Neurobiology of Disease

Seizures Accelerate Functional Integration of Adult-Generated Granule Cells

Linda S. Overstreet-Wadiche,[1] Daniel A. Bromberg,[1,2] AeSoon L. Bensen,[1] and Gary L. Westbrook[1]

[1]Vollum Institute, L474, Oregon Health and Science University, Portland, Oregon 97239, and [2]Biology Department, Reed College, Portland, Oregon 97202

In humans and experimental animals, structural and functional changes in neural circuits can accompany the development of epilepsy. In the dentate gyrus, seizures enhance adult neurogenesis, but it is unclear to what extent newborn granule cells participate in seizure-induced synaptic reorganization. During the first weeks of their existence, mouse newborn granule cells labeled with enhanced green fluorescent protein have only short dendrites that lack excitatory input. We report that pilocarpine-induced seizures accelerated the morphological development of labeled granule cells, causing their dendrites to extend through the molecular layer. In whole-cell recordings 5–16 d after seizure induction, perforant-path stimulation now evoked glutamatergic input to newborn granule cells. These synaptic responses were mediated by monosynaptic as well as recurrent polysynaptic input. Thus, seizures facilitated functional integration of adult-generated granule cells. One month later, subsequent generations of newborn cells also showed alterations in dendrite morphology, suggesting persistent effects of seizures on granule cell maturation. The sensitivity of newborn granule cells to seizures could contribute to hyperexcitability during the latent period.

Key words: **adult neurogenesis; recurrent EPSCs; epilepsy; dentate gyrus; synaptic transmission; mossy fiber sprouting**

Introduction

Neurogenesis in the dentate gyrus continues in adulthood. The neural circuitry of the dentate gyrus can also be profoundly altered in epilepsy. Many models of experimentally induced epilepsy increase the proliferation of neural progenitors that generate newborn granule cells (Parent and Lowenstein, 2002). In principle, enhanced neurogenesis could provide a compensatory mechanism to replace damaged neurons. However, recent attention has focused on pro-epileptogenic effects of adult-generated granule cells. For example, after epileptogenesis, some newborn cells migrate into the hilus where aberrant connectivity promotes synchronous discharges with surviving CA3 pyramidal cells (Scharfman et al., 2000; Scharfman, 2004). Indeed, blocking neurogenesis after seizure induction attenuates the subsequent development of spontaneous recurrent seizures (Jung et al., 2004).

Experimentally induced seizures alter molecular expression patterns and network connectivity in the granule cell layer. Many changes occur during the latent period, the relatively seizure-free interval before the generation of spontaneous recurrent seizures. Mossy fiber sprouting represents one of the most dramatic changes initiated during this time (Nadler, 2003). Glutamatergic mossy fibers invade the granule cell and inner molecular layer, regions mostly populated by GABAergic synaptic boutons, and form recurrent synapses between granule cells. Little is known about how the changing environment affects neurite outgrowth and synaptogenesis of newborn granule cells. Until recently, this question was impossible to address because adult-generated granule cells could only be identified by incorporation of thymidine analogs that label nuclei and require fixation. Viral vectors and developmentally regulated promoter sequences that drive green fluorescent protein (GFP) expression have facilitated identification of adult-generated cells in living preparations (Ming and Song, 2005). These techniques are beginning to reveal how newborn neurons integrate into the circuitry of the adult brain (van Praag et al., 2002; Esposito et al., 2005; Ge et al., 2005).

Here, we investigate the functional integration of newborn granule cells after pilocarpine-induced seizures. We used pro-opiomelanocortin–enhanced GFP (POMC-EGFP) transgenic mice, in which newborn granule cells in the dentate gyrus transiently express EGFP during the first weeks after cell birth (Overstreet et al., 2004). Seizures enhanced the dendritic arbors of EGFP-labeled newborn granule cells and triggered excitatory synaptogenesis. Our results indicate that seizure-induced changes in the local environment accelerate the functional integration of adult-generated granule cells. Thus, the developmental stages following progenitor proliferation are also dynamically regulated.

Materials and Methods

Animals. Transgenic −13/+8POMC-EGFP mice were generated as described previously (Cowley et al., 2001). Experiments were performed in heterozygous −13/+8POMC-EGFP mice maintained by out-breeding homozygous males with wild-type C57BL/6J females. All animal procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and the United States Public Health Service and were approved by the Oregon Health and Science University Institutional Animal Care and Use Committee.

Received Sept. 7, 2005; revised Feb. 9, 2006; accepted March 8, 2006.

This work was supported by National Institutes of Health Grant NS-26494 (G.L.W.). We thank the members of the Westbrook laboratory and Jacques Wadiche for helpful comments and suggestions and Ilia Halatchev for participating in preliminary experiments.

Correspondence should be addressed to Dr. Linda S. Overstreet-Wadiche, Vollum Institute, L474, Oregon Health and Science University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97239. E-mail: overstre@ohsu.edu.

DOI:10.1523/JNEUROSCI.5508-05.2006

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Seizure induction. Adult mice (2–4 months of age) were pretreated with scopolamine methyl nitrate (1 mg/kg, s.c.; Sigma, St. Louis, MO) 30 min before the induction of seizures with pilocarpine hydrochloride (300–325 mg/kg, i.p.; Bausch & Lomb, Tampa, FL). Seizures were scored using the Racine scale for at least 2 h after pilocarpine injections. Age-matched control mice received scopolamine and vehicle injections (0.9% NaCl; $n = 3$) or no treatment ($n = 7$). After initial experiments to assess the effect of seizure severity on newborn granule cells, only mice that had multiple level III–V seizures within 2 h of pilocarpine injection (status epilepticus) (Shibley and Smith, 2002) were used. After seizures, assisted feeding and hydration were provided for all mice.

Bromodeoxyuridine labeling and immunohistochemistry. Bromodeoxyuridine (BrdU) was administered to age-matched adult mice for 1 d via an osmotic pump (8 μl/h, 25 mg/ml in 0.9% saline and 0.008N NaOH, s.c., day 0; 2001D, Alzet, Cupertino, CA). The pump was then removed (day 1), and seizures were induced in the test mice (day 2). On day 16, control and test mice were anesthetized with 2,2,2-tribromoethanol (Avertin; Aldrich, St. Louis, MO) and perfused transcardially with 4% paraformaldehyde in PBS. The brains were removed and postfixed overnight. Horizontal sections through the hippocampus (50 μm) were cut on a vibratome and stored at −20°C in cryoprotecting buffer (30% ethylene glycol, 20% glycerin, and 0.05 M PBS). BrdU immunohistochemistry was performed simultaneously on sections from all mice. Free-floating sections were washed twice in potassium PBS (KPBS), incubated in 2N HCl (30 min at 37°C), and rinsed in 0.1 M borate buffer (pH 8.4, 10 min). Sections were incubated in KPBS, 0.4% Triton X-100, and 5% normal goat serum for 30 min, followed by overnight incubation with primary anti-BrdU antibody (monoclonal rat, 1:200; ImmunologicalsDirect.com, Kidlington, Oxfordshire, UK). After rinsing, sections were incubated for 1 h in Texas Red-conjugated goat anti-rat IgG (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were then incubated for 1 h in AlexaFluor 488 rabbit anti-GFP IgG (1:500; Molecular Probes, Eugene, OR). Sections were mounted with Prolong Antifade (Molecular Probes, Eugene, OR) and imaged with a confocal microscope (Olympus, Tokyo, Japan). Cell counts were performed blind to the experimental condition, and results were confirmed with partial counts by a second blinded investigator. The number of $BrdU^+$ nuclei positioned within or adjacent to the granule cell layer (>50 cells per animal) and the number of cells that colocalized BrdU and EGFP were counted. For morphometric measurements, we reconstructed biocytin-filled cells or EGFP-labeled cells with maximum *z*-projections of confocal stacks (0.5–1.0 μm intervals) using ImageJ. EGFP-labeled cells that were relatively isolated from other labeled cells were chosen randomly for reconstruction. Total dendritic length and branch point measurements were made with NeuronJ (Meijering et al., 2004).

Electrophysiology. Mice were anesthetized with Avertin and perfused intracardially with ice-cold modified artificial CSF containing the following (in mM): 110 choline chloride, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 20 dextrose, 2.4 pyruvate, and 1.3 ascorbic acid, bubbled with 95%O_2/5% CO_2. The brains were removed, and horizontal slices from the hippocampus (350 μm) were incubated in a solution containing the following (in mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 25 D-glucose, bubbled with 95% O_2/5% CO_2. Patch pipettes were filled with the following (in mM): 113 Cs-gluconate, 10 HEPES, 10 EGTA, 17.5 CsCl, 8 NaCl, 2 Mg_2ATP, 0.3 NaGTP, and 10 phosphocreatine, pH 7.3, 305 mOsm, 4–8 MΩ. Biocytin (0.2%) was included in the pipette solution for some experiments. The extracellular solution contained SR95531 (gabazine; 10 μM). Differential interference contrast and fluorescent images were combined (PIX/2; MicroImage Video Systems, Boyertown, PA) for simultaneous viewing of $EGFP^+$ and unlabeled cells. Series resistance (8–30 MΩ) was compensated 50–80%, and experiments were discarded if substantial changes were observed. Currents were filtered at 2 kHz and sampled at 10 kHz (Axopatch 200B; Molecular Devices, Union City, CA). Voltages were not corrected for junction potentials. Synaptic responses were evoked by a pipette filled with extracellular solution placed in the medial perforant path (MPP). Data are expressed as mean ± SEM. ANOVA with Bonferroni's multiple comparisons tests and two-tailed paired or unpaired *t* tests were used to determine statistical significance at the $p < 0.05$ level. Unless noted, *n* values refer to the number of cells sampled. All drugs and chemicals were obtained from Sigma or Tocris (Ellisville, MO).

Results

Dendrite outgrowth after seizures

To follow the effects of seizures on newborn neurons, we took advantage of POMC-EGFP transgenic mice (Young et al., 1998; Cowley et al., 2001). Adult-generated granule cells in these animals are transiently labeled with EGFP when they are ~12 d postmitotic (Overstreet et al., 2004). At this developmental stage, labeled granule cells have immature membrane properties including high input resistance and small broad action potentials. They also have immature morphology, with short dendrites that do not project through the molecular layer where the primary afferent input is located (the medial and lateral perforant path). Throughout the life span of the mouse, EGFP-labeled newborn cells have similar morphology and exclusively GABAergic synaptic input (Overstreet-Wadiche et al., 2005, 2006). However, pilocarpine-induced seizures dramatically altered the morphology of EGFP-expressing newborn granule cells. Two weeks after seizures, labeled dendrites were clearly visible in the middle and outer molecular layer (Fig. 1*a*), whereas in control mice, dendrites were restricted to the inner molecular layer (Fig. 1*b*). The dendritic length of newborn granule cells increased from 270 ± 11 μm ($n = 25$) to 429 ± 44 μm ($n = 27$; $p = 0.001$) in mice with level III–V seizures (Fig. 1*c*). Branch point number also increased from 3.8 ± 0.3 in controls to 6.1 ± 0.7 after seizures ($p = 0.002$). Increased dendrite length was also observed in biocytin-filled cell reconstructions after whole-cell recordings (638 ± 116 μm; $n = 5$; see below). Dendritic outgrowth was variable across the dentate gyrus, with some regions showing little change (Fig. 1*a*, asterisk). Dendrite outgrowth correlated with the behavioral severity of the initial seizures. Newborn granule cells in mice with level I–II seizures ($n = 4$ mice; data not shown) were similar to non-injected and vehicle-injected controls ($n = 10$ mice). Only mice that displayed multiple level III–V seizures were used for subsequent experiments.

Consistent with previous work using BrdU labeling (Parent et al., 1997), the number of newborn granule cells expressing EGFP also appeared to increase (Fig. 1). To determine whether seizures enhanced the dendritic arbors of existing adult-generated cells, we labeled S-phase cells with BrdU 2 d before seizure induction. Mice were killed 2 weeks later (Fig. 2*a*). After seizures, some BrdU-labeled cells had elongated dendrites (Fig. 2*b*), indicating that pre-existing adult-generated cells contributed to the population of cells with enhanced dendritic outgrowth. The degree of colocalization between BrdU and EGFP was 57.8 ± 3.0% in seizure mice ($n = 3$) and 57.6 ± 2.0% in control mice ($n = 3$), indicating that the timing of EGFP expression was not altered. Colocalization between BrdU and EGFP at a single interval of 16 d is sufficient to detect accelerated expression of EGFP in young mice (Overstreet-Wadiche et al., 2006). Furthermore, after seizures, essentially all EGFP-labeled granule cells expressed the immature marker PSA-nCAM (95.9 ± 0.8%; $n = 3$ mice) (Fig. 2*c*), similar to what was observed in control mice (97%) (Overstreet et al., 2004). Thus, seizures specifically enhanced dendritic outgrowth of adult-generated granule cells, not just their proliferation.

Seizures accelerate the functional integration of newborn granule cells

The dendrites of EGFP-labeled newborn granule cells normally do not receive synaptic input from the perforant path (Overstreet

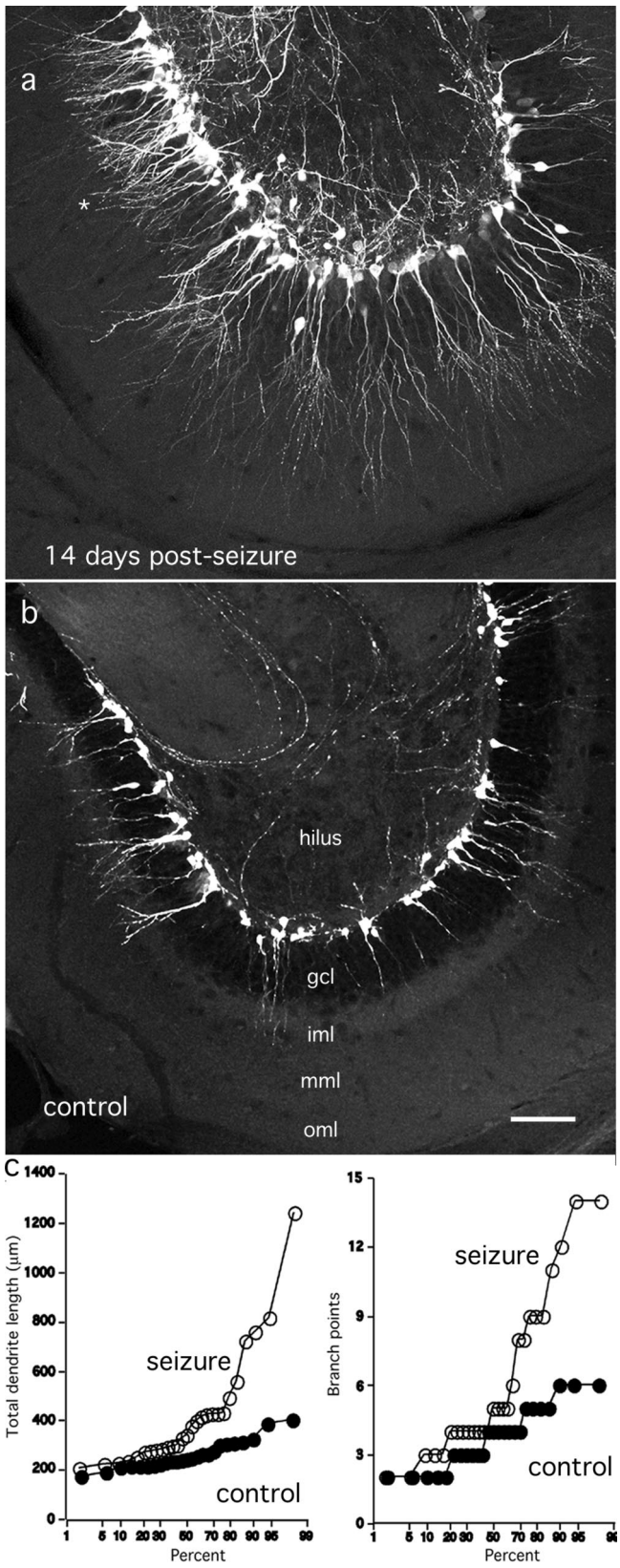

Figure 1. Seizures enhance dendrite outgrowth of newborn granule cells. ***a***, Two weeks after pilocarpine-induced seizures, many EGFP-labeled newborn granule cells had dendrites that extended into the middle and outer molecular layer. ***b***, In control mice, the dendrites of all newborn granule cells terminated in the inner molecular layer. Some newborn granule cells after seizures remained unaffected (***a***, asterisk). Seizures also appeared to increase the number of labeled newborn cells. Images are maximum intensity *z*-projections through 50 μm sections from perfusion-fixed POMC-EGFP brains. Image intensity was increased to visualize the layers. Scale bar, 100 μm. gcl, Granule cell layer; iml, inner molecular layer; mml, middle molecular layer; oml, outer molecular layer. ***c***, Seizures increased the total dendritic length and number of branch points of EGFP-labeled newborn granule cells. The cumulative probability distribution of measured values is shown. The *x*-axis indicates the percentage of values that fall below and above each measurement, such that the median falls at 50%. Twenty-five cells from 15 sections were measured in control mice ($n = 10$), and 27 cells from 12 sections were measured in mice that had level III–V seizures after pilocarpine injections ($n = 3$).

et al., 2004; Overstreet-Wadiche et al., 2005). To test whether dendritic outgrowth allowed newborn granule cells to receive perforant-path input, we made whole-cell recordings from labeled cells in acute slices 5–16 d after seizure induction. In 17 of 33 newborn granule cells, stimulation of the MPP evoked EPSCs mediated by AMPA and NMDA receptors, whereas EPSCs were never evoked in slices from control mice ($n = 24$) (Fig. 3*a*,*b*). MPP stimulation evoked EPSCs in all neighboring mature granule cells in both conditions ($n = 26$). Consistent with seizure-induced synaptogenesis, reconstructions of biocytin-filled cells in acute slices and EGFP-labeled cells in fixed slices revealed that the dendrites of newborn granule cells were covered with spines, whereas in control mice, newborn cells were essentially spine free (Fig. 3*c*).

The input resistance of granule cells inversely correlates with granule cell maturity (Liu et al., 2000; Schmidt-Hieber et al., 2004). Newborn granule cells with synaptic input after seizures had lower input resistance (2.5 ± 0.2 GΩ; $n = 17$ cells) compared with newborn cells from control mice (5.1 ± 0.5 GΩ; $n = 8$; $p = 0.008$). The decreased input resistance was specific to newborn granule cells because the input resistance of mature granule cells was unchanged after seizures (266 ± 29 MΩ, $n = 9$, compared with 315 ± 39 MΩ, $n = 14$; $p = 0.39$). The appearance of MPP-evoked EPSCs and dendritic spines, along with the reduction in input resistance, demonstrates that seizures accelerated the maturation and functional integration of adult-generated granule cells.

Recurrent synaptic activity in mature granule cells

Several months after pilocarpine-induced seizures, granule cells display EPSCs arising from newly formed recurrent mossy fiber synapses (Okazaki et al., 1999; Tu et al., 2005). As shown in Figure 4*a*, mossy fiber sprouting occurred as early as 5 d after seizure induction. This biocytin-filled mature granule cell had an axon collateral contacting the dendrite of a second filled mature granule cell. More importantly, mature granule cells had asynchronous synaptic events on the decay phase of EPSCs evoked by MPP stimulation (Fig. 4*b*, asterisk), consistent with polysynaptic activity. These delayed events contrasted with the smooth monotonic decay in control slices and caused a 44% prolongation of the EPSC half-width (12.2 ± 0.9 ms, $n = 9$ cells in control and 17.6 ± 2.2 ms, $n = 17$ cells after seizure; $p = 0.04$). To confirm that this asynchronous activity was mediated by polysynaptic or recurrent synapses rather than delayed release at monosynaptic perforant-path synapses, we recorded NMDA receptor-mediated EPSCs at +40 mV (NMDA EPSCs) and blocked polysynaptic activity with 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonoamide (NBQX). After seizures, NBQX accelerated the decay of NMDA EPSCs (half-width reduced to 68 ± 9% of control; $n = 8$; $p = 0.007$). In some cases, NBQX also reduced the NMDA EPSC amplitude (Fig. 4*b*). Thus, in mature granule cells, a component of the NMDA EPSC was attributable to glutamate released by recurrent synapses. In contrast, NMDA EPSCs in mature granule cells were unaffected by NBQX in control slices

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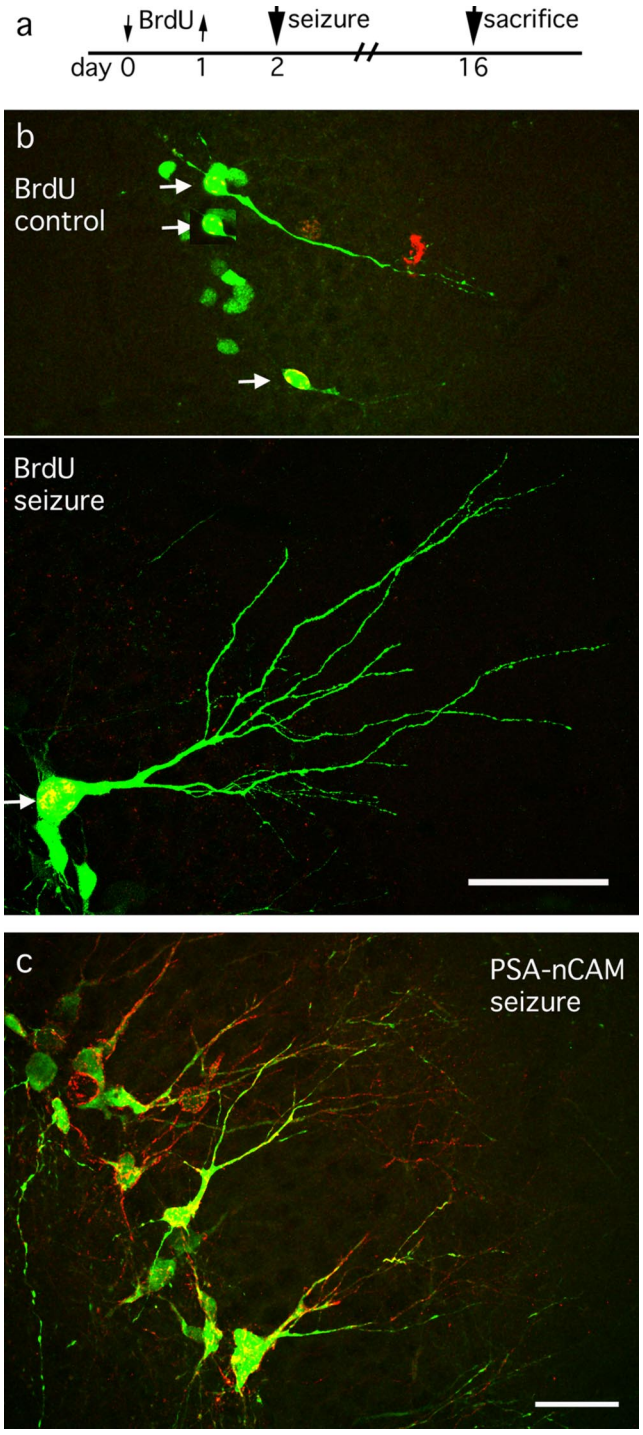

Figure 2. EGFP labels newborn granule cells after seizures. ***a***, Diagram of the BrdU experiment to birth date newborn granule cells before seizure induction. ***b***, EGFP and BrdU immunolabeling from control (top) and seizure (bottom) mice perfused 16 d after BrdU incorporation. After seizures, some EGFP-expressing cells with elongated dendrites were labeled with BrdU. Colabeled cells are indicated with arrows. The percentage of BrdU-positive cells that expressed EGFP was the same in seizure and control mice, indicating there was no change in the temporal pattern of EGFP expression after seizures. ***c***, After seizures, almost all EGFP-labeled granule cells expressed PSA-nCAM, indicating EGFP was selective for newborn granule cells. Scale bars: ***b***, 50 μm; ***c***, 20 μm.

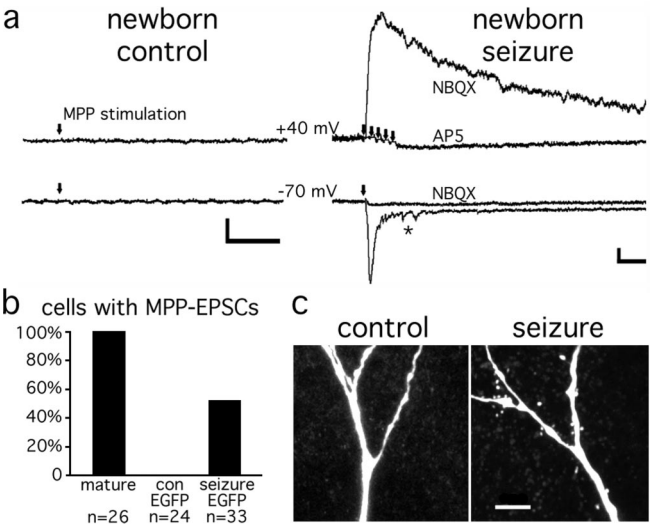

Figure 3. Seizures promote functional integration of newborn granule cells. ***a***, MPP stimulation failed to evoke EPSCs in newborn granule cells in slices from control mice. After seizures, single MPP stimuli evoked EPSCs in newborn granule cells. At a holding potential of −70 mV, inward EPSCs were blocked by NBQX. At +40 mV in NBQX, outward EPSCs were blocked by AP-5, even after multiple stimuli (arrows). The asterisk indicates asynchronous synaptic events on the decay of the EPSC at −70 mV. Calibration: 10 pA, 50 ms. These synaptic responses were recorded in the biocytin-filled cell shown in supplemental Figure 1 (available at www.jneurosci.org as supplemental material). ***b***, The percentage of mature and newborn granule cells with MPP-evoked EPSCs in control (con) and seizure mice. There were significant differences among groups ($p < 0.0001$; χ^2 test). *Post hoc* comparisons showed that newborn granule cells in control were different from the other two groups ($p < 0.001$; Fisher's exact test). ***c***, After seizures, newborn granule cells had dendritic spines that were not present in control mice. Scale bar, 5 μm.

(105 ± 7% of control; $n = 7$; $p = 0.69$) (Fig. 4*c*), indicating that the MPP-evoked control response was entirely monosynaptic. The sensitivity of the NMDA EPSC to NBQX thus provides a measure of seizure-induced synaptic reorganization. We then used this measure to determine whether EGFP-labeled newborn granule cells participate in synaptic reorganization.

Newborn granule cells receive direct and recurrent perforant-path input

Similar to mature cells, MPP stimulation often resulted in asynchronous synaptic events in newborn granule cells (Figs. 3*a*, 5*a*, asterisks). The delayed EPSCs at −70 mV could be as large as the short-latency component (18 ± 4 pA; $n = 9$ cells; 14–16 d after seizure) (Fig. 5*a*). NBQX blocked EPSCs recorded at −70 mV and reduced the half-width of the NMDA EPSC at +40 mV (to 53 ± 11% of control; $n = 10$; $p = 0.02$) (Fig. 5*a*, normalized traces), indicating newborn granule cells also received polysynaptic recurrent input. With polysynaptic activity blocked, the remaining NMDA EPSC (23 ± 8 pA; $n = 10$) was mediated by monosynaptic perforant-path input. The weighted decay time constant of monosynaptic NMDA EPSCs was more than twofold greater in newborn cells (172 ± 2 ms; $n = 6$) compared with neighboring mature cells (78 ± 0.6 ms; $n = 8$; $p = 0.001$) (Fig. 5*b*). The ratio of NMDA/AMPA peak amplitudes was also greater in newborn granule cells (2.1 ± 0.5; $n = 6$) compared with mature cells (1.0 ± 0.2; $n = 11$; $p = 0.03$). Thus, MPP synapses on newborn granule cells had prolonged and large NMDA receptor-mediated components characteristic of immature excitatory synapses (Carmignoto and Vicini, 1992; Tovar and Westbrook, 1999). Similar to previous reports (Sayin et al., 1999; Behr et al., 2001; Scimemi et al., 2006), mature granule cells also had larger NMDA EPSCs after seizures, even when polysynaptic input was blocked (242 ± 43 pA, $n = 8$, vs 69 ± 16 pA, $n = 7$; $p = 0.004$) (Fig. 4*b*,*c*). AMPA receptor-mediated EPSCs were not significantly increased (225 ± 55 pA after seizures, $n = 8$, compared

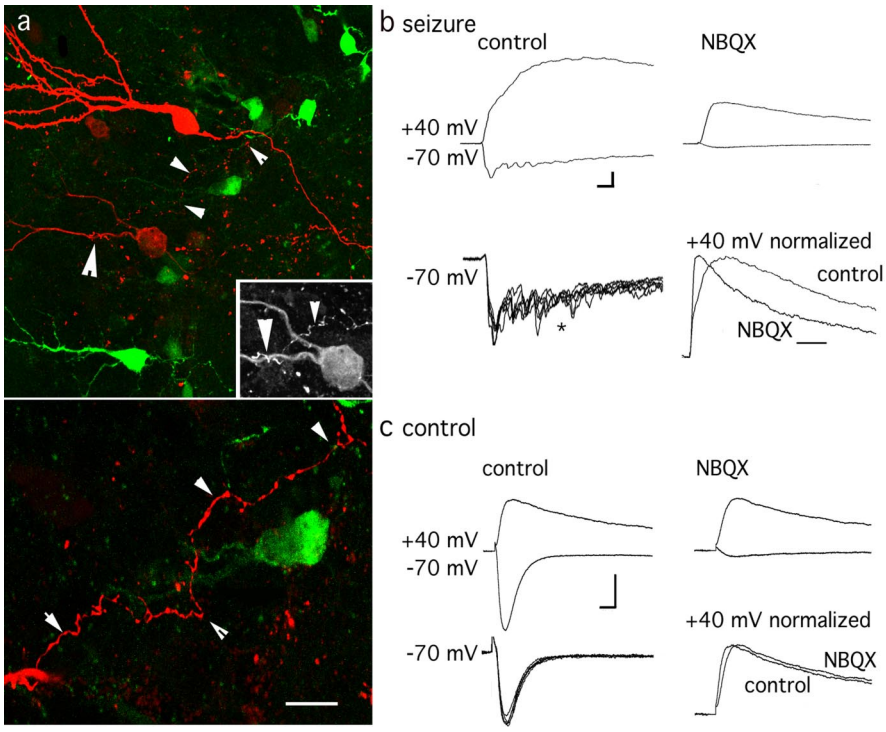

Figure 4. Recurrent polysynaptic input to mature granule cells. ***a***, Morphological evidence for mossy fiber sprouting 5 d after seizures. An axon collateral (small arrowheads) of a biocytin-filled mature granule cell (red, top cell) appears to terminate on the dendritic shaft of a second biocytin-filled mature granule cell (large arrowhead, enlarged in inset). EGFP-labeled newborn granule cells (green) are also shown. The axon collateral is enlarged in the bottom panel. Scale bar, 10 μm. ***b***, After seizures, MPP-evoked EPSCs in mature granule cells had asynchronous events detectable in individual traces (−70 mV, asterisk, bottom traces; calibration: 50 pA, 10 ms) that prolonged the duration of the averaged EPSC (top trace; calibration: 100 pA, 10 ms). When polysynaptic activity was blocked by NBQX, the amplitude and duration of the NMDA receptor-mediated EPSC (+40 mV) was reduced (right traces; calibration: 100 pA, 10 ms), indicating recurrent polysynaptic input contributed to the synaptic response. Normalized traces at +40 mV are also shown. Scale bar, 50 ms. Stimulus artifacts are blanked for clarity. ***c***, In contrast, polysynaptic activity did not contribute to EPSCs in control slices. At −70 mV, individual (bottom) and averaged (top) traces had monotonic decay phases. Blocking polysynaptic activity with NBQX did not alter the NMDA-EPSC at +40 mV (right and normalized traces). Calibration: 100 pA, 10 ms.

with 134 ± 30 pA in control, $n = 7$; $p = 0.17$), suggesting the larger NMDA responses were not attributed entirely to greater numbers of activated inputs.

The NBQX sensitivity of MPP-evoked responses indicates that polysynaptic synapses constitute a major input to newborn granule cells (Fig. 5*a*, histogram). Consistent with this idea, 5 of the 17 newborn granule cells with synaptic input had exclusively recurrent synapses, a situation never seen in mature granule cells. Recurrent synapses had small evoked EPSCs with variable long latencies and were completely blocked by NBQX (Fig. 5*c*). At −70 mV, recurrent EPSCs had small amplitudes (9.5 ± 0.7 pA) with fast rise (20–80% rise time; 0.29 ± 0.03 ms) and decay phases (weighted τ, 1.9 ± 0.1 ms; $n = 5$). The mean EPSC latency was 18 ± 3.7 ms. At +40 mV, recurrent EPSCs were usually too small and prolonged to detect individually, although in one exceptional case, the averaged detected response had characteristics typical of NMDA synaptic responses (Fig. 5*c*).

Polysynaptic activity in newborn granule cells after seizures could be generated by sprouted mossy fibers (Tauck and Nadler, 1985; Scharfman et al., 2003; Tu et al., 2005). To address this possibility, we directly stimulated the mossy fiber pathway in the stratum lucidum (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In three of five newborn granule cells, hilar stimulation produced EPSCs that were similar to those generated by MPP-evoked recurrent input (Fig. 5*c*). In one additional newborn granule cell, hilar stimulation evoked a small short-latency EPSC (data not shown). These results are consistent with the idea that sprouted mossy fiber synapses target newborn, as well as more mature, granule cells.

Granule cell basal dendrites could promote hyperexcitability by providing additional targets for mossy fiber recurrent collaterals (Ribak et al., 2000; Austin and Buckmaster, 2004). The frequency of recurrent basal dendrites increases the first week after seizure induction (Dashtipour et al., 2003), and newborn granule cells have longer recurrent basal dendrites after seizures (Shapiro et al., 2005). Indeed, 6–14 d after seizures, many EGFP-labeled granule cells had prominent basal dendrites that extended into the molecular layer (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). EGFP-labeled cells in control mice had small neurites projecting from the cell body, but prominent basal dendrites like those seen after seizures were not apparent. Basal dendrites were also not observed in biocytin-filled mature granule cells ($n = 5$; data not shown). Thus, seizures seem to trigger a combination of appropriate (monosynaptic MPP input) and inappropriate (recurrent polysynaptic and basal dendrite) synaptogenesis in newborn granule cells.

Accelerated integration persists during recurrent spontaneous seizures

We wondered whether the accelerated functional integration of newborn neurons was a transient phenomenon or whether seizures induced a persistent change that affected subsequent generations of newborn cells. Thus, we examined the dentate gyrus 30–40 d after pilocarpine administration. Because of the transient EGFP expression, at this time point, all EGFP-labeled granule cells represent neurons born after seizure induction (Overstreet et al., 2004). Dendrite outgrowth in most mice was even more dramatic than at 2 weeks ($n = 7$ of 9 mice) (Fig. 6*a*). In the remaining two mice, newborn-labeled neurons appeared similar to controls. Dendrite outgrowth in cells born after seizure was associated with the loss of calretinin-positive mossy cells, an indicator of seizure-induced damage (Nadler, 2003). In contrast, calretinin-positive cells were present in mice that did not display seizure-induced outgrowth (Fig. 6*b*). This suggests dendritic outgrowth correlated with the degree of seizure-induced damage and/or the development of spontaneous seizures. Indeed, affected mice displayed tremors, and behavioral seizures could be observed during routine handling. Interestingly, at these later time points, the number of newborn granule cells was quite variable across sections. Some sections from affected mice had many labeled cells (Fig. 6*a*, right), whereas other sections had few or none (not shown).

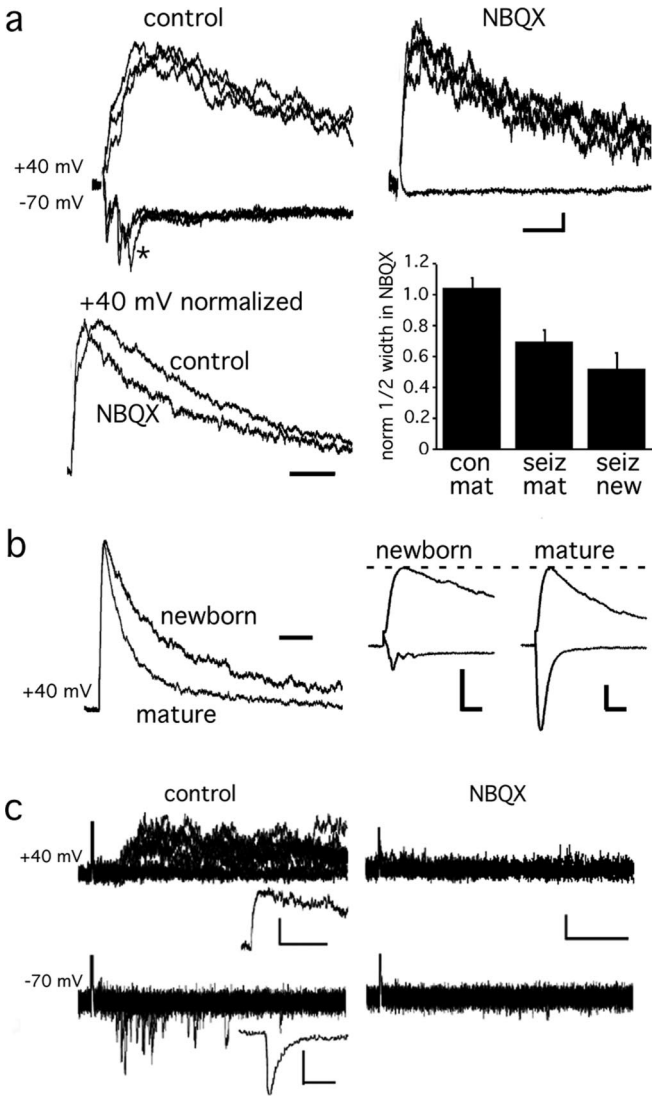

Figure 5. Newborn granule cells receive direct and recurrent EPSCs after seizures. ***a***, MPP-evoked EPSCs in newborn granule cells were recorded at −70 and +40 mV. Calibration: 10 pA, 50 ms. Blocking polysynaptic activity with NBQX reduced the half-width of NMDA receptor-mediated EPSCs (normalized traces; scale bar, 100 ms), indicating polysynaptic activity contributed to the synaptic response. The remaining NMDA receptor-mediated EPSC (right) revealed monosynaptic input from the MPP. Stimulus artifacts are blanked. The histogram illustrates the relative contribution of polysynaptic activity in each condition. NBQX had no effect on EPSCs in mature (mat) granule cells in control (con) slices ($n = 7$), whereas after seizures (seiz), polysynaptic activity contributed to EPSCs in both mature ($n = 11$) and newborn (new; $n = 10$) granule cells. ***b***, Synapses on newborn granule cells had prolonged and large NMDA receptor-mediated components. Examples of monosynaptic NMDA receptor-mediated EPSCs in a newborn and neighboring mature granule cell are shown normalized to the peak currents (left). Scale bar, 100 ms. The ratio of NMDA/AMPA receptor-mediated components was larger in newborn cells (right). Ratios were measured as the peak current at +40 mV (in NBQX)/the peak current at −70 mV. Calibration: 50 pA, 20 ms. ***c***, Exclusively polysynaptic responses were recorded in a subset of newborn neurons after seizures ($n = 5$). These EPSCs had long and variable latencies that were completely blocked by NBQX at both voltages. Calibration: 5 pA, 50 ms. The insets show individual events that were aligned and averaged, with kinetics characteristic of NMDA (+40 mV) and AMPA (−70 mV) receptor-mediated responses. Calibration: top: 10 pA, 100 ms; bottom: 4 pA, 5 ms.

Discussion

Here, we demonstrate that pilocarpine-induced seizures altered the functional integration of adult-generated granule cells. We focused on dendrite outgrowth and synaptogenesis in individual newborn neurons rather than the increased numbers of newborn

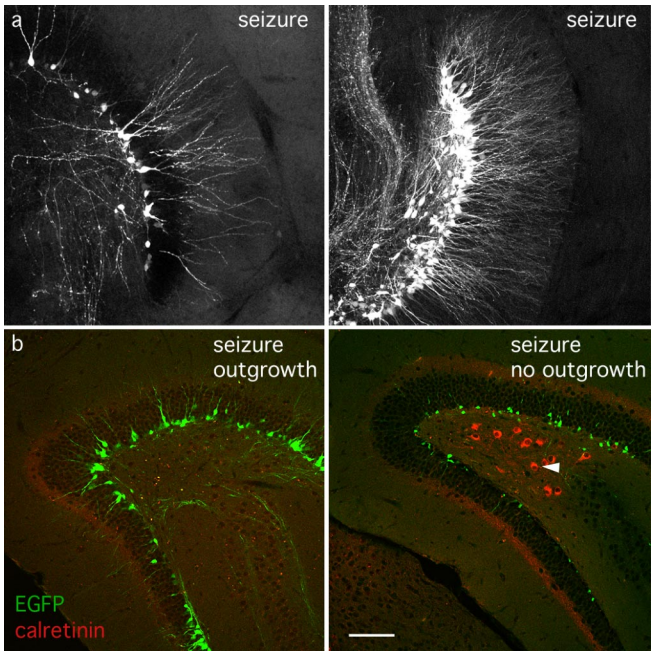

Figure 6. Accelerated development persists for subsequent generations of newborn granule cells. ***a***, Examples of newborn granule cells with elongated dendrites in mice perfused 30–40 d after seizure induction. At this time, all labeled cells were generated after the initial seizure event. Seven of nine mice had obvious dendrite elongation compared with control mice. In affected mice, some sections of the dentate had large numbers of labeled newborn cells (right), whereas other sections had fewer or no newborn cells (left). ***b***, Mice that displayed dendrite outgrowth 30–40 d after seizure induction (left) also had reduced numbers of calretinin-positive hilar interneurons (arrowhead) compared with the two of nine mice with dendrites similar to controls (right).

cells that has been shown previously (Parent et al., 1997). This approach was made possible by POMC-EGFP transgenic mice that label newborn granule cells within their first weeks after cell division (Overstreet et al., 2004). This expression pattern was unaltered by seizure induction, allowing us to compare the effects of seizures on the maturation of newborn granule cells. Dendrite length, input resistance, and synaptic input are all measures of granule cell maturity (for review, see Overstreet-Wadiche and Westbrook, 2006). After seizures, newborn granule cells had elongated spiny dendrites and developed excitatory synaptic input in response to MPP stimulation. Monosynaptic input was likely generated on dendrites that now reached the outer two-thirds of the molecular layer. Newborn granule cells also received polysynaptic input that was likely generated in the granule cell and inner molecular layer. These alterations indicate seizures accelerated the functional integration of adult-generated neurons. It is likely that accelerated maturation is not limited to the pilocarpine model, because dendrite outgrowth is also observed in transgenic mice with spontaneous seizure activity (Overstreet-Wadiche et al., 2006).

Newborn granule cells and epileptogenesis

The hippocampus constitutes a principal site of temporal lobe epileptogenesis. Although seizure-induced alterations have been studied extensively, the mechanisms that translate initial seizure induction into spontaneous recurrent seizures have not been fully established. Experimental models of epilepsy cause structural changes in the hippocampus that are also observed in human temporal lobe epilepsy (Turski et al., 1989), including loss of specific populations of principal cells and interneurons (Houser,

1992; Magloczky and Freund, 2005) and mossy fiber sprouting (Nadler, 2003). Although some aspects of synaptic reorganization are controversial (Buckmaster et al., 2002; Ratzliff et al., 2002; Cossart et al., 2005; Harvey and Sloviter, 2006), there is a consensus across many experimental models that excitability in the dentate gyrus is enhanced during the "latent" or "silent" period (Tauck and Nadler, 1985; Kobayashi and Buckmaster, 2003; Zappone and Sloviter, 2004; Harvey and Sloviter, 2006). Mossy fiber sprouting (Tauck and Nadler, 1985), alterations in GABAergic inhibition (Kobayashi and Buckmaster, 2003; Sayin et al., 2003), and changes in intrinsic excitability (Bernard et al., 2004) could contribute to this hyperexcitability.

There is emerging evidence that newly generated granule cells could also play a role in dentate hyperexcitability. During the silent period, neurogenesis is enhanced, and some newborn granule cells migrate inappropriately to CA3 where they display synchronized bursting activity with surviving pyramidal cells (Scharfman et al., 2000; Scharfman, 2004). Seizure-induced newborn granule cells also retain basal dendrites that may be targets for recurrent mossy fiber synapses (Shapiro and Ribak, 2005; Shapiro et al., 2005). Consistent with a pro-epileptogenic role, suppression of adult neurogenesis during the latent period reduces the frequency and duration of spontaneous recurrent seizures (Jung et al., 2004). Our results indicate that newborn granule cells in the granule cell layer receive perforant-path input earlier than newborn granule cells in normal brains and suggest that they are targets for polysynaptic innervation, likely from mossy fiber recurrent collaterals. The high input resistance, slow membrane time constant (Overstreet et al., 2004; Schmidt-Hieber et al., 2004), and preponderance of the NMDA component of the synaptic response all tend to promote temporal summation of excitatory inputs. Thus, seizure-induced synaptogenesis in newborn granule cells could contribute to overall hyperexcitability.

The effects of seizures on newborn granule cells

The increased number of EGFP-labeled cells 2 weeks after seizure induction is consistent with enhanced proliferation (Parent et al., 1997). The distribution of labeled newborn granule cells throughout the granule cell layer and nearby hilus confirms the seizure-induced dispersion of granule cells (Houser, 1992; Parent et al., 1997). Although we did not observe newborn granule cells clustered at the border between the hilus and CA3, transient EGFP expression and the short observation interval could mask this population (Scharfman, 2004). Indeed, EGFP-labeled cells in the hilus near the granule cell layer (Figs. 1*a*, 6*a*) could reflect the initial stages of ectopic migration. It has been proposed that synapses formed by sprouted mossy fibers stabilize basal dendrites on newborn granule cells (Shapiro and Ribak, 2005). Although we did not specifically record EPSCs from basal dendrites, we did observe newborn granule cells with prominent basal dendrites and recurrent EPSCs that were not present before seizures. Finally, we could identify EGFP-labeled axons of newborn granule cells that targeted the hilar/CA3 region, but these axons did not appear to innervate the granule cell and inner molecular layer. Thus, the axons of adult-generated granule cells at this early developmental stage may not contribute to synaptic reorganization. This is consistent with previous reports that mossy fiber sprouting emanates from pre-existing granule cells (Parent et al., 1999; Jung et al., 2004) (but see Parent et al., 1997).

The development of glutamatergic synaptic input in newborn granule cells was particularly striking because EGFP-labeled newborn cells in control mice completely lacked glutamatergic responses. Although adult-generated granule cells express functional AMPA and NMDA receptors, they first receive exclusively GABAergic synaptic input (Overstreet et al., 2004; Esposito et al., 2005; Ge et al., 2005; Overstreet-Wadiche et al., 2005; Song et al., 2005; Wang et al., 2005; Overstreet-Wadiche and Westbrook, 2006). This presumably occurs because newborn granule cell dendrites are confined initially to the granule cell layer and inner molecular layer, two regions with significant GABAergic innervation but sparse glutamatergic input. Perisomatic inhibition in the granule cell layer is maintained after seizure induction (Magloczky and Freund, 2005), therefore GABAergic synaptic input will likely shape the excitatory responses of newborn neurons.

The morphological and synaptic changes we observed were present within 2 weeks of seizure induction. Because EGFP is transiently expressed, we were unable to determine the long-term fate of the affected cells. However, the changes we observed persisted for a subsequent generation of newborn granule cells. It is possible that pilocarpine-induced status epilepticus produces sufficient damage that all succeeding generations of newborn granule cells would undergo accelerated and inappropriate integration. Alternatively, accelerated integration may be a short-term phenomenon that occurs with each cycle of spontaneous seizure activity. In the long run, accelerated integration of newborn granule cells may be overshadowed by their reduced proliferation and survival after chronic severe seizures (Hattiangady et al., 2004; Mohapel et al., 2004).

Local environment and granule cell maturation

The short-term net effect of seizures was to accelerate the development of newborn granule cells. Accelerated maturation could result directly from enhanced activity in newborn granule cells, or indirectly via seizure-induced alterations in their local environment. Direct depolarization of neural stem cells upregulates neurogenesis by promoting neuronal fate specification (Deisseroth et al., 2004). Depolarizing GABAergic input to neural progenitors (Tozuka et al., 2005; Wang et al., 2005) could thus contribute to seizure-induced enhanced neurogenesis. Depolarizing $GABA_A$ receptor-mediated input to adult-generated granule cells also promotes their maturation and synaptic integration (Ge et al., 2005). Thus, seizure-induced hyperexcitation in the hippocampal network could accelerate granule cell maturation via enhanced GABAergic signaling. In this manner, newborn granule cells may be directly sensitive to network activity despite a lack of perforant-path input (Overstreet-Wadiche et al., 2005) and seizure-induced c-Fos activation (Jessberger and Kempermann, 2003).

Alternatively, changes in the local molecular environment of newborn neurons could contribute to accelerated maturation. Indeed, gene profiling suggests that expression patterns during neonatal development are reiterated in response to seizures (Elliott et al., 2003). Upregulation of neurogenic factors, such as neurotrophins (Ernfors et al., 1991; Isackson et al., 1991), in surrounding mature granule cells could promote the rapid development of newborn granule cells in a non-cell autonomous manner. Interestingly, BDNF mRNA and protein accumulates in proximal granule cell dendrites after acute seizure activity (Tongiorgi et al., 2004). Proximal dendrites of mature granule cells in the inner molecular layer also exhibit increased thickness, length, and spine number in the weeks after seizure induction (Suzuki et al., 1997). Thus, newborn granule cells with dendrite arbors confined to the inner molecular layer may be particularly sensitive to upregulation of trophic factors in proximal dendrites of neighboring mature granule cells. Similarly, BDNF overexpression in-

creases the number of basal dendrites in granule cells (Danzer et al., 2002). The variability in dendrite growth that we observed suggests that the signals involved, whether direct activity or molecular expression patterns, are not uniformly distributed throughout the dentate gyrus.

Pilocarpine-induced status epilepticus generates intense granule cell activity during behavioral seizures and subsequent hyperexcitability that persists for several weeks (Harvey and Sloviter, 2006). This pathological level of activity could trigger TrkB receptor-mediated signaling that is necessary for epileptogenesis in some model systems (He et al., 2004). Physiological levels of activity may promote qualitatively similar but less dramatic alterations in the dentate gyrus, thus directing the functional integration, as well as proliferation, of newborn granule cells.

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