High Ratio of Synaptic Excitation to Synaptic Inhibition in Hilar Ectopic Granule Cells of Pilocarpine-Treated Rats

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Zhan R-Z, Timofeeva O, Nadler JV. High ratio of synaptic excitation to synaptic inhibition in hilar ectopic granule cells of pilocarpine-treated rats. J Neurophysiol 104: 3293–3304, 2010. First published September 29, 2010; doi:10.1152/jn.00663.2010. After experimental status epilepticus, many dentate granule cells born into the postseizure environment migrate aberrantly into the dentate hilus. Hilar ectopic granule cells (HEGCs) have also been found in persons with epilepsy. These cells exhibit a high rate of spontaneous activity, which may enhance seizure propagation. Electron microscopic studies indicated that HEGCs receive more recurrent mossy fiber innervation than normotopic granule cells in the same animals but receive much less inhibitory innervation. This study used hippocampal slices prepared from rats that had experienced pilocarpine-induced status epilepticus to test the hypothesis that an imbalance of synaptic excitation and inhibition contributes to the hyperexcitability of HEGCs. Mossy fiber stimulation evoked a much smaller GABA_A-receptor–mediated inhibitory postsynaptic currents (IPSC) in HEGCs than in normotopic granule cells from either control rats or rats that had experienced status epilepticus. However, recurrent mossy fiber-evoked excitatory postsynaptic currents (EPSCs) of similar size were recorded from HEGCs and normotopic granule cells in status epilepticus–experienced rats. HEGCs exhibited the highest frequency of miniature excitatory postsynaptic currents (mEPSCs) and the lowest frequency of miniature inhibitory postsynaptic currents (mIPSCs) of any granule cell group. On average, both mEPSCs and mIPSCs were of higher amplitude, transferred more charge per event, and exhibited slower kinetics in HEGCs than in granule cells from control rats. Charge transfer per unit time in HEGCs was greater for mEPSCs and much less for mIPSCs than in the normotopic granule cell groups. A high ratio of excitatory to inhibitory synaptic function probably accounts, in part, for the hyperexcitability of HEGCs.

INTRODUCTION

A unique feature of temporal lobe epilepsy is the anatomical reorganization of the dentate gyrus (reviews: Nadler 2003, 2009). One component of this reorganization is the seizure-induced enhancement of granule cell replication. Dentate granule cells are unusual in that they continue to be born and differentiate throughout life. After pilocarpine-induced status epilepticus, most of the newly born granule cells migrate into the dentate cell body layer and differentiate. However, an estimated 21–25% of these cells, accounting for ~1% of the total granule cell population, migrate aberrantly into the dentate hilus (Kron et al. 2010; Walter et al. 2007). Only a few granule cells are located in the hilus normally (Jiao and Nadler 2007; Marti-Subirana et al. 1986; Scharfman et al. 2003). Hilar ectopic granule cells (HEGCs) survive for at least months after status epilepticus (Jessberger et al. 2007; Jiao and Nadler 2007; McCloskey et al. 2006), and some percentage of newborn granule cells continue to migrate aberrantly even after the replication rate normalizes (Boné et al. 2006). Thus the fraction of granule cells that is ectopically located may increase with time after the initial insult. Granule cell neurogenesis may be enhanced in humans with temporal lobe epilepsy as well. Some findings support the hypothesis that seizures induce neurogenesis in young patients (Siebzehrubl and Blümcke 2008), and HEGCs have been found in tissue resected from persons with epilepsy (Houser et al. 1992; Parent et al. 2006). The increased frequency and duration of spontaneous seizures with time after status epilepticus in rats with neuronal death and mossy fiber sprouting (the “progression of seizures”) has been linked to enhanced granule cell neurogenesis (Jung et al. 2004, 2006). Seizure-related neurogenesis also seems to disrupt hippocampus-dependent learning (Jessberger et al. 2007a; Pekceıı et al. 2008). It is uncertain whether these adverse outcomes relate to postseizure-generated granule cells that migrate normally, aberrantly, or both. Many HEGCs burst spontaneously (Scharfman et al. 2000; Zhan and Nadler 2009), and they are active during experimental limbic seizures (Scharfman et al. 2002). In addition, the nucleus of HEGCs is indented, unlike that of normal granule cells, consistent with a high rate of activity (Dashtipour et al. 2001). These findings suggest that HEGCs contribute to circuit hyperexcitability. HEGCs may thus be important for seizure propagation through the dentate gyrus.

Electron microscopic studies suggest that one reason for the hyperexcitability of HEGCs may be a relative excess of excitatory innervation. The somata and proximal apical dendrites of these cells are contacted by numerous boutons having the typical ultrastructure of mossy fiber boutons, symmetric terminals of dentate granule cell axons, and some have been positively identified as such by retrograde labeling with biocytin or by ZnT3 immunocytochemistry (Dashtipour et al. 2001; Pierce et al. 2005). HEGCs are more densely innervated by other granule cells than normotopic granule cells in the same animals (Pierce et al. 2005). Furthermore, the somata and proximal dendrites of HEGCs seem practically devoid of inhibitory innervation, as evidenced by the apparent lack of symmetric synapses (Dashtipour et al. 2001). This finding is consistent with the location of HEGCs at a considerable distance from most dentate basket cells, which innervate the somata and proximal dendrites of normotopic granule cells.

If HEGCs receive a high ratio of excitatory to inhibitory innervation relative to normotopic granule cells, this difference should be reflected in a similarly high ratio of evoked and
miniature excitatory to inhibitory synaptic currents. This study used whole cell patch-clamp recording to show such a difference.

METHODS

Pilocarpine-induced status epilepticus

Male Sprague-Dawley rats (150–200 g; Zivic Laboratories, Pittsburgh, PA) received a single injection of pilocarpine hydrochloride (340–380 mg/kg, ip) 30 min after pretreatment with scopolamine methyl bromide and terbutaline hemisulfate (both 2 mg/kg, ip). Status epilepticus, defined as a continuous limbic motor seizure of stage 2 or higher (Racine 1972), was allowed to self-terminate after 6–8 h. Rats treated in this way develop extensive and consistent hilar lesions followed by consistently robust mossy fiber sprouting, the accumulation of HEGCs, and spontaneous seizures (Jiao and Nadler 2007; Sloviter et al. 2003). Some rats pretreated with methylscopolamine and terbutaline and then injected with pilocarpine exhibited only a few brief behavioral seizures but not status epilepticus. They were used as controls to account for any possible action of pilocarpine not mediated by status epilepticus. Histological tests showed no evidence of neuronal degeneration or mossy fiber sprouting in these animals (Okazaki et al. 1999), and their electrophysiological responses were not significantly different from those of age-matched untreated rats (Hardison et al. 2000; Molnár and Nadler 1999; Okazaki and Nadler 2001; Okazaki et al. 1999). All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved in advance by the Duke University Institutional Animal Care and Use Committee.

Hippocampal slice preparation

Hippocampal slices were prepared 10–40 wk after pilocarpine administration. Control rats were studied 97 ± 66 days and rats that had developed status epilepticus 93 ± 67 (SD) days after pilocarpine administration. Animals were decapitated under deep ether anesthesia, and the brain was removed to ice-cold high-Mg2+ ACSF; in mM): 112 NaCl, 25 NaHCO3, 3.1 KCl, 1.8 CaCl2, 11.2 MgSO4, 0.4 KH2PO4, 1 ascorbic acid, and 10 α-glucose, equilibrated with 95% O2–5% CO2. Transverse 420-μm-thick slices of the caudal hippocampus were prepared with a vibratome, incubated at 34°C in high-Mg2+ ACSF for 30 min, and maintained in standard ACSF (122 mM NaCl, 1.2 mM MgSO4) at room temperature (22–24°C).

Electrophysiology

A slice was submerged in a recording chamber mounted on the stage of a Nikon Eclipse E600FN microscope equipped with far infrared-differential interference contrast optics, a CCD camera, and a 40× water-immersion objective. The slice was superfused with standard ACSF at room temperature (22–24°C) and a rate of 3 ml/min. Criteria for selecting HEGCs were 1) soma located within the hilus and of a size and shape indistinguishable from granule cells in the cell body layer and 2) no more than three dendrites emerged from the soma. In rats studied after status epilepticus, numerous cells scattered throughout the dentate hilus met these criteria, comprising an average of 62% of the total hilar neuron population (Jiao and Nadler 2007). Recorded HEGCs were located in all parts of the hilus except for the deep region adjacent to the end of area CA3. Cell identity was confirmed by intracellular dialysis with biocytin and subsequent visualization of cellular morphology (Zhan and Nadler 2009). Results obtained from putative HEGCs were included in this study only if cellular morphology appeared identical to previous descriptions of HEGCs (Dashitipour et al. 2001; Scharfman et al. 2000, 2003): small (8–12 μm diam) soma located within the dentate hilus, one to two apical dendrite(s) penetrating into or directed toward the dentate molecular layer, and axon with giant boutons in area CA3 and extensive branches within the hilus. Examples of HEGCs recorded in this study are shown in Fig. 1. More than 90% of the recorded hilar neurons were confirmed to be HEGCs; data obtained from hilar neurons not confirmed to be HEGCs were discarded. The normotopic dentate granule cells selected for recording were located in the granule cell body layer at the apex of the granule cell arch or in the supragranular blade close to the apex.

Borosilicate patch electrodes used for whole cell recording had tip resistances of 4.5–6.5 MΩ. Recordings were made with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Series resistances (<20 MΩ) were compensated 75%. Recordings were rejected if the series resistance varied by >20%. Voltage-clamp recordings were filtered at 2 kHz, digitized at 20 kHz, and stored for analysis off-line with pClamp8.1 (Molecular Devices) or MiniAnalysis (Synaptosoft, Decatur, GA) software.

To record compound synaptic currents evoked by mossy fiber stimulation, the recording electrode was filled with an internal solution that contained (in mM) 120 cesium gluconate, 10 HEPES, 2 MgATP, 1 EGTA, 5 creatine phosphate, 20 U/ml creatine phosphokinase, 10 QX-314 (N-ethyl lidocaine) and 1% (wt/vol) biocytin, pH 7.25–7.30 and 293–297 mosm. The liquid junction potential was determined to be 10 mV with use of the method described by Neher (1992), and this value was subtracted from all membrane potentials.

FIG. 1. Morphology of representative hilar ectopic granule cells (HEGCs). Cells were filled with biocytin during whole cell patch-clamp recording, fixed slices were cut into serial 60-μm-thick sections, biocytin was visualized with nickel-intensified avidin-horseradish peroxidase-diaminobenzidine (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA), and cell morphology was reconstructed with use of NeuroLucida (MicroBrightField, Williston, VT). Criteria for confirmation of HEGC identity included hilar location of the soma, apical dendrite(s) penetrating into or directed toward the dentate molecular layer, and axon with giant boutons in area CA3 and extensive branches within the hilus. A: the apical dendrite (AD) of this HEGC crosses the granule cell body layer (G) and penetrates the molecular layer. The mossy fiber (MF) branches within the hilus. This cell also has a basal dendrite (BD) directed into the hilus. Scale bar, 500 μm. B: HEGC mossy fiber courses through stratum lucidum of area CA3 adjacent to the pyramidal cell body layer (P). Arrow indicates a giant bouton from which a filopodium originates. Scale bar, 200 μm. C: reconstructed morphology of an HEGC from which miniature inhibitory postsynaptic currents (mIPSCs) were recorded. D: reconstructed morphology of an HEGC from which miniature excitatory postsynaptic currents (mEPSCs) were recorded. The apical dendrites of both cells reached the outer edge of the dentate molecular layer (ML) and the cell in D had a short basal dendrite directed into the hilus (H). The main branch of the mossy fiber reached stratum lucidum of area CA3.
The monopolar stimulating electrode was a 25-μm-diam nichrome wire insulated to the tip with a polymerized polyvinyl resin. It was placed in stratum lucidum at the junction of areas CA3b and CA3c. Rectangular electrical stimuli of 100-μs duration were delivered every 30 s at an intensity (500–800 μA) that evoked an antidromic population spike of just-maximal amplitude in a portion of the granule cell body layer close to where whole cell patch-clamp recordings were made. Under each experimental condition, 10 stimuli were delivered, and the responses were averaged. First, the mossy fiber–evoked GABA<sub>A</sub> receptor–mediated inhibitory postsynaptic current (IPSC) was recorded at a holding potential of 0 mV. The IPSC was defined as the outward current obtained by subtracting electronically the averaged response recorded in the presence of 30 μM bicuculline from the averaged response recorded before addition of bicuculline to the superfusion medium. Activation of postsynaptic GABA<sub>A</sub> receptors was prevented by the use of a cesium-based internal solution that contained QX-314 but not GTP. Then the holding potential was changed to −30 mV. The N-methyl-D-aspartate (NMDA) receptor–mediated excitatory postsynaptic current (EPSC) was defined as the inward current obtained by subtracting electronically the averaged response recorded in the presence of 50 μM d-2-aminooxy-5-phosphonopentanoate (d-AP5) and 30 μM bicuculline from the averaged response recorded in the presence of bicuculline alone. Finally, the holding potential was changed to −80 mV. The AMPA/kainate receptor–mediated EPSC was defined as the inward current obtained by subtracting electronically the averaged response recorded in the presence of 10 μM 2,3-dihydroxy-6-nitro-7-sulfamyl-benzo(F)quinoxaline-2,3-dione (NBQX), 50 μM d-AP5, and 30 μM bicuculline from the averaged response recorded in the presence of d-AP5 and bicuculline alone.

To record miniature synaptic currents, the recording electrode was filled with an internal solution that contained (in mM) 126 CsCl, 10 HEPES, 5 EGTA, 2 MgATP, 1 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 5 creatine phosphate, 20 U/ml creatine phosphokinase, and 1% (wt/vol) biocytin, pH 7.25–7.30 and 293–297 mosm. Miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 30 μM bicuculline and 1 μM TTX, whereas miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of 10 μM NBQX, 50 μM d-AP5, and 1 μM TTX. Miniature events were recorded for 2.5 min at a holding potential of −70 mV. The amplitude threshold for detecting these events was 5 pA, the amplitude of the first event from all recorded granule cells in each group were combined, and between-group differences were analyzed for statistical significance by the Kolmogorov-Smirnov test. Between-group differences in event frequency were analyzed by one-way ANOVA followed by the Newman-Keuls post hoc test. Grouped data are expressed as mean ± SE unless otherwise indicated.

**Materials**

TTX, d-AP5, and NBQX were obtained from Tocris Bioscience (Ellisville, MO). Bicuculline methiodide, MgATP, creatine phosphate, creatine phosphokinase, d-gluconic acid lactone, cesium hydroxide (99.9%; 50% by weight), CsCl, HEPES, EGTA, biocytin, pilocarpine hydrochloride, (−)-scopolamine methyl bromide, and terbutaline hemisulfate were purchased from Sigma (St. Louis, MO). QX-314 was obtained from Alomone Laboratories (Jerusalem, Israel).

**RESULTS**

**Membrane properties of HEGCs and normotopic dentate granule cells**

Resting membrane potential was assessed on break-in, and input resistance and membrane capacitance were determined after intracellular dialysis with the CsCl-based internal solution. In agreement with our previous report (Zhan and Nadler 2009), HEGCs had a significantly less polarized resting membrane potential than normotopic granule cells [dentate granule cells from control rats (CGCs): −76 ± 0 mV; n = 15; normotopic granule cells from rats subjected to pilocarpine-induced status epilepticus (GC-SEs): −74 ± 1 mV; n = 16; HEGCs: −69 ± 1 mV; n = 17; P < 0.001 compared with CGCs and P < 0.005 compared with GC-SEs by Newman-Keuls test after 1-way ANOVA yielded P < 0.001]. There was no significant between-group difference in input resistance (CGC: 190 ± 20 MΩ; GC-SE: 180 ± 20 MΩ; HEGC: 200 ± 20 MΩ) or membrane capacitance (CGC: 21 ± 2 pF; GC-SE: 23 ± 3 pF; HEGC: 27 ± 3 pF).

**Mossy fiber stimulation evokes small IPSCs, but normal-size recurrent EPSCs, in HEGCs**

Stimulation of the mossy fibers at the CA3b–CA3c border evoked a GABA<sub>A</sub> receptor–mediated compound IPSC in HEGCs, GC-SEs, and CGCs (Fig. 2). Both the peak amplitude of and charge transferred by mossy fiber–evoked IPSCs were much smaller in HEGCs than in either normotopic granule cell population. In HEGCs, the average size of the mossy fiber–evoked IPSC, measured by the charge transferred, was an order of magnitude smaller than the mossy fiber-evoked IPSC in CGCs (HEGC: 1265 ± 355 fC, GC-SE: 11,276 ± 2863 fC; P < 0.001 by Newman-Keuls test). The largest mossy fiber–evoked IPSCs were recorded from GC-SEs. The average charge transferred was 45% greater than in CGCs (GC-SE: 11,276 ± 2863 fC, CGC: 6143 ± 600 fC; P < 0.05 by Newman-Keuls test). Because peak IPSC amplitudes did not differ significantly (P > 0.4 by Newman-Keuls test), the greater charge transferred to GC-SEs was accounted for mainly by a more prolonged response (cf. left and middle of Fig. 2, top).

Mossy fiber stimulation evoked an AMPA/kainate receptor–mediated EPSC only in granule cells from rats that had experienced status epilepticus (Fig. 2). This finding can probably be explained by the sprouting of mossy fibers after status epilepticus and the subsequent formation of functional synapses with other granule cells (Nadler 2003, 2009). Mossy fiber stimulation evoked AMPA/kainate receptor–mediated EPSCs of similar size in HEGCs (mean: 1489 fC, range: 164–5127 fC) and GC-SEs (mean: 2184 fC, range: 266–9127 fC). There was also no significant between-group difference in the ratio between the sizes of NMDA receptor– and AMPA/kainate receptor–mediated components of the evoked EPSC.

Because AMPA/kainate receptor–mediated EPSCs were about the same size in HEGCs and GC-SEs, whereas the GABA<sub>A</sub> receptor–mediated IPSC was much smaller in HEGCs, the ratio between the two responses was an order of magnitude greater in HEGCs (Fig. 2; P = 0.01 by Student’s t-test).

Although mossy fiber stimulation did not evoke an AMPA/kainate receptor–mediated EPSC in granule cells from control...
rats, it did evoke a small NMDA receptor–mediated response (Fig. 2, top left). Most likely, glutamate released at mossy fiber synapses on nearby interneurons overflowed those synapses and activated extrasynaptic NMDA receptors on the recorded cell.

Electrical stimulation in stratum lucidum at the Ca3b–CA3c border could possibly activate projections to dentate granule cells other than recurrent mossy fibers, especially when using a large stimulus current. We tested the specificity of our stimuli by varying the position of the stimulating electrode. Moving the stimulating electrode perpendicular to the pyramidal cell body layer by as little as 50 μm abolished both the antidromic response and any evoked compound EPSC. This observation suggests that the electrical stimuli activated predominantly mossy fibers. They probably also activated some CA3 pyramidal cells located close to the electrode. The firing of CA3 pyramidal cells can evoke an excitatory synaptic response in dentate granule cells through a disynaptic pathway that involves hilar mossy cells (Scharfman 1994). If this pathway contributed significantly to compound EPSCs in this study, a compound EPSC should have been recorded in CGCs. No such response was observed in those cells. Furthermore, little synaptic excitation could have been relayed through hilar mossy cells from rats that had experienced pilocarpine-induced status epilepticus, because status epilepticus induced by the method used in this study consistently kills >90% of mossy cells (Jiao and Nadler 2007). It is possible that status epilepticus causes CA3 pyramidal cells to form monosynaptic feedback connections with dentate granule cells. If so, synapses made by axons of CA3 pyramidal cells could have accounted for some portion of the excitatory synaptic response evoked by stimulating in stratum lucidum. However, there is currently no evidence to support the formation of these synapses.

Spontaneous EPSCs were recorded from dentate granule cells with a CsCl-based internal solution in the presence of bicuculline and TTX at a holding potential of −70 mV (Fig. 3). mEPSCs were recorded more frequently from HEGCs than from CGCs and only one third as great compared with GC-SEs. The interval between these events was less than one half as great compared with GC-SEs or CGCs; mEPSC amplitude is generally greater in HEGCs and GC-SEs than in CGCs. The traces shown are from representative experiments. Recordings were made with a CsCl-based internal solution, and the membrane potential was clamped at −70 mV. Individual events that met our criterion are indicated by asterisks.
with CGCs (Fig. 4; Table 1; \(P < 0.001\) by Kolmogorov-Smirnov test in each case). Otherwise, mEPSCs recorded from HEGCs resembled those recorded from GC-SEs. Compared with CGCs, a substantially greater average charge was transferred by each event (\(P < 0.001\) by Kolmogorov-Smirnov test for both GC-SEs and HEGCs). In both HEGCs and GC-SEs, the mean peak amplitude was greater than control, and on average, the events decayed more slowly (\(P < 0.001\) by Kolmogorov-Smirnov test in each instance). The mean rise time was longer than control only in HEGCs, however (\(P < 0.001\) by Kolmogorov-Smirnov test). Thus mEPSCs were recorded most frequently from HEGCs, and in those cells, they were relatively large and exhibited relatively slow kinetics.

**TABLE 1. Properties of mEPSCs in dentate granule cells after pilocarpine-induced status epilepticus**

<table>
<thead>
<tr>
<th>Property</th>
<th>CGC</th>
<th>GC-SE</th>
<th>HEGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency, Hz</td>
<td>0.16 ± 0.02</td>
<td>0.22 ± 0.03</td>
<td>0.38 ± 0.08*†</td>
</tr>
<tr>
<td>Interevent interval, s</td>
<td>6.0 ± 0.4</td>
<td>4.6 ± 0.3</td>
<td>2.0 ± 0.1‡§</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>8.48 ± 0.18</td>
<td>9.86 ± 0.24‡</td>
<td>10.38 ± 0.35‡</td>
</tr>
<tr>
<td>10–90% rise time, ms</td>
<td>1.89 ± 0.07</td>
<td>2.19 ± 0.08</td>
<td>2.52 ± 0.08‡</td>
</tr>
<tr>
<td>(\tau), ms</td>
<td>2.22 ± 0.09</td>
<td>3.51 ± 0.17‡</td>
<td>4.15 ± 0.16‡</td>
</tr>
<tr>
<td>Charge transfer, FC</td>
<td>22.83 ± 0.93</td>
<td>38.73 ± 2.23‡</td>
<td>57.74 ± 3.42‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. mEPSC frequency was determined for 9 (CGC) or 10 (GC-SE and HEGC) cells per group. Other parameters were determined for 268 (CGC), 397 (GC-SE), or 785 (HEGC) events recorded from the indicated number of cells. mEPSCs were recorded in the presence of 30 \(\mu M\) bicuculline and 1 \(\mu M\) TTX for 2.5 min at 22–24°C. Recordings were made with a CsCl-based internal solution at a holding potential of −70 mV. *Significantly different from CGC at \(P < 0.05\) (Newman-Keuls test after 1-way ANOVA yielded \(P < 0.025\)). †Significantly different from GC-SE at \(P < 0.05\) (Newman-Keuls test). ‡Significantly different from CGC at \(P < 0.001\) (Kolmogorov-Smirnov test). §Significantly different from GC-SE at \(P < 0.001\) (Kolmogorov-Smirnov test). mEPSC, miniature excitatory postsynaptic current; CGC, normotopic granule cells from control rats; GC-SE, normotopic granule cells from rats subjected to pilocarpine-induced status epilepticus; HEGC, hilar ectopic granule cells.

Between-group comparisons of mean interevent interval and charge transfer per event showed an inverse relationship between these properties (Table 1). The more frequently mEPSCs were recorded from these granule cell populations, the larger they were. Thus the total charge transferred per unit time in HEGCs was on average 2.6 times as great as in GC-SEs and 6.1 times as great as in CGCs.

To determine whether there was a selective increase in fast- or slow-rising mEPSCs in HEGCs, we constructed histograms that included the events recorded from every cell (Fig. 5, top). There was no between-group difference in the proportion of mEPSCs with 10–90% rise times <1 ms (CGC: 18.3%; GC-SE: 17.6%; HEGC: 17.7%). However, 16.4% of the mEPSCs recorded from HEGCs had 10–90% rise times >4 ms compared with 8.6% in GC-SEs and 4.5% in CGCs. These differences were statistically significant (\(P < 0.001\) by \(\chi^2\) test comparing HEGCs to each of the other granule cell groups).

**mIPSC frequency in HEGCs is very low**

Spontaneous mIPSCs were recorded from dentate granule cells with a CsCl-based internal solution in the presence of NBQX, \(\alpha\)-AP5, and TTX at a holding potential of −70 mV (Fig. 6). mIPSCs were recorded from GC-SEs and CGCs about an order of magnitude more frequently than from HEGCs (Fig. 7; Table 2). The interval between these events was about four times as great in HEGCs compared with CGCs and about three times as great compared with GC-SEs (\(P < 0.001\) by Kolmogorov-Smirnov test in each case). Recordings made at 34–35°C produced similar differences in interevent intervals (HEGC: 1.51 ± 0.09 s, GC-SE: 0.93 ± 0.07 s, CGC: 0.56 ± 0.01 s, \(n = 496, 716, 496, 716, 716, 1041\), and 2418 mIPSCs recorded from 5–9 cells, respectively; \(P < 0.001\) for all between-group comparisons by Kolmogorov-Smirnov test). However, each mIPSC recorded from HEGCs transferred on average 58% more charge than the average mIPSC in CGCs (Table 2; \(P < 0.001\) by Kolmogorov-Smirnov test). The mean charge transferred by mIPSCs recorded from GC-SEs was inter-

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**FIG. 4.** Cumulative probability plots show lower interevent intervals (higher frequency) of mEPSCs in HEGCs and differences from control in mEPSC amplitude, rise time, decay time constant, and charge transfer per event. Results were computed from the number of events given in Table 1, which also presents the grouped data. Averaged mEPSCs recorded from a representative CGC, GC-SE, and HEGC are shown at the top left.
mediate between mean values from the other groups, significantly
larger \( (P < 0.005 \text{ by Kolmogorov-Smirnov test}) \) than in CGCs
but significantly smaller \( (P < 0.001 \text{ by Kolmogorov-Smirnov}
\) test) than in HEGCs. These differences can be explained, in part,
by a small (10\%–11\%), but statistically significant \( (P < 0.001 \text{ by}
\) Kolmogorov-Smirnov test), increase in the mean peak amplitude
of mIPSCs recorded from HEGCs and GC-SEs compared with
those recorded from CGCs. mIPSCs recorded from HEGCs, but
not those recorded from GC-SEs, decayed significantly more
slowly on average than those recorded from CGCs \( (P < 0.001 \text{ by}
\) Kolmogorov-Smirnov test). This finding largely explains the
greater charge transfer per event in HEGCs than in GC-SEs.
Mean rise times were significantly longer in GC-SEs than in CGCs
and longer still in HEGCs \( (P < 0.001 \text{ by Kolmogorov-Smirnov test}
\) for all between-group comparisons). Thus mIPSCs were recorded
from HEGCs with low frequency, but in those cells, these events
were relatively large and exhibited relatively slow kinetics.

Comparisons of mean interevent interval and charge transfer
per event showed a direct relationship between these properties
(Table 2). The less frequently mIPSCs were recorded from
these granule cell populations, the larger they were. Thus the
relatively large size of individual mIPSCs in HEGCs compensated
to some degree for their infrequency. Nevertheless, the
total charge transferred per unit time was still only 37\%–38\% as
great as in GC-SEs or CGCs.

Histograms of mIPSC rise times showed between-group
differences in the proportions of both rapidly rising and slowly
rising events (Fig. 5, bottom). The proportion of mIPSCs having a
10\%–90\% rise time \(<1 \text{ ms}\) was much smaller in
granule cells from survivors of status epilepticus (CGC: 31.8\%;
GC-SE: 15.4\%; HEGC: 13.5\%; \( P < 0.001 \text{ for both}
\) GC-SEs and HEGCs compared with CGCs by \( \chi^2 \) test). How-
ever, only HEGCs had an unusually large proportion of
mIPSCs with \( 10 \%–90\% \) rise times \( >4 \text{ ms}\) (CGC: 6.3\%;
GC-SE: 7.8\%; HEGC: 13.0\%; \( P < 0.001 \text{ compared with CGCs}
and \( P = 0.005 \text{ compared with GC-SEs by } \chi^2 \text{ test})\).

Comparison of mEPSCs and mIPSCs shows a much higher
ratio of synaptic excitation to synaptic inhibition in HEGCs
than in normotopic granule cells

The frequency and charge transfer/s of mEPSCs or mIPSCs
were calculated for each granule cell from which recordings
were made and the group ratios were compared (Fig. 8).
HEGCs exhibited far higher mEPSC/mIPSC ratios by either
measure. Compared with granule cells from control rats, the
frequency ratio was 10.3 times as great and the charge trans-
fer/s ratio was 16.1 times as great. Compared with GC-SEs, the
frequency ratio was 5.9 times as great and the charge transfer/s
ratio was 6.8 times as great.
Our results show a marked difference between HEGCs and normotopic dentate granule cells in the balance between afferent synaptic excitation and synaptic inhibition. In HEGCs, this balance strongly favors synaptic excitation, whether assessed by mossy fiber–evoked compound synaptic responses or by miniature synaptic events. mEPSCs are larger and more frequently recorded than in CGCs. Although mIPSCs are also larger than control, they are recorded much less frequently. The resulting >60% reduction in mIPSC charge transfer per unit time correlates with a relatively small mossy fiber–evoked compound IPSC. Thus both enhanced synaptic excitation and reduced synaptic inhibition contribute to the high synaptic compound IPSC. Thus both enhanced synaptic excitation and synaptic inhibition. In HEGCs, this.

**DISCUSSION**

HEGCs most differences from control in the properties of miniature synaptic events. However, these differences are generally more pronounced in HEGCs. In addition, the frequencies of mEPSCs and mIPSCs recorded from GC-SEs differ only subtly or insignificantly from control compared with the several-fold differences observed in HEGCs. Finally, mossy fiber stimulation evokes a significantly larger compound IPSC in GC-SEs than in CGCs. These observations may be explained at least partly by seizure-related loss of innervation followed by axon sprouting, changes in the expression and composition of postsynaptic receptors, the unique anatomical location of HEGCs, and the different ages of granule cells in the three experimental groups. Model-dependent variables, including the time between slice preparation and the last spontaneous seizure and the extent of seizure-related brain damage and axon sprouting, may also influence some of our outcome measures. We have not assessed these variables, however.

**Excitatory synaptic responses in HEGCs**

Pilocarpine-induced status epilepticus destroys the great majority of hilar mossy cells (Buckmaster and Jongen-Rêlo 1999; Jiao and Nadler 2007; Sloviter et al. 2003), an average of 95% when performed by the method used in this study (Jiao and Nadler 2007). The associational–commissural axons of mossy cells provide excitatory innervation to the proximal third of the granule cell apical dendrite (Ribak et al. 1985). When these connections degenerate or fail to form because of the seizure-induced death of mossy cells, granule cell mossy fibers sprout collaterals that establish synapses on the denervated (GC-SEs) or noninnervated (HEGCs) dendritic region of other granule cells, thus replacing the synapses that had been lost. Both GC-SEs and HEGCs are synaptic targets of recurrent mossy fibers. In addition, HEGCs and those GC-SEs that were born either after or within 5 wk before status epilepticus have been identified as the source of those fibers (Kron et al. 2010). Both granule cell types receive mossy fiber synapses on their.

**TABLE 2. Properties of mIPSCs in dentate granule cells after pilocarpine-induced status epilepticus**

<table>
<thead>
<tr>
<th></th>
<th>CGC</th>
<th>GC-SE</th>
<th>HEGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency, Hz</td>
<td>1.44 ± 0.21</td>
<td>1.19 ± 0.25</td>
<td>0.34 ± 0.06†</td>
</tr>
<tr>
<td>Interevent interval, s</td>
<td>0.69 ± 0.02</td>
<td>0.91 ± 0.04‡</td>
<td>2.69 ± 0.22§</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>27.61 ± 0.55</td>
<td>30.24 ± 0.85‡</td>
<td>30.78 ± 1.40‡</td>
</tr>
<tr>
<td>10–90% rise time, ms</td>
<td>1.71 ± 0.03</td>
<td>2.05 ± 0.04‡</td>
<td>2.39 ± 0.07§</td>
</tr>
<tr>
<td>τ, ms</td>
<td>8.66 ± 0.16</td>
<td>8.44 ± 0.17</td>
<td>10.80 ± 0.46§</td>
</tr>
<tr>
<td>Charge transfer, fC</td>
<td>226 ± 7</td>
<td>271 ± 10†</td>
<td>358 ± 25§</td>
</tr>
</tbody>
</table>

Values are means ± SE. mIPSC frequency was determined for 6 (CGC and GC-SE) or 7 (HEGC) cells per group. Other parameters were determined for 1,293 (CGC), 1,073 (GC-SE), or 362 (HEGC) events recorded from the indicated number of cells. mIPSCs were recorded in the presence of 10 μM NBQX, 50 μM D-AP5, and 1 μM TTX for 2.5 min at 22–24°C. Recordings were made with a CsCl-based internal solution at a holding potential of −70 mV. *Significantly different from CGC at P < 0.01 (Newman-Keuls test after 1-way ANOVA yielded P < 0.005). †Significantly different from CGC at P < 0.01 (Newman-Keuls test). ‡Significantly different from CGC at P < 0.001 (Kolmogorov-Smirnov test). §Significantly different from GC-SE at P < 0.001 (Kolmogorov-Smirnov test). ¶Significantly different from CGC at P < 0.005 (Kolmogorov-Smirnov test). mIPSC, miniature inhibitory postsynaptic current. See Table 1 for other abbreviations.
FIG. 8. Analysis of miniature synaptic events shows an excess of excitation over inhibition in HEGCs compared with the normotopic granule cell groups. mEPSCs and mIPSCs were recorded from different cells. The numbers of cells from which each were recorded are given in Tables 1 and 2. Event frequency and charge transfer/s were computed for each granule cell studied, and the results obtained from all cells in each group were averaged. The mean mEPSC frequency or charge transfer/s was divided by the mean mIPSC frequency or charge transfer/s. The mEPSC/mIPSC ratio for both measures was much greater in HEGCs.

mEPSCs were recorded about twice as frequently from HEGCs as from either normotopic or GC-SEs (Pierce et al. 2005). Mossy fibers also contact the soma of HEGCs (Dashtipour et al. 2001). Moreover, granule cell basal dendrites are innervated predominantly by excitatory afferent projections (Thind et al. 2008), at least some of which are mossy fibers (Ribak et al. 2000). Granule cells normally have a basal dendrite during their early development, but it either retracts or evolves into an apical dendrite as the cell migrates into the cell body layer and differentiates (Shapiro and Ribak 2005). HEGCs and those GC-SEs that entered their final division after or within 5 wk before status epilepticus often retain a bilaral dendrite (Jessberger et al. 2007b; Kron et al. 2010; Shapiro and Ribak 2005; Walter et al. 2007). Thus HEGCs may be expected to receive more excitatory innervation, on average, than normotopic granule cells and, in particular, more innervation from recurrent mossy fibers.

mEPSCs recorded from HEGCs because their mean peak amplitude was larger, causing a smaller percentage of mEPSCs to be undetected because they were obscured by electrical noise. However, the mean peak amplitude of mEPSCs recorded from GC-SEs was also greater than control and not significantly different from that of mEPSCs recorded from HEGCs. However, mEPSC frequency was significantly less than in HEGCs and not significantly different from control. Thus between-group differences in peak mEPSC amplitude did not correlate with differences in mEPSC frequency. Although further studies are needed, the relatively high frequency of mEPSCs recorded from HEGCs seems to be explained best by a greater density of excitatory synapses compared with normotopic granule cell populations, that is, more excitatory synapses per unit dendritic length. The greater excitatory synaptic density may be related not only to HEGCs having a greater number of recurrent mossy fiber synapses than GC-SEs, but possibly also to their receiving innervation from neurons that do not project to normotopic granule cells. Again, further studies are needed to assess this possibility.

The establishment of mossy fiber synapses with HEGCs and GC-SEs most likely explains the larger average size of mEPSCs in these groups. Mean peak amplitude, decay time constant, and charge transfer per event were significantly greater than in CGCs, with no significant difference between them. Unitary postsynaptic responses at recurrent mossy fiber synapses can be quite large relative to unitary responses produced by activation of other excitatory pathways (Molnár and Nadler 1999; Simmons et al. 1997). In part, these findings can be explained by the proximity of these synapses to the soma and by the simultaneous or overlapping release of glutamate from the multiple active zones of each synaptic bouton. In addition, recurrent mossy fiber synapses differ from the perforant path and associational–commissural synapses normally present on dentate granule cells in that released glutamate activates postsynaptic kainate and AMPA receptors (Epsztein et al. 2005). In GC-SEs, AMPA and kainate receptors mediate synaptic transmission at largely separate populations of recurrent mossy fiber synapses. Each population contributes about one half the total recurrent mossy fiber innervation. Importantly, kainate receptor–mediated synaptic responses exhibit slower kinetics than AMPA receptor–mediated responses. Thus the activation of kainate receptors contributes to postsynaptic responses in GC-SEs, but not in CGCs, and would be expected to do so in HEGCs as well. Kainate receptor activation at recurrent mossy fiber synapses could account for the slower average decay of mEPSCs in both GC-SE and HEGCs. It could also account for the longer average rise time of mEPSCs in HEGCs than in CGCs. However, GC-SEs did not...
differ significantly from control in this regard. Possibly, kainate receptor activation contributes to mEPSCs more prominently in HEGCs than in GC-SEs. Rise times are also influenced by the location of the synapse relative to the recording electrode at the soma. All else being equal, the greater the distance between the synapse and the soma, the longer the apparent rise time. The apical dendrite(s) of most HEGCs extends from within the dentate hilus to the outer edge of the molecular layer, and some HEGCs have an elongated basal dendrite (Scharfman et al. 2000, 2003; Zhan and Nadler 2009). Thus the average distance of excitatory synapses from the soma is probably greater for HEGCs than for normotopic granule cells.

Inhibitory synaptic responses in HEGCs

Electron micrographs suggested that the somata and proximal dendrites of HEGCs are practically devoid of inhibitory innervation, as evidenced by the apparent lack of symmetric synapses (Dastipour et al. 2001). This finding is consistent with the location of HEGCs at a considerable distance from most dentate basket cells, which innervate the somata and proximal dendrites of normotopic granule cells. In addition, we estimate that pilocarpine-induced status epilepticus kills \(~70\)–\(80\)% of hilar interneurons in our animals (Jiao and Nadler 2007). In contrast, status epilepticus kills few interneurons located at the hilar border of the granule cell body layer and apparently none in the molecular layer. The most vulnerable inhibitory neurons are the large multipolar somatostatin/neuropetide Y-immunoreactive HIPP cells (Buckmaster and Jon- gen-Rêlo 1999). Basket cells and HIPP cells are the major interneuron populations that contribute to mossy fiber–evoked inhibition of dentate granule cells. We therefore predicted that HEGCs receive little inhibition of this type. Our results confirmed this prediction.

Another mechanism that might explain, in part, the relatively small mossy fiber–evoked IPSCs is that the inhibitory projections to HEGCs are more likely to be transected during slice preparation than those of normotopic granule cells. The coincidence of a small evoked IPSC with a low mIPSC frequency argues more strongly for a deficit of inhibitory synapses, however. In addition, a higher probability of inhibitory afferent transection would predict that HEGCs exhibit a lower frequency of sIPSCs (spontaneous inhibitory postsynaptic currents recorded in the absence of TTX) than GC-SEs, even if the spontaneous firing rate of inhibitory interneurons increases after status epilepticus (Shao and Dudek 2005). Our recordings of sIPSCs did not support this prediction (Zhan and Nadler 2009).

Recordings of mIPSCs further supported the hypothesis that HEGCs receive much less synaptic inhibition than normotopic granule cells. mIPSC frequency and charge transfer per unit time were much lower in HEGCs than in normotopic granule cells. Because spontaneous inhibitory events recorded from dentate granule cells in control rats originate mainly from GABA \(_A\) receptors (Gibbs et al. 1997; Nusser et al. 1998; Otis et al. 1994). Similarly, all studies have shown at least some long-term deficit in mIPSC frequency, although the decrease was not statistically significant in the study of Cohen et al. (2003). In contrast, the effect of status epilepticus on rise and decay times has varied considerably. Different outcomes may relate to different recording conditions, analytical methods, animal model, survival times, and/or proportion of each interneuron population that was lost.

HIPP cells project to the distal dendrites of dentate granule cells (Han et al. 1993). Their degeneration after status epilepticus therefore predicts a loss of slowly rising mIPSCs, resulting in a downward shift in the mean rise time. In contrast, we found a small increase in mean rise time associated with loss of some of the fastest rising events. Although a loss of slowly rising mIPSCs was found shortly after status epilepticus in an electrical stimulation model of epilepsy, there seemed to have been substantial recovery before spontaneous seizures appeared (Sun et al. 2007a). No change in rise time was reported previously in pilocarpine-treated (Cohen et al. 2003; Kobayashi and Buckmaster 2003) or kainic acid–treated (Shao and Dudek 2005) animals. These unexpected findings may be explained by replacement of the HIPP cell→granule cell synapses on and near the soma suggests that the mean distance of these synapses from the soma is greater for HEGCs than for either normotopic granule cell population. Thus greater dendritic filtering may account for the relatively slow kinetics of mIPSCs recorded from these cells. Cell age may also be a factor. The earliest inhibitory synaptic currents to appear in adult-generated dentate granule cells exhibit slow rise and decay (Espósito et al. 2005; Karten et al. 2006; Overstreet-Wadiche et al. 2005). These responses seem to be generated by dedicated inputs that produce a relatively low concentration of GABA at postsynaptic receptors (Markwardt et al. 2009). These dedicated inputs probably originate from neogliaform cells (Karayannis et al. 2010). Because HEGCs continue to be produced for months at least after status epilepticus (Bonde et al. 2006), the birthdates of the HEGCs recorded in this study could not be estimated. Some of the recorded HEGCs could have been young enough to retain the slow inhibitory synaptic currents characteristic of immature granule cells. Differences in the number, clustering, and composition of postsynaptic GABA \(_A\) receptors may also contribute to differences in mIPSC properties. Further studies are needed to evaluate these possibilities.
mIPSCs from inhibitory synapses located more proximal to the soma, changes in the composition of GABA$_A$ receptors, and/or the young age of some recorded GC-SEs. The lost synapses may be replaced by axon sprouting from surviving HIPP cells (Zhang et al. 2009). In this way, the initial loss of slowly rising mIPSCs would be compensated. Furthermore, the dentate basket cell circuit seems dysfunctional in pilocarpine-treated rats (Zhang and Buckmaster 2009). Basket cells receive less excitatory drive, and transmission failure at basket cell→granule cell synapses is increased. Basket cell dysfunction may explain why fewer rapidly rising mIPSCs were recorded from GC-SEs than from CGCs. The increased mean rise time we observed could be explained by a greater loss of mIPSCs associated with basket cell synapses than with HIPP cell synapses. Studies of epilepsy models showed increased expression by dentate granule cells of the $\alpha_2$ subunit of the GABA$_A$ receptor at the expense of the more abundant $\alpha_1$ subunit (Brooks-Kayal et al. 1998; Nishimura et al. 2005; Peng et al. 2004). Many of these $\alpha_2$ subunits are inserted in the postsynaptic membrane of inhibitory synapses (Sun et al. 2007b). Studies of recombinant receptors indicate that $\alpha_2$$\beta_2$$\gamma_2$ GABA$_A$ receptors activate more slowly than $\alpha_1$$\beta_2$$\gamma_2$ GABA$_A$ receptors (Lagrange et al. 2007). Thus a longer mean rise time could involve a change in the subunit composition of synaptic GABA$_A$ receptors. Finally, an unknown percentage of GC-SEs could involve a change in the subunit composition of synaptic GABA$_A$ receptors (Lagrange et al. 2007).

Mosdy fiber–evoked compound IPSCs were significantly larger in GC-SEs than in CGCs, despite the degeneration of most hilar interneurons and dysfunction of the basket cell circuit. Their relatively slow decay supports the hypothesis that reverberating excitation among granule cells allows inhibitory interneurons to be driven repeatedly by a single mossy fiber volley rather than just once. Other mechanisms that may contribute to this overcompensation include the sprouting of inhibitory axons (Andre et al. 2001; Davenport et al. 1990; Mathern et al. 1995; Wittner et al. 2001; Zhang et al. 2009), enhanced interneuron excitability (Shao and Dudek 2005), and increased mossy fiber innervation of basket cells (Sloviter et al. 2006). Along with enhanced tonic GABA inhibition (Zhan and Nadler 2009), larger inhibitory feedback responses may contribute to the normalization of granule cell excitability with time after status epilepticus (Buckmaster and Dudek 1997; Sloviter et al. 2006; Urano et al. 1994; Wilson et al. 1998; Wu and Leung 2001).

Possible implications for the role of HEGCs in promoting excitability of the granule cell network

The high ratio of excitatory to inhibitory synaptic function shown in this study probably accounts, in part, for the hyperexcitability of HEGCs. Excitatory synaptic activity may drive the bursting and action potential firing of these cells with little opposition from synaptic inhibition. HEGCs become tightly integrated into dentate gyrus circuitry. The progression of seizures after status epilepticus has been linked to enhanced granule cell neurogenesis (Jung et al. 2004, 2006). It has been suggested that postseizure-generated granule cells that migrate normally into the granule cell body layer are less excitable than preexisting granule cells (Jakubs et al. 2006). If so, the postseizure-generated granule cells associated with seizure progression must be HEGCs. To the extent that hyperexcitable HEGCs enhance synchronous granule cell firing during seizures, their unique innervation may be important for impairing the filtering function of the dentate gyrus and thus facilitating seizure propagation through the limbic circuit (Gabriel et al. 2004; Hardison et al. 2000; Okazaki and Nadler 2001; Patrylo and Dudek 1998).

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

References


DashtiPour K, Tran PH, Okazaki MM, Nadler JV, Ribak CE. Ultrastructural features and synaptic connections of hilar ectopic granule cells in the rat dentate gyrus are different from those of granule cells in the granule cell layer. Brain Res 890: 261–271, 2001.


