

Stereological methods reveal the robust size and stability of ectopic hilar granule cells after pilocarpine-induced status epilepticus in the adult rat

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Keywords: dentate gyrus, epilepsy, hippocampus, neurogenesis, seizures

Abstract

Following status epilepticus in the rat, dentate granule cell neurogenesis increases greatly, and many of the new neurons appear to develop ectopically, in the hilar region of the hippocampal formation. It has been suggested that the ectopic hilar granule cells could contribute to the spontaneous seizures that ultimately develop after status epilepticus. However, the population has never been quantified, so it is unclear whether it is substantial enough to have a strong influence on epileptogenesis. To quantify this population, the total number of ectopic hilar granule cells was estimated using unbiased stereology at different times after pilocarpine-induced status epilepticus. The number of hilar neurons immunoreactive for Prox-1, a granule-cell-specific marker, was estimated using the optical fractionator method. The results indicate that the size of the hilar ectopic granule cell population after status epilepticus is substantial, and stable over time. Interestingly, the size of the population appears to be correlated with the frequency of behavioral seizures, because animals with more ectopic granule cells in the hilus have more frequent behavioral seizures. The hilar ectopic granule cell population does not appear to vary systematically across the septotemporal axis, although it is associated with an increase in volume of the hilus. The results provide new insight into the potential role of ectopic hilar granule cells in the pilocarpine model of temporal lobe epilepsy.

Introduction

The subgranular zone of the adult rat dentate gyrus contains progenitor cells that differentiate into granule cells, migrate into the granule cell layer and incorporate into the existing circuitry (Hastings & Gould, 1999; Markakis & Gage, 1999). Both the number of existing progenitors and the likelihood that they differentiate into neurons are influenced by various conditions, including stress, aging, stroke and seizures (Kuhn *et al.*, 1996; Parent *et al.*, 1997; Gray & Sundstrom, 1998; Covolan *et al.*, 2000; Nakagawa *et al.*, 2000; Scharfman *et al.*, 2000; Czeh *et al.*, 2001; Jin *et al.*, 2001; Kee *et al.*, 2001; Tanapat *et al.*, 2001; Malberg & Duman, 2003). In particular, status epilepticus – a state of severe, continuous seizures – is a robust neurogenic stimulus (Parent *et al.*, 1997; Gray & Sundstrom, 1998; Covolan *et al.*, 2000; Nakagawa *et al.*, 2000). However, following status epilepticus, many new granule cells of subgranular zone origin (Parent *et al.*, 2006) migrate away from the granule cell layer into the hilus (Parent *et al.*, 1997, 2006; Scharfman *et al.*, 2000; Dashtipour *et al.*, 2001; Bonde *et al.*, 2006). Although rare, these ‘ectopic’ hilar granule cells have also been identified in the rat under normal conditions (Amaral, 1978; Seress & Pokorny, 1981; Gaarskjaer & Laurberg, 1983; Marti-Subirana *et al.*, 1986; Scharfman *et al.*, 2003).

Similar to conventional granule cells, located within the granule cell layer, ectopic granule cells in the hilus develop mossy fiber axons (Dashtipour *et al.*, 2001), become incorporated into pre-existing circuitry (Scharfman *et al.*, 2000; Dashtipour *et al.*, 2001; Pierce *et al.*, 2005), and demonstrate classic granule cell membrane properties and firing behavior (Scharfman *et al.*, 2000). However, granule cells in the hilus differ from conventional granule cells in ways that may predispose them to increased excitability. For example, ectopic hilar granule cells have an increased proportion of somatic and dendritic asymmetric (presumably excitatory) synapses (Dashtipour *et al.*, 2001; Pierce *et al.*, 2005), increased mossy fiber innervation (Pierce *et al.*, 2005) and a distinct pattern of activation during spontaneous seizures (Scharfman *et al.*, 2002a). Furthermore, spontaneous epileptiform bursts have been recorded from ectopic hilar granule cells, but not conventional granule cells, *in vitro* (Scharfman *et al.*, 2000).

It has been suggested that ectopic hilar granule cells could contribute to the development of spontaneous seizures following status epilepticus (Parent & Lowenstein, 2002; Scharfman, 2002, 2004; Jung *et al.*, 2004; Shapiro & Ribak, 2005). However, until the size of the ectopic hilar granule cell population has been determined quantitatively, it is difficult to argue that these cells can exert a functional effect. Therefore, the present study used unbiased stereology to estimate the size of the hilar ectopic granule cell population following pilocarpine-induced status epilepticus. Hilar ectopic granule cells were distinguished by their immunoreactivity for

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Received 24 May 2006, revised 27 July 2006, accepted 8 August 2006

Prox-1, a marker of dentate gyrus granule cells (Pleasure *et al.*, 2000; Brandt *et al.*, 2003; Parent *et al.*, 2006).

Materials and methods

Experimental subjects

Animal care and use was approved by the Helen Hayes Hospital IACUC in accordance with the guidelines set by the National Institutes of Health and the New York State Department of Health. Adult, male Sprague–Dawley rats (Charles River; Wilmington, MA, USA) were group-housed using a 12-h light/dark cycle and provided food and water *ad libitum*. At 48 ± 2 days of age (mean \pm SEM, $n = 19$; 180–230 g), animals were treated with either pilocarpine or saline, as described below.

Seizure induction

Animals treated with pilocarpine received a single injection of atropine methylbromide (1 mg/kg s.c.), followed after 30 min by an s.c. injection of 380 mg/kg pilocarpine hydrochloride. Status epilepticus was defined as stage 5 seizures (Racine, 1972) that did not cease for at least 5 min, and usually developed in the first hour after pilocarpine administration. One hour after the onset of status epilepticus, animals received an injection of diazepam (5 mg/kg i.p.; Henry Schein, Melville, NY, USA), to suppress the seizures. During the hour before diazepam injection, animals were similar in behavior, typically prone and exhibiting head bobbing and limb twitching. After diazepam injection, these movements were attenuated, but did not completely stop until 5–6 h later. In previous reports (Scharfman *et al.*, 2000, 2002b), we have found that this procedure leads to a consistent lesion of layer III of the medial entorhinal cortex, loss of hilar neurons, robust mossy fiber sprouting in ventral hippocampus, and a population of ectopic granule cells in the hilus. Approximately 6 h after the start of status epilepticus, animals received an injection of 5% dextrose in lactate Ringer's solution (2.5 mL s.c.) to ensure adequate hydration during the recovery from status epilepticus. In addition, an apple that was cut open was placed in each cage every day, for 1 week after injection. Control rats were treated identically, i.e. they received atropine methylbromide, diazepam, lactate Ringer's solution and apple, but they were administered a 3-mL/kg injection of phosphate-buffered saline (pH 7.4) instead of pilocarpine.

Observation of spontaneous behavioral seizures

After pilocarpine-induced status epilepticus, animals were housed in clear cages and monitored for stage 4 or 5 behavioral seizures between 09:00 and 17:00 h (Monday–Friday), by blinded investigators, until the animals were killed. Observations were made for periods of 30 min or less, 1–3 times per day, at random times throughout the 8-h day. The date of any stage 4 or 5 behavioral seizure was noted for each animal. All animals were housed in the same room and monitored under identical conditions, and therefore had the same likelihood of being observed during a seizure. Therefore, the daily seizure observation methods used here are likely to provide an unbiased estimate of the frequency of stage 4–5 behavioral seizures. However, because animals were not monitored 24 h per day, and EEG recording was not conducted, the number of observed behavioral seizures is likely to be an underestimate of the number of actual behavioral and electrographic seizures.

Tissue processing

Animals were anesthetized with urethane (1.25 g/kg i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (pH 7.4; Invitrogen, Carlsbad, CA, USA). Both hippocampi were carefully removed, extended and embedded flat in a type of agar that has a low melting point (Fisher, BP165-25). Agar blocks were post-fixed overnight in a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C.

Embedded hippocampi were measured along the longitudinal axis, and cut into six equal length blocks using a McIlwain tissue chopper (Brinkman Instruments, Westbury, NY, USA). One block was randomly selected from each hippocampus, mounted onto an agar cube and serially sectioned, transverse to the longitudinal axis, at a thickness of 75 μ m using a vibratome (Ted Pella, Redding, CA, USA).

Immunohistochemistry

To identify granule cells located in the hilus, nickel-enhanced diaminobenzidine peroxidase immunohistochemistry was conducted using an antibody to prospero-like homeobox protein 1 (Prox-1). Prox-1 antibody has been shown to have specificity for granule cells in the adult mouse and rat hippocampus (Pleasure *et al.*, 2000; Brandt *et al.*, 2003; Parent *et al.*, 2006), and has been used previously to identify ectopic granule cells following pilocarpine-induced status epilepticus (Parent *et al.*, 2006).

Each section from a selected block was collected into a single culture dish well. For immunohistochemistry to detect Prox-1 expression, sections were first washed four times each for 5 min in 0.1 M Tris buffer, and then treated with 1% hydrogen peroxide made in 0.1 M Tris buffer for 30 min, followed by washing in 0.1 M Tris buffer for 5 min and then in 0.1% Triton X-100 in 0.1 M Tris buffer (Tris A) for 10 min followed by a 10-min wash in Triton X-100 and 0.005% bovine serum albumin in 0.1 M Tris buffer (Tris B). Sections were then incubated in 10% normal goat serum (Vector, Burlingame, CA, USA) in Tris B for 1 h, followed by incubation in anti-rabbit Prox-1 antibody, made in goat (1 : 60 000; Covance, Berkeley, CA, USA), for 72 h. Sections were then washed in Tris A for 10 min, then Tris B for 10 min, followed by incubation in biotinylated goat anti-rabbit IgG diluted in Tris B (1 : 400; Vector) for 45 min. Sections were then washed in Tris A for 10 min followed by 10 min in 0.1% Triton X-100 and 0.005% bovine serum albumin in 0.5 M Tris buffer (Tris D). This was followed by incubation in avidin–biotin–horseradish peroxidase complex made in Tris D for 2 h (ABC Elite kit; 1 : 1000; Vector). Sections were then washed three times each for 5 min in 0.1 M Tris buffer, and developed in a Tris buffer solution made of 0.022% 3,3'-diaminobenzidine (DAB), containing 5 mM NiCl₂. This was followed by three washes each for 5 min in Tris buffer. Sections from pilocarpine-treated animals were always developed concurrently with sections from saline-treated animals.

Stereological procedures

Slide-mounted serial sections from each randomly selected block were viewed with an Olympus BX-51 microscope interfaced with a computer running StereoInvestigator 6.0 software (MicroBrightfield, Williston, VT, USA). An experimenter blinded to the treatment conditions traced the extent of the hilus with a 100- μ m-diameter circle placed on the interior of the granule cell layer to avoid the subgranular zone (Fig. 1). For the purposes of counting ectopic hilar granule cells, the hilus was defined as the region between the upper and lower blades of the granule cell layer, extending from the vertex of the upper and

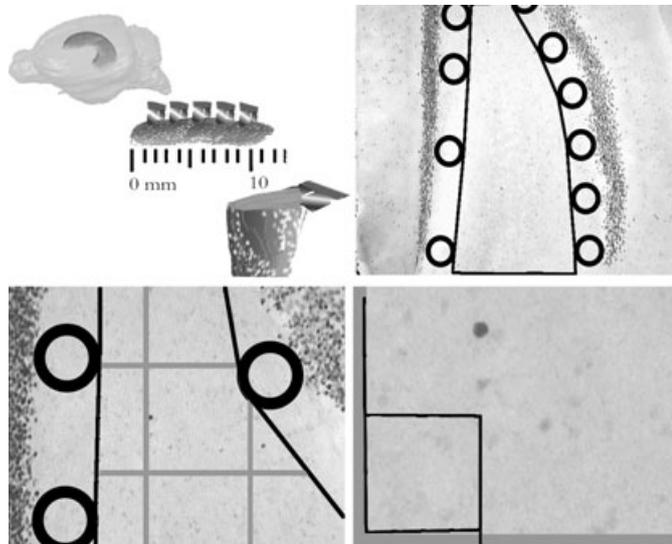


FIG. 1. Schematic illustration of stereological methods used for ectopic hilar granule cell quantification. Upper left: hippocampi were extracted, extended longitudinally, measured and cut into six equal blocks. One randomly selected block was sectioned exhaustively, transverse to the longitudinal axis, into 75 μm sections. Sections were labeled with Prox-1 antibody and viewed under a microscope interfaced with StereoInvestigator software. Upper right: several 100- μm -diameter circles were placed on the hilar border of the granule cell layer to avoid counting the subgranular zone. The contour of the hilus was traced along the hilar border of the circles from the vertex of the granule cell layer to a straight line drawn between the lateral tips of the upper and lower granule cell blades. Bottom left: a 150- μm^2 xy step grid was randomly placed within the traced hilar contour. Bottom right: a 50- μm^2 counting frame was placed at systematic points within the grid.

lower blades, to a straight line drawn between the lateral tips of the upper and lower blades. This operational definition of the hilus was used to avoid the difficulty of distinguishing the border of area CA3c and the hilus, which is particularly problematic when non-granule cells are unstained. It is, admittedly, an overestimation of the hilar area (for the classic definition of the hilus, see Amaral, 1978), but would ensure the inclusion of ectopic granule cells that cluster at the hilar/CA3c border (Scharfman *et al.*, 2000). For each section, the area of the traced hilar region was used to estimate total hilar volume (see below).

Within each traced contour of the hilus, a 150- μm^2 xy step grid was used, with a 50- μm^2 counting frame (Fig. 1). The height of the counting frame was equal to the thickness of the section, minus a total guard zone of 10 μm (5 μm from the top and bottom of the section). At each randomly placed counting frame site, granule cells were counted only when they came into focus. Granule cells were defined as darkly labeled Prox-1-immunopositive spheres (Fig. 2). Cells touching two predetermined sides of the counting frame were not counted, whereas cells touching the remaining two sides were counted. The number of counted cells per block was used to estimate the total ectopic hilar granule cell number using the formula developed by West *et al.* (1991):

$$N = \Sigma_Q^- \times (1/\text{ssf}) \times (1/\text{asf}) \times (1/\text{tsf})$$

where the estimated total number of granule cells (N) is derived from multiplying the number of counted cells (Σ_Q^-) by the reciprocal of the sampling probability (section sampling fraction, area sampling fraction and thickness sampling fraction).

To estimate total hilar volume for each hippocampus, the following formula was used:

$$V = \Sigma(A_{\text{hilus}} \times T) \times (1/\text{ssf})$$

where the estimated total hilar volume (V) is determined by multiplying the sum of the products of hilar area (A_{hilus}) and measured thickness per section (T) by the inverse of the proportion of the total hilar sections that were examined ($1/\text{ssf}$).

Statistical analysis

To determine differences in estimated ectopic granule hilar cell number, estimated hilar volume, or hippocampal length between groups, between hemispheres or at different survival times, a Student's t -test was used. To compare differences in estimates along the septotemporal axis of the hippocampus, a one-way analysis of variance was used. In all other cases, a Pearson's correlation coefficient was used to compute the strength of the relationship between two variables.

Results

Ectopic granule cells in the hilus of control animals

Figure 2 shows representative examples of Prox-1 immunolabeling in the hippocampal formation following saline treatment (control rats). Prox-1-immunoreactive cells were distributed densely and uniformly throughout the granule cell layer, demonstrating its specificity for granule cells (Pleasure *et al.*, 2000; Brandt *et al.*, 2003; Parent *et al.*, 2006). Similar immunoreactive cells were also scattered throughout the hilus, but were rare. Table 1 provides a summary of the estimates of Prox-1-immunoreactive cells in the hilus from all of the animals used in the study. The estimated total number of these 'ectopic hilar granule cells' in control animals was 1167 ± 193 (mean \pm SEM; $n = 9$). Figure 3 illustrates the small degree of variability across animals in the saline-treated controls. Ectopic hilar granule cell number was not influenced by hemisphere ($t_{15} = -1.36$, $P = 0.19$) or septo-temporal location ($F_{2,8} = 0.57$, $P = 0.59$). The number of ectopic hilar granule cells also appeared to be stable over time, because there was no significant correlation between number and survival time (survival time = time from saline treatment to the animal being perfused; $r = -0.29$, $P = 0.45$). Also, when animals with shorter survival times (2–6 months) were compared with animals with longer survival times (11–17 months), the estimated number of ectopic hilar granule cells was not significantly different ($t_7 = -1.15$, $P = 0.29$). Finally, it was not evident that there was a relationship between ectopic hilar granule cells and estimated hilar volume ($r = -0.47$, $P = 0.19$), or hippocampal length ($r = 0.29$, $P = 0.07$).

Ectopic granule cells in the hilus of pilocarpine-treated animals

Figure 2 shows a representative example of a Prox-1-immunoreactive section from a rat that was perfused 12 months after pilocarpine-induced status epilepticus. As described above for saline-treated rats, the Prox-1 antibody labeled the granule cell layer uniformly and consistently, reflecting its specificity for granule cells in control and epileptic rats. As reported previously (Scharfman *et al.*, 2000), ectopic hilar granule cells were located throughout the hilus. Table 1 provides a summary of the estimates of ectopic hilar granule cell number for each pilocarpine-treated animal. Pilocarpine-treated animals had an estimated 9842 ± 1692 ectopic hilar granule cells per hippocampus ($n = 10$), significantly more than saline-treated control animals ($t_{17} = 4.82$, $P = 0.00015$). There was a large range in ectopic hilar granule cell number per animal within the pilocarpine-treated group

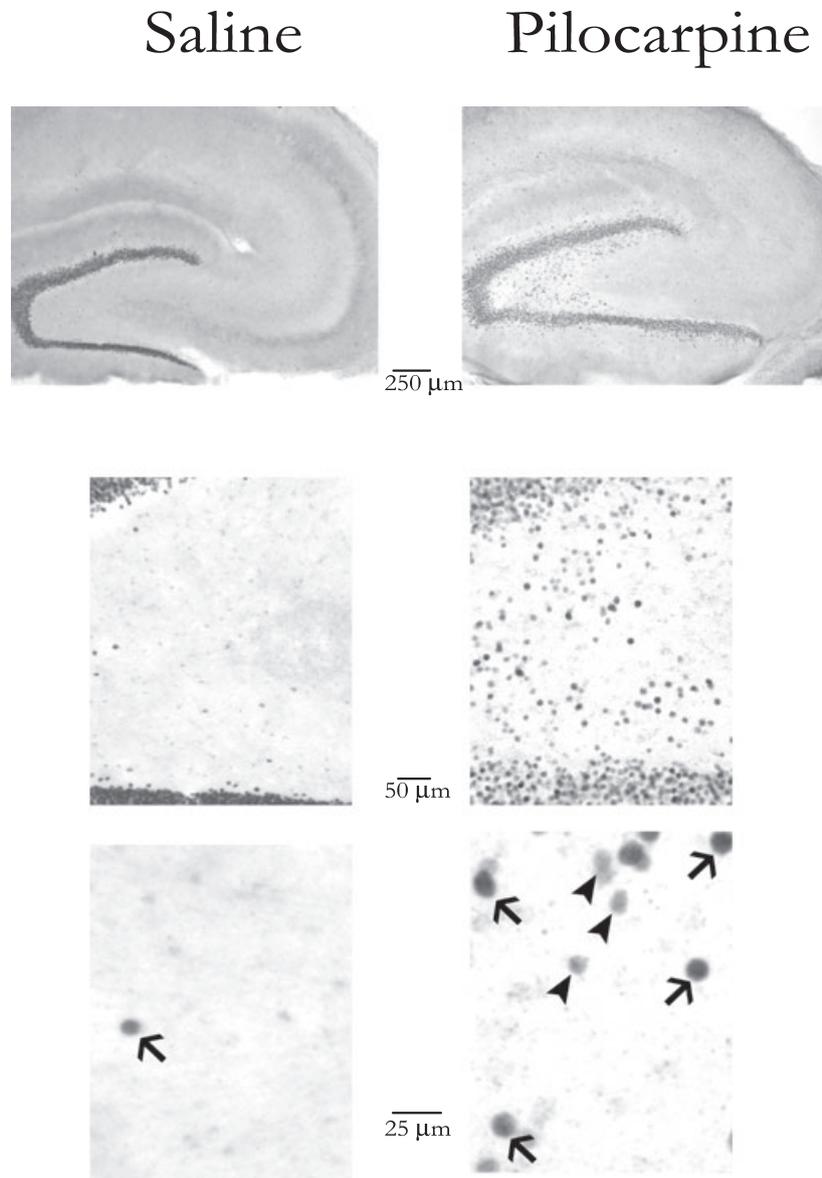


FIG. 2. Prox-1 immunohistochemical detection of ectopic granule cells in the hilus of the rat. Representative sections from the dentate gyrus of saline-treated controls (left) and pilocarpine-treated animals with status epilepticus (right) illustrate the immunoreactive profiles used to define ectopic granule cells. In a focal plane, cells were counted only when they came into focus (arrows), whereas out-of-focus profiles were not counted (arrowheads).

(3474–19 974 cells, $n = 10$; Fig. 3). To address the variability in the ectopic hilar granule cell population across animals, a number of factors were analysed.

Variation in the distribution of hilar ectopic granule cells

Figure 4 shows the distribution of ectopic hilar granule cells in pilocarpine-treated animals by hemisphere and septotemporal location. Estimates from left and right hippocampi showed no significant difference ($t_{18} = 0.73$, $P = 0.48$). Estimated neuronal density, determined as the estimated number of cells per hippocampal block divided by the total hilar area of the hippocampal block, showed no significant effect of septotemporal position. This analysis was based on estimates from the pairs of blocks in the septal, middle and temporal thirds of the hippocampus, followed by a one-way analysis of variance ($F_{2,13} = 1.60$, $P = 0.24$).

Variation in ectopic hilar granule cells as a function of hilar volume or hippocampal length

Following status epilepticus, ectopic hilar granule cell number was significantly correlated with estimated hilar volume ($r = 0.71$, $P = 0.022$), but not hippocampal length ($r = 0.17$, $P = 0.64$). Because ectopic hilar granule cell number was not related to hilar volume in control animals, a comparison of hilar volume was made between control and pilocarpine-treated animals. Figure 5 shows that pilocarpine-treated animals had significantly larger hilar volumes than control animals ($t_{17} = -2.133$, $P = 0.048$), without having a significant difference in hippocampal length ($t_{17} = 1.350$, $P = 0.195$). When pilocarpine-treated animals with shorter survival times (2–6 months after status epilepticus) were compared with animals with longer survival times (11–17 months after status), no age-related difference in hilar volume was detected ($t_8 = 0.26$, $P = 0.80$). Together, this suggests that pilocarpine-induced status epilepticus

TABLE 1. Estimation of ectopic hilar granule cells and hilar volume in saline-treated controls and after pilocarpine-induced status epilepticus

Animal	Months after injection	Observed spontaneous seizures	Hemisphere	Block (septal to temporal)	<i>N</i> (EGC)	Estimated CE	<i>V</i> (mm ³)
Saline							
p961	11	0	Right	5	585.55	0.015	0.26
p972	11	0	Right	5	1143.11	0.024	0.70
p979	11	0	Right	6	845.35	0.015	0.09
p1074	3	0	Right	1	1341.84	0.006	0.38
p1075	3	0	Right	6	533.13	0.015	1.38
p1076	6	0	Left	1	1944.00	0.025	0.49
p1077	6	0	Left	2	556.95	0.041	0.81
p1141	5	0	Right	2	1558.42	0.004	0.38
p1142	5	0	Right	5	1998.00	0.010	0.49
Mean	—	—	—	—	1167.37*	0.019†	0.55 ± 0.13*
CV	—	—	—	—	0.497	—	—
Pilocarpine							
p627	12	18	Right	4	16960.88	0.013	1.83
p799	17	1	Left	5	5207.50	0.011	0.63
p803	14	0	Right	1	7060.03	0.018	0.73
p959	14	2	Left	3	10814.44	0.011	0.58
P991	11	1	Left	4	3474.15	0.014	1.27
p1000	12	4	Right	5	19974.87	0.011	1.34
p1099	2	2	Left	3	12645.72	0.006	2.63
p1124	6	1	Right	3	5186.14	0.008	0.60
p1136	6	2	Left	2	9160.79	0.012	0.90
p1137	6	2	Left	2	7941.15	0.003	0.60
Mean	—	—	—	—	9842.57*	0.011†	1.11 ± 0.21*
CV	—	—	—	—	0.544	—	—

Estimates of total hilar ectopic granule cell number (*N*) and total hilar volume (*V*) were made from randomly selected hemispheres and septo-temporal blocks for each animal. The estimated coefficient of error (CE; calculated as standard error/mean), an estimate of the degree of intra-animal variance, was calculated for the ectopic hilar granule cell number estimates for each animal, and determined to contribute very little to the observed group variance, or coefficient of variation (CV calculated as group standard deviation/ group mean). Group means and coefficients of variation are reported at the bottom of each group column. *The ectopic granule cell number and estimated hilar volume in pilocarpine-treated animals were significantly higher than control values (Student's *t*-test, $P > 0.05$). †Mean CE is calculated as $\sqrt{\text{Mean CE}^2}$.

may alter the shape of the dentate gyrus, perhaps by widening the space between the upper and lower granule cell layer blades, or by extending the length of the blades further in the direction of area CA3.

cells in the hilus ($r = 0.28$, $P = 0.43$), and comparison of shorter (2–6 months) to longer (11–17 months) survival times after status epilepticus did not reveal a significant difference in the size of the

Variation in ectopic granule cells during the period of chronic spontaneous seizures following pilocarpine-induced status epilepticus

Figure 6 shows the estimated number of ectopic granule cells in the hilus as a function of time after pilocarpine-induced status epilepticus. The survival time did not correlate with the number of ectopic granule

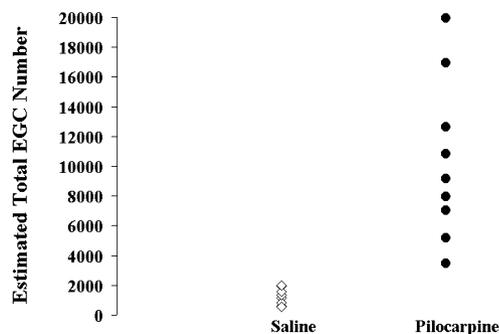


FIG. 3. Pilocarpine-induced status epilepticus significantly increases hilar ectopic granule cell number. Comparison of the estimated total ectopic hilar granule cell (EGC) number in saline-treated controls (white diamonds) and pilocarpine-treated rats that had status epilepticus (black circles) showed a large increase in ectopic hilar granule cell number following pilocarpine-induced status epilepticus.

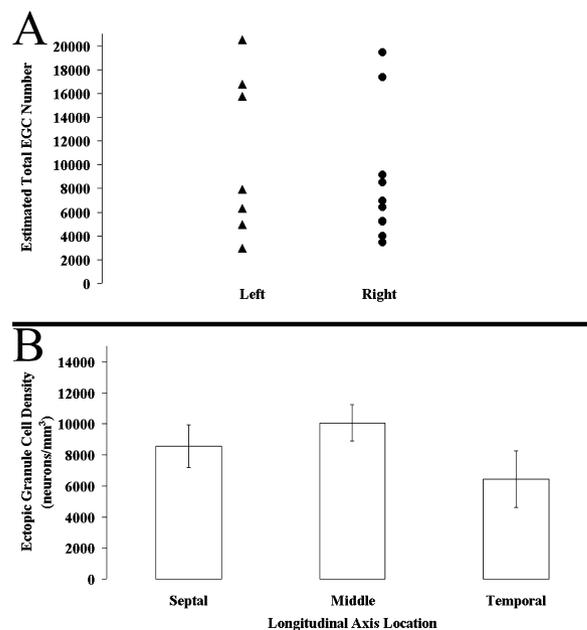


FIG. 4. Ectopic hilar granule cell formation does not have a hemispheric or septotemporal bias. (A) There were no differences when comparisons were made of the estimated number of ectopic hilar granule cells (EGC) in the left and right hippocampus (Student's *t*-test, $P < 0.05$), (B) or the septal, middle and temporal thirds of the hippocampus (one-way analysis of variance, $P < 0.05$).

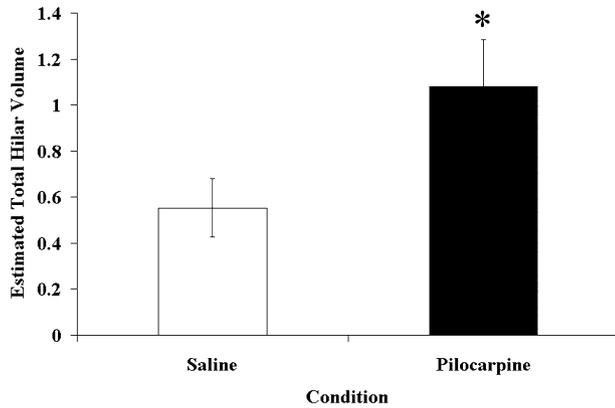


FIG. 5. Status epilepticus increases hilar volume. Measurements of the estimated total hilar volume, determined by the hilar contour area, section thickness and number of sections per block, showed a significant increase in hilar volume after pilocarpine-induced status epilepticus. A lack of difference in hippocampal or block length between saline- and pilocarpine-treated animals suggests this increase may be the result of granule cell layer widening or lengthening. *Student's *t*-test, $P < 0.05$.

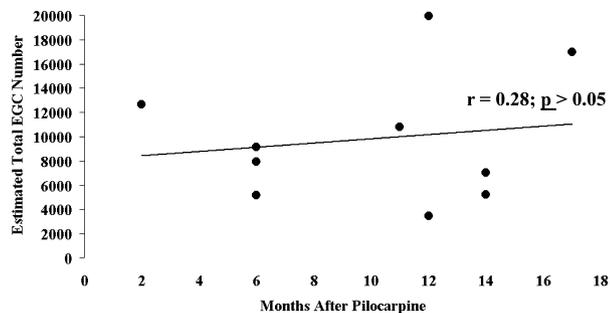


FIG. 6. The ectopic hilar granule cell population (EGC) is stable over time. Ectopic hilar granule cell number (per hippocampus) is plotted as a function of survival (in months) after pilocarpine-induced status epilepticus. The relationship between survival time and ectopic hilar granule cell number was not significant (Pearson's correlation coefficient, $P > 0.05$).

hilar ectopic granule cell population ($t_8 = 0.11$, $P = 0.92$), suggesting that the population is stable over time. When the estimate of stage 4–5 behavioral seizures per month was compared with the total estimated ectopic hilar granule cell number, there was a significant correlation (Fig. 7; $r = 0.71$, $P = 0.02$), suggesting that animals with more ectopic granule cells in the hilus exhibited more frequent spontaneous behavioral seizures. There was also a significant correlation between the estimated stage 4–5 behavioral seizure frequency and estimated hilar volume ($r = 0.86$, $P = 0.01$), suggesting that animals with greater hilar volumes had more frequent seizures. The relationship between both ectopic hilar granule cell number and hilar volume with the spontaneous behavioral seizure frequency suggests that these measures are related to epileptogenesis, and are not just the result of pilocarpine exposure.

Discussion

Summary

The main finding of the present study was that pilocarpine-induced status epilepticus is followed by the formation of a robust population of ectopic granule cells in the hilus. Although an average of ~10 000

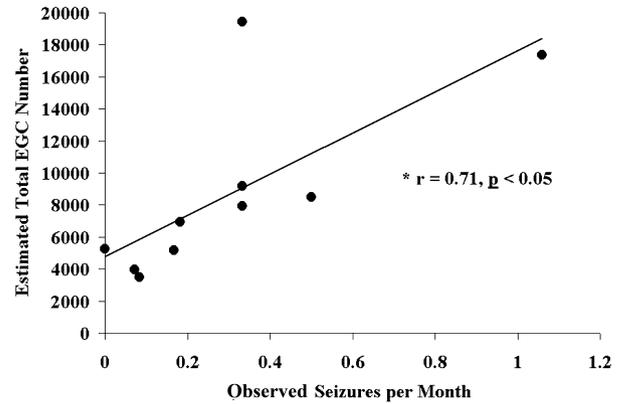


FIG. 7. Ectopic hilar granule cell number is related to estimated seizure frequency. There was a significant correlation between the number of hilar ectopic granule cells (EGC) per hippocampus and the number of observed behavioral seizures per month (Pearson's correlation coefficient, $P < 0.05$).

ectopic granule cells in the hilus of pilocarpine-treated rats is less than 1% of the total granule cell population (West *et al.*, 1991), it may represent as much as 25% of the total hilar neuron population (West *et al.*, 1991; Buckmaster & Dudek, 1997), suggesting it could have a profound impact on hilar circuitry. The influence of ectopic hilar granule cell formation may be particularly important in the context of pilocarpine-induced status epilepticus, when many of the original hilar neurons are lost (Buckmaster & Dudek, 1997). Under such conditions, the ectopic hilar granule cells may become a more substantial fraction of the residual hilar cell population, possibly becoming the predominant hilar cell type.

An important point, however, is that our results may not predict the ectopic hilar granule cell population that results from other methods to induce status, such as kainic acid. Variation could occur even within the pilocarpine model, depending on the dose, age of the animal and other factors. For example, we administered diazepam 1 h after the onset of status epilepticus, but neither the type of anticonvulsant nor the time of administration are standardized across laboratories. This may change the severity and duration of status, and the result could be a difference in the degree of seizure-induced neurogenesis, survival of newly born cells, and many other factors. It would be helpful to conduct a systematic stereological evaluation of ectopic hilar granule cells as a function of these variables, which could lead to insight into the relationship between status epilepticus and ectopic hilar granule cells.

Relation of ectopic hilar granule cells to hilar volume and seizure frequency after status epilepticus

There was a large range in estimates of the size of the ectopic hilar granule cell population. Estimates ranged from ~3500 cells to ~20 000 cells for pilocarpine-treated rats. Differences between hemispheres used for stereological estimation, or septo-temporal location, could not account for this variability, nor could the survival time after status epilepticus. However, two factors were found that appeared to be related to the number of ectopic hilar granule cells in the hippocampi of pilocarpine-treated rats: hilar volume and estimated behavioral seizure frequency.

Hilar volume increased significantly after pilocarpine-induced status epilepticus, and the increase in the volume of the hilus was related to the number of ectopic granule cells in the hilus. Interestingly, an increase in hilar volume has been reported in some other animal

models of epilepsy (Cavazos & Sutula, 1990; Bertram & Lothman, 1993; Watanabe *et al.*, 1996), but not in all (Cadotte *et al.*, 2003). Although a causal relationship between hilar volume and ectopic granule cell development is not known, hilar volume could contribute to ectopic granule cell formation, or vice versa. It is also possible that a distinct mechanism could lead, in parallel, to an increase in hilar volume and ectopic granule cells. For example, astrogliosis, which is related to inflammation, has been linked to hilar enlargement in the kindling model (Adams *et al.*, 1998). Altered size or number of astrocytes may influence the fate of progenitors and their migratory path, facilitating a granule cell fate and ectopic destination. Alternatively, an increase in the number of granule cells in the granule cell layer, which occurs after pilocarpine-induced status epilepticus (Parent *et al.*, 1997; Covolán *et al.*, 2000; Nakagawa *et al.*, 2000), may cause the blades of the granule cell layer to extend, resulting in a lengthening of the dentate gyrus. Regardless of the mechanism involved, the change in hilar volume is interesting because it may reflect a lengthening or a widening of the dentate gyrus that, to our knowledge, has not been previously considered.

The second factor that correlated with ectopic hilar granule cell number, observed seizure frequency, is even more intriguing because it supports the hypothesis that ectopic granule cells contribute to epileptogenesis. The current study is not definitive, however, for two reasons. First, the correlative data do not prove that ectopic granule cells caused recurrent spontaneous seizures in the pilocarpine model. Second, the evaluation of seizure frequency used here provided only an estimate of behavioral seizures, and did not include mild behavioral seizures or electrographic seizures without behavioral manifestations.

It is important to consider that spontaneous recurrent seizures could influence the ectopic hilar granule cell population, leading to a positive feedback mechanism that would influence seizure frequency. However, animals with longer survival times, and therefore with a higher total accumulation of spontaneous seizures, did not appear to have more ectopic granule cells in the hilus. Therefore, it seems unlikely that the spontaneous seizures played a major role in ectopic hilar granule cell number, or the influence of this population on seizure threshold.

Potential impact of ectopic hilar granule cells on the hippocampal network

It is interesting to consider that the ectopic granule cells may replace hilar cells that are lost after status epilepticus. Although ectopic granule cells have membrane properties like conventional granule cells, they are similar to hilar neurons in several ways. For example, they exhibit abnormal spontaneous network bursts after pilocarpine-induced status epilepticus (Scharfman *et al.*, 2000). The ectopic neurons are also similar to hilar neurons in the time course of c-fos-like immunoreactivity after a spontaneous seizure (Scharfman *et al.*, 2002a). Anatomical evidence shows that, like other types of hilar neurons, ectopic hilar granule cells receive dense mossy fiber innervation (Pierce *et al.*, 2005). Furthermore, ectopic granule cells in the hilus are likely to be innervated by CA3 pyramidal cells because they exhibit closely synchronized burst discharges in slices from pilocarpine-treated rats (Scharfman *et al.*, 2000). This is similar to hilar mossy cells and hilar GABAergic interneurons, which are synchronized with CA3 burst discharges in slices from pilocarpine-treated rats (Scharfman *et al.*, 2001).

Therefore, it is interesting to consider the consequence of adding neurons to the hilus with the firing properties of granule cells but the characteristics of hilar neurons. Granule cells trigger larger depolar-

izations in their postsynaptic targets than hilar neurons, when unitary EPSPs are compared (Scharfman, 1995; Scharfman *et al.*, 2003). Therefore, substituting a granule cell for a hilar cell could have a major impact on the excitability of the network.

Implications for human temporal lobe epilepsy

If it were indeed the case that an increase in ectopic granule cell formation is a driving force behind epileptogenesis in the pilocarpine model of epilepsy, and we assume that pilocarpine-induced epileptogenesis is a model of human temporal lobe epilepsy (TLE), then it would follow that humans with TLE would have an increase in ectopic granule cells. Yet, the data thus far are inconclusive. In a recent study (Parent *et al.*, 2006), Prox-1-labeled granule cells were found in the hilar region of hippocampal tissue resected from humans with intractable TLE. However, another study noted the lack of cells expressing nestin, an early indicator of neuronal differentiation, in hippocampal tissue resected from adults with intractable TLE (Blumcke *et al.*, 2001). Perhaps this discrepancy can be explained by the evidence that ectopic granule cells appear to migrate into the hilus after they differentiate into neurons (Parent *et al.*, 2006). Yet, it appears that in some cases there is an increase in neuronal precursors in the hilus of patients with pharmacoresistant TLE (Crespel *et al.*, 2005; Thom *et al.*, 2005). Interestingly, one of those studies suggested that the appearance of neuronal precursors in the hilus is related to dispersion of the granule cell layer (Thom *et al.*, 2005), which indicates that ectopic granule cell formation and granule cell dispersion may depend on similar mechanisms. More work will be needed to quantify the number of ectopic granule cells in human epileptic tissue to determine whether the population is as prevalent as it is in the rat pilocarpine model.

Conclusions

The data presented here confirm that the growth of the population of ectopic granule cells in the hilus is substantial after pilocarpine-induced status epilepticus. It is, on average, eight times larger than the population of ectopic granule cells in the hilus under control conditions. When one considers the potential for reciprocal innervation of ectopic hilar granule cells with both granule cells located normally, in the granule cell layer, and CA3 pyramidal cells (Scharfman *et al.*, 2000), it is easy to see how this population could contribute to a maladaptive reverberatory circuit within the epileptic hippocampal formation (Parent, 2002; Scharfman, 2004). However, it is also important to evaluate the potential role of GABAergic neurons in these circuits, and indeed the GABAergic neurons modulate the activity of ectopic hilar granule cells (Scharfman *et al.*, 2003). Methods specifically to reduce ectopic granule cells in the epileptic hippocampus would have great potential in elucidating the role of ectopic hilar granule cells in the dentate gyrus.

Acknowledgements

This work was supported by National Institutes of Health Grant NS 41490, the Epilepsy Foundation and the Helen Hayes Hospital Foundation. We greatly appreciate the gift of Prox-1 antibody from Dr Sam Pleasure, which was used to generate preliminary data for this study.

Abbreviations

CA3, cornu ammonis region of the hippocampus 3; Prox-1, the prospero-like homeobox protein 1; TLE, temporal lobe epilepsy.

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