticus

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Abstract
Granule cells with hilar basal dendrites (HBDs) are found after status epilepticus (SE) in three rat models of temporal lobe epilepsy. These granule cells are commonly located at the hilar border and could be newly born granule cells based on their location. The aim of this study was to determine how long it takes for HBDs to form on granule cells after SE. Pilocarpine was injected to induce SE and rats were killed at different times: 3 days, 1, 2, and 3 weeks after SE. Biocytin was injected into CA3 stratum lucidum of hippocampal slices to label granule cells with HBDs. The number, morphology, and length of HBDs were analyzed at the different time points. Basal processes of granule cells from rats killed 3 days after pilocarpine injection were judged not to be HBDs because they were short in length and did not ramify in the hilus. “True” HBDs were detected as early as 7 and 8 days after pilocarpine-induced SE. Similar frequencies of granule cells with HBDs were observed at the later time points. This study shows that HBDs can form on granule cells as early as 1 week following SE. These results are consistent with the hypothesis that HBDs on granule cells may be generated from seizure-induced, de novo granule cells, however, alternative explanations that some or all HBDs arise from pre-SE generated granule cells cannot be ruled out at this time and will require further examination.

1. Introduction
Granule cells in the rat hippocampal dentate gyrus display an important morphological change following status epilepticus (SE), the formation of hilar basal dendrites (HBDs). These HBDs were shown to extend from the base of granule cell bodies into the hilus for variable distances in three different models of epilepsy in rats (Buckmaster and Dudek, 1999; Ribak et al., 2000; Spigelman et al., 1998) and an epileptic mouse mutant (Wenzel et al., 2001). It is important to note that HBDs were not found on granule cells in control adult rats (Buckmaster and Dudek, 1999; Ribak et al., 2000; Spigelman et al., 1998). However, HBDs on...
Recent studies have addressed the function of HBDs by analyzing these dendrites for synapses. The presence of spines on HBDs suggested that they were postsynaptic to axon terminals (Buckmaster and Dudek, 1999; Spigelman et al., 1998). More recently, using electron microscopy, Ribak et al. (2000) observed HBDs that were postsynaptic to mossy fibers making asymmetric synapses. These findings showed that HBDs provide another neuroplastic change in addition to that of sprouted mossy fibers that synapse with dendrites in the inner molecular layer of the dentate gyrus (Okazaki et al., 1995; Sutula et al., 1998; Zhang and Houser, 1999). Therefore, HBDs are similar to apical dendrites of granule cells in that they contribute to additional recurrent excitatory circuitry via sprouted mossy fibers in the epileptic brain.

Granule cells with HBDs after SE are commonly located at the hilar border (Buckmaster and Dudek, 1999; Ribak et al., 2000; Spigelman et al., 1998). It should be noted that granule cell neurogenesis occurs in adults and the newly generated granule cells are located at the hilar border (Cameron and McKay, 2001; Kempermann and Gage, 1999; Parent et al., 1997, 1998). These neurons were labeled using markers for newly generated granule cells or their incorporation of newly synthesized deoxynucleotides. Following SE, the rate of dentate granule cell neurogenesis is increased (Parent et al., 1997, 1998; Scott et al., 1998). It needs to be pointed out that most of the labeled newly generated granule cells were found at the same location as normally found, at the hilar border. However, it was reported that some newly generated granule cells following SE also migrate into the deep hilus and are referred to as hilar ectopic granule cells (Dashtipour et al., 2001; Parent et al., 1997, 1998).

A previous study showed that HBDs formed as early as 1 month after SE (Spigelman et al., 1998). The goal of the present study was to determine how long it takes for HBDs to form after SE. Hastings and Gould (1999) showed that newly generated granule cells need about 10 days to grow their axons into distal CA3. Thus, we hypothesize that granule cells with HBDs are newly generated granule cells and may be present within 10 days after SE. We used biocytin-loaded granule cells in hippocampal slices from adult rats after pilocarpine-induced SE to analyze when HBDs form after seizures. This method was previously shown to label HBDs of granule cells (Ribak et al., 2000).

2. Materials and methods

2.1. Induction of status epilepticus

Sixteen adult male Sprague–Dawley rats (200–250 g; Simonsen, Gilroy, CA) were used in these studies. The Institutional Animal Care and Use Committees at the University of California at Irvine and Los Angeles approved animal protocols in advance of the experiments. Rats were injected with pilocarpine hydrochloride (320–340 mg/kg, i.p.) preceded 30 min earlier by methylscopolamine (1 mg/kg, i.p.) to develop SE, a continuous limbic motor seizure of stage 2 or higher (Turski et al., 1987). SE was terminated after 3.5–4.5 h with a single injection of diazepam (10 mg/kg, i.p.). Animals were anesthetized and euthanized at different times: 3, 7, 8, 15, 19, and 20 days after SE (two rats at each time point). Pilocarpine-treated rats that did not develop continuous SE ($n = 4$) were not used for this study.

2.2. Granule cell labeling with biocytin

At various times after SE, rats were anesthetized with 4% halothane and decapitated. Brain slices (400 μm thick) obtained from the caudal one-third of the hippocampal formation (mid-ventral hippocampus), transverse to the long axis, were cut with a vibrating blade slicer (Campden Instruments). Six to seven hippocampal slices were obtained from each rat. They were then transferred to a custom-made recording chamber during perfusion with artificial cerebrospinal fluid (ACSF) composed of (in mM): 124 NaCl, 3.5 KCl, 1.25 NaH2PO4, 2.0 CaCl2, 2.0 MgCl2, 26 NaHCO3, and 10 dextrose. The ACSF was continuously bubbled with a 95/5% mixture of O2/CO2 to ensure adequate oxygenation of slices and a pH of 7.4. A 10–15% solution of biocytin was iontophoretically ejected into stratum lucidum of CA3b of the hippocampus to label the mossy fibers of dentate granule cells (Okazaki et al., 1995). A glass micropipette with a filament (WPI, 1.5 mm external diameter, 10–50 μm tip diameter) was filled...
with a freshly prepared solution of 10–15% biocytin in 0.9% NaCl. The tip of the glass micropipette was about 200 μm deep to the surface. Biocytin was ejected into the extracellular space with intermittent positive current pulses (15 μA, 3 s on, 3 s off) for 15–25 min. Three hours following biocytin injection, slices were immersed in a fixative solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4 and stored overnight at 4°C.

To visualize the biocytin-labeled granule cells, the fixed slices were re-sectioned at a thickness of 50 μm. The sections were collected in 0.1 M PB and treated with 1% H2O2 to suppress endogenous peroxidase activity. Then, the sections were washed in 0.1 M PB for 30 min and incubated in avidin-biotin horseradish peroxidase solution (Vectastain Elite ABC Kit, Vector Labs) for 3–5 h at room temperature and overnight at 4°C. Finally, they were washed several times in 0.1 M PB and incubated in 0.025% diaminobenzidine (DAB) and 0.01% nickel ammonium sulfate for 15–20 min.

2.3. Data analysis

The granule cell layer was analyzed using light microscopic observation. The frequency of labeled granule cells with HBDs among total labeled granule cells was compared between rats killed 3 days, 1, 2, and 3 weeks after pilocarpine injection. Hippocampal slices, where the injection site was outside the stratum lucidum of CA3 were not used because granule cells were not labeled. Also, slices where labeling was not found in apical dendrites of granule cells were excluded from this analysis.

3. Results

The appearance of biocytin-labeled granule cells with HBDs in sections of the dentate gyrus has been described previously (Ribak et al., 2000). A brief description of these granule cells is provided here as a basis for comparison with the data obtained after different time points after SE. Granule cells with HBDs were mainly located in the granule cell layer at the border with the hilus or one cell away from the border (Ribak et al., 2000). Labeled processes arising from the hilar pole of granule cells were considered HBDs based on the anatomical features described by Spigelman et al. (1998). HBDs were distinctly separate from the axons, and extended into the subgranular region of the hilus (first 50 μm subjacent to the granule cell layer). The diameter of HBDs was as thick as that of the apical dendrites that arose from the opposite pole of the granule cell body. HBDs varied between 20–200 μm in length, and densely packed spines were present on the surface of the HBD throughout its length.

3.1. Three days after pilocarpine-induced SE

No HBDs were found in biocytin preparations obtained 3 days after pilocarpine-induced SE. Processes that extended from the hilar surface of granule cells were found, but they did not satisfy the HBD criteria of extending into the hilus, being longer than the length of the cell body, and possessing many spines. Instead, these processes were considered growth cones, which are rudimentary projections from the granule cell body that could eventually form a basal dendrite. Fig. 1A depicts a growth cone sprouting at the granule cell layer border with the hilus. Because of its thickness and its location, it shows the potential to become an HBD. The growth cone in Fig. 1D shows similar characteristics and is located one granule cell above the hilus. The process of the granule cell in Fig. 1B has less potential to become an HBD because it is less thick, and would more likely develop into an axon. The process in Fig. 1C is too short to identify as a dendrite or to make a prediction on its later fate. Overall, the processes emanating from the basal surface of granule cells 3 days after pilocarpine-induced SE were judged not to be HBDs.

3.2. One week after pilocarpine-induced SE

Granule cell processes that fit the HBD criteria were found in sections that were obtained from rats 1 week after pilocarpine-induced SE. This is the earliest time point in which developed HBDs were found in this study. Fig. 2B and C show HBDs that are thick, extend into the hilus, and are longer than the granule cell body length. Note that these two granule cells with HBDs in Fig. 2B and C are also located in the granule cell layer at the border with the hilus. In addition, the HBDs shown in Fig 2B and C were measured and
found to be 32 and 36 μm in length, respectively. The basal process in Fig. 2A has the morphological characteristics of a HBD, but curves back into the granule cell layer, thereby falling into a different category of basal dendrite, a recurrent basal dendrite (RBD). This RBD extends for a length of 51 μm. Fig. 2D (bottom) shows a labeled granule cell body with many short thin processes that are identified as growth cones in their later stage of development because they are longer and more developed than the growth cones found in the sections from rats 3 days after pilocarpine-induced SE. It should also be noted that perhaps one of its growth cones could develop into a HBD based on their relative length and thickness. This latter granule cell, in Fig. 2D, is located slightly beneath the granule cell layer.

Several examples of granule cells with HBDs were observed in preparations obtained 8 days following pilocarpine-induced SE. Fig. 3A is an example of a HBD ramifying in the hilus, extending longer than the cell body length (29 μm), and being thick. Fig. 3C and D show three granule cells, with overlapping HBDs at different planes of focus. The granule cell with a HBD on the right in Fig 3C extends for 47 μm into the hilus (out of the picture), while the one on the left in Fig. 3D extends for 32 μm (perpendicular to the other two HBDs). All three granule cells were found in the granule cell layer at the border with the hilus.
Fig. 2. High magnification photomicrographs of biocytin-labeled granule cells from rats at 7 days after pilocarpine injection. (A) Granule cell at the hilar border with the granule cell layer (GL) shows a thick dendrite (arrow) running parallel to the border, then curving away from the hilus (H). Notice how this dendritic thickens at a distal part (arrowhead). (B) & (C) Hilar basal dendrites (arrows) extending from granule cells into the hilus (H). (D) Granule cell with many dendritic growth cones (arrows) sprouting at different angles into the hilus. More superficial in the GL is a thick basal dendrite extending towards the hilus (arrowheads). Scale bars: 10 μm.

The granule cell in Fig 3B (left) was found at the hippocampus end blade. It shows a branching RBD that curves back to the granule cell layer and an additional thin process directed towards the hilus. The RBD runs in the hilus for 20 μm before branching.

Table 1

| Time (after SE) | HBDs | Length of HBDs (μm) | RBDs | Total granule cells |
|-----------------|------|---------------------|------|---------------------|
| 3 days          | 0    | –                   | 2 (0.77%) | 281                |
| 1 week          | 26 (0.27%) | 31.0               | 19 (0.20%) | 9483               |
| 2 weeks         | 1 (0.20%) | 29.0               | 3 (0.03%) | 361                |
| 3 weeks         | 6 (0.30%) | 39.2               | 9 (0.45%) | 1979               |

Twenty-six HBDs were found out of 9483 granule cells analyzed in the sections obtained 7 and 8 days after pilocarpine-induced SE. This gives a HBD frequency of 0.27% of stained granule cells (Table 1).
3.3. Two and three weeks after pilocarpine-induced SE

Granule cells found in sections obtained from rats 2 weeks (15 days) and 3 weeks (19 and 20 days) after pilocarpine-induced SE showed well-developed HBDs. These HBDs were thicker and extended deeper into the hilus than the HBDs from rats with shorter intervals after pilocarpine-induced SE. For example, Fig. 4B and C show thick and long HBDs (55 and 43 μm, respectively) that appear to be at more advanced stages of development than those in Figs. 2 and 3. Fig. 4D shows a granule cell at the hilar border with a RBD that runs into the hilus for 19 μm, and then makes a U-turn back towards the granule cell layer for a total RBD length of 30 μm. This RBD was similar to some of those described by Dashtipour et al. (2002). In contrast to these examples, the HBD in Fig. 4A appears to be a less developed HBD, extending only 13 μm into the hilus.

Fewer granule cells were stained in the preparations obtained 2 and 3 weeks after SE. As a result, fewer HBDs were counted. Nevertheless, the 2-week sections had a comparable HBD frequency to the 1-week sections. One HBD was stained from 361 granule cells for an HBD frequency of 0.28% (Table 1). The 3-week sections showed a HBD frequency of 0.30%.
4. Discussion

The aim of the present study was to determine how long it takes for HBDs to form on granule cells after SE. The data from the time series experiment showed that rats at 3 days after SE induced by pilocarpine had no HBDs on their granule cells. In contrast, rats at least a week after SE displayed many granule cells with HBDs. These data indicate that it takes about a week for granule cells to form HBDs following SE. Together with previous data on the location of granule cells with HBDs, the present results are consistent with the hypothesis that HBDs on granule cells arise from seizure-induced, de novo generated granule cells and not from mature granule cells generated prior to SE.
4.1. Technical considerations

The use of biocytin retrograde labeling in hippocampal slices provides an effective method of labeling the cell body and dendrites of dentate granule cells following injections into stratum lucidum of CA3 (Okazaki et al., 1995). In the preparations used in the present study, granule cells were labeled with different intensities. Thus, some granule cells were very darkly labeled with excellent labeling of their dendrites while others were moderately or lightly labeled and their dendrites were not as prominent. This difference in the extent of labeling of granule cells may explain why the percentage of granule cells with HBDs in the present study (about 0.3%) is less than that found in our previous study for rats surviving 6–14 months after SE (5.4% in Ribak et al., 2000). In the present study, HBDs were not usually observed because many lightly and moderately labeled granule cells were not seen in their entirety. However, these latter granule cell bodies were included in the total number of labeled cells for calculating the percentages of granule cells with HBDs. Nevertheless, the dark labeling of a smaller percentage of granule cells in the present study still allowed for the identification of timepoints when HBDs were formed on granule cells.

It needs to be noted that Hastings and Gould (1999) suggested that newly generated granule cells take about 10 days to grow their axons into CA3. Their study used injections into the most distal part of CA3, while the present study injected biocytin into more proximal parts of CA3. Therefore, it is likely that the labeled granule cells with HBDs at the second timepoint analyzed (1 week after SE was induced) are newly generated granule cells because these cells could have grown their axons into the injection site used in the present study by this time.

4.2. Distinguishing growth cones from dendrites

The present study focused attention on the biocytin-labeled granule cells located in the granule cell layer at the hilar border, the site where newly generated granule cells are found (Kempermann and Gage, 1999). Many granule cells at this location in preparations from rats 3 days after pilocarpine-induced SE had small processes arising from their cell body. Some of these processes arising from the base of granule cells were most likely growth cones that either would regress or develop into HBDs or RBDs. In contrast to these granule cells, the granule cells from rats 7 days following SE often showed thick, basal processes that entered the hilus and were identified as HBDs (Fig. 2B and C). Also, granule cells with several short processes were found in these preparations (see Fig. 2D). Such granule cells lacked a large thick process and therefore had not yet grown a dendrite, either from their apical or basal poles. The fact that the cell was labeled due to its axon projecting into stratum lucidum where biocytin was injected indicated that this cell was a granule cell based on its axonal projection. This finding is consistent with developmental data for neurons showing that axons develop before dendrites (see spinal cord development in Cajal, 1911).

More recently, Dotti et al. (1988) showed that fetal hippocampal cells grown in culture first establish several short processes and one of these begins to grow very rapidly to become the axon. The remaining processes elongate a few days later at a slower rate, and they become dendrites. Taken together, these observations are consistent with the hypothesis that newly generated granule cells following SE are involved in forming HBDs.

It needs to be emphasized that not all dendritic processes arising from the base of granule cells are HBDs. As shown in several figures (Figs. 2A, 3B and 4D), some of these processes enter the hilus, curve back into the granule cell layer and often enter the molecular layer. Because they joined the region of the apical dendrite, this type of basal dendrite was called a RBD (Dashtipour et al., 2002; Ribak et al., 2000; Yan et al., 2001). These latter dendrites are found in normal rats as well as epileptic ones but are more common in epileptic rats (Ribak et al., 2000). The factors involved in controlling the growth orientation of the basal process of granule cells to form either a RBD or HBD are still unknown.

4.3. HBDs and epilepsy

As mentioned in Section 1, HBDs are spiny dendrites that enter the hilus where they are in a position to be postsynaptic to many types of axon terminals, including mossy fibers arising from granule cells (Buckmaster and Dudek, 1999, Spigelman et al., 1998). Indeed, we previously showed using electron
microscopy that HBDs were postsynaptic to identified mossy fibers (Ribak et al., 2000). This point is important because granule cells normally do not innervate each other. The data from the present study show that HBDs need at least 7 days to arise from granule cells following pilocarpine-induced SE in rats. Taken together, this raises an important question as to whether the formation of HBDs correlates with the development of spontaneous seizures. The development of seizures in the pilocarpine model has a mean latency of 14–15 days according to Turski et al. (1987) and Cavalheiro et al. (1991). Thus, the presence of HBDs before this timepoint and the fact that HBDs contribute to additional recurrent excitatory circuitry (see below) may provide a basis for these spontaneous seizures. The formation of HBDs appears to occur faster than another neuroplastic change that provides additional recurrent excitatory circuitry in epileptic brains, sprouted mossy fibers in the inner molecular layer. It should be noted that the frequency of HBDs 1 week after SE was similar to the HBD frequency in rats 2 and 3 weeks after pilocarpine-induced SE. In contrast, mossy fiber sprouting in the inner molecular layer is first observed about 3 weeks after pilocarpine-induced SE and continues to increase in frequency for 6–10 months (Okazaki et al., 1995).

The relationship of HBDs and epilepsy in humans must also be discussed because previous studies showed that HBDs appear on some granule cells in normal primates and humans (Seress, 1992; Seress and Mrzljak, 1987). Approximately 20–25% of granule cells from normal humans have HBDs while two reports showed an increase in their frequency to 43% in epileptic human brains (Franck et al., 1995; von Campe et al., 1997). Therefore, the epileptic human brain shows the potential for more granule cell to granule cell synaptic circuitry than the normal human brain.

The functional significance of this circuitry is only recently being defined. In recordings from granule cells with HBDs in monkey brain slices, Austin and Buckmaster (2002) reported that antidromic stimulation resulted in excitatory synaptic potentials in all granule cells with HBDs compared to only 50% of cells without HBDs, indicating the presence of a robust recurrent excitatory pathway for cells with HBDs. Furthermore, they showed that the amplitude of excitatory currents in granule cells with HBDs is much larger than in granule cells without HBDs. Together, these studies provide strong evidence for the functional incorporation of granule cells with HBDs into hippocampal circuitry and add further support for their involvement in recurrent excitation.

It should be noted that this circuitry may not be exclusively recurrent excitation because Sloviter et al. (1996) showed immunolabeling for GAD and GABA in small amounts in the mossy fibers of normal animals, including primates. This result suggested that the new granule cell to granule cell connections in the epileptic brain might provide a substrate for recurrent inhibition. In addition, mossy fibers from epileptic brains show increased expression of GAD and GABA (Schwarzer and Sperk, 1995; Sloviter et al., 1996). Although this phenomenon may represent a compensatory mechanism to calm the epileptic brain (Sloviter, 2003), no functional data have been reported to support this view.

4.4. HBDs and adult neurogenesis of granule cells

Previous studies on HBDs indicated that they arose from granule cells that were mainly located in the granule cell layer at the border with the hilus or one cell away from the hilus (Buckmaster and Dudek, 1999; Ribak et al., 2000; Spiegelman et al., 1998). Because granule cell neurogenesis occurs in adults and the newly generated granule cells are added to the granule cell layer at the hilar border, we hypothesized that granule cells with HBDs were newly generated granule cells. One could argue that pre-existing granule cells could grow a dendrite into the hilus in at least 3 days after SE. The lack of HBDs at this timepoint in the present study is consistent with the hypothesis that HBDs form on newly generated granule cells because it might take longer than 3 days for the axons of such granule cells to reach the biocytin injection site in CA3. The presence of HBDs on granule cells from rats 1 week after SE is also consistent with this hypothesis because such granule cells would have had the time to be born and send their axons to CA3 for retrograde labeling with biocytin by this time. However, the finding by Parent et al. (1999) that destruction of nearly all newly born cells by x-irradiation does not prevent either mossy fiber sprouting or spontaneous seizure development, raises the possibility that HBDs...
do not necessarily arise from newly generated granule cells. Thus, alternative explanations that some or all HBDs arise from pre-SE generated granule cells cannot be ruled out at this time and will require further examination.

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