Differential Epilepsy-Associated Alterations in Postsynaptic GABA\textsubscript{A} Receptor Function in Dentate Granule and CA1 Neurons

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Gibbs, John W., III, Melissa D. Shumate, and Douglas A. Coulter. Differential epilepsy-associated alterations in postsynaptic GABA\textsubscript{A} receptor function in dentate granule and CA1 neurons. *J. Neurophysiol.* 77: 1924–1938, 1997. Alterations in GABAergic function associated with the development of temporal lobe epilepsy (TLE) were examined with the use of patch-clamp recording techniques in dentate granule (DG) and CA1 neurons acutely isolated from control and spontaneously epileptic rats in which TLE was elicited by pilocarpine injection 3–17 wk before use. The maximal efficacy of \(\gamma\)-aminobutyric acid (GABA) in activating whole cell GABA currents increased significantly in epileptic DG neurons relative to controls. This efficacy increase was due to a 78% enhancement in the functional capacitance-normalized GABA\textsubscript{A} receptor (GABA\textsubscript{A}R) current density in epileptic DG neurons. Increased DG GABA\textsubscript{A}R current density was not accompanied by alterations in GABA potency (EC\textsubscript{50}). However, the maximal sensitivity of DG GABA-evoked currents to blockade by zinc increased 190% in epileptic neurons. Augmentation of electrical DG neuron GABA-evoked currents by the broad-spectrum anticonvulsant benzodiazepine clonazepam (100 nM) was enhanced 114% relative to controls, whereas augmentation by the benzodiazepine, (BZ),-selective agonist zolpidem (100 nM) was decreased by 66%. In contrast to DG neurons, maximal efficacy of GABA in activating GABA currents decreased in epileptic CA1 neurons relative to controls, due to a 52% decrease in functional capacitance-normalized GABA\textsubscript{A}R current density. This altered efficacy of GABA was accompanied by an increased GABA potency (GABA EC\textsubscript{50} was 35.8 and 24.5 \(\mu\text{M}\) in control and epileptic neurons, respectively). Sensitivity of GABA-evoked currents to blockade by zinc was unchanged in epileptic CA1 neurons, whereas clonazepam (100 nM) augmentation of CA1 GABA-evoked currents decreased 81% relative to controls. These regionally distinct epilepsy-associated modifications in hippocampal GABAergic function may be due to discrete structural alterations in postsynaptic GABA\textsubscript{A}R\textsubscript{s} accompanying epileptogenesis, could be therapeutically important, and undoubtedly could contribute to the enhanced limbic excitability underlying TLE.

INTRODUCTION

The hippocampal formation is integrally involved in the generation of partial seizures underlying temporal lobe epilepsy (TLE). The factors involved in triggering these recurrent spontaneous seizures within the limbic system are a subject of active investigation. There are characteristic patterns of pathology evident in hippocampal tissue resected from patients with chronic TLE (reviewed in Babb and Pretorius 1993). This pattern of pathology has been termed mesial temporal sclerosis. Considerable attention has been paid to these anatomic correlates of TLE, with detailed examinations of the patterns and nature of neuronal loss and circuit rearrangements accompanying development of TLE being a focus of vigorous research both in surgically resected tissue from TLE patients (Babb et al. 1991; Houser et al. 1990; Sutula et al. 1989) and in animal models of TLE (Mello et al. 1993; Sutula et al. 1989; Tauck and Nadler 1985). The role of these anatomic changes in the generation of limbic seizure susceptibility is an area of lively debate. However, somewhat lost in this anatomic debate is the potential epileptogenic role played by functional alterations in postsynaptic intrinsic properties and excitatory and inhibitory neurotransmitter receptor expression patterns that may occur in the surviving neurons in epileptic hippocampus.

Alterations in neurotransmitter-mediated responses have been described in various hippocampal neuronal populations both as acute consequences of limbic seizure activity and as a chronic epilepsy-associated change in animals with TLE. In physiological studies, excitatory synaptic responses mediated through activation of \(N\)-methyl-\(D\)-aspartate receptors are altered as a consequence of kindling in dentate granule (DG) cells (Köh and Mody 1994; Mody and Heineman 1987), and after the development of TLE in both CA1 and DG in the “super-kindling” model of Lothman et al. (1995). In addition, the binding properties of \(N\)-methyl-\(D\)-aspartate receptors in CA3 neurons are altered as a long-term consequence of kindling (Martin et al. 1992).

Inhibitory synaptic responses mediated through activation of \(\gamma\)-aminobutyric acid-A receptors (GABA\textsubscript{A}Rs) have also been shown to be altered in CA1 and dentate gyrus by development of an epileptic condition. Pared pulse depression was enhanced by kindling (deJonge and Racine 1987; Oliver and Miller 1985), and miniature inhibitory postsynaptic currents (mIPSCs) were significantly larger in the dentate gyrus of kindled animals relative to controls (Otis et al. 1994). In binding studies, both \({^3}\text{H}\text{muscimol binding and muscimol-stimulated synaptosomal Cl}^-\text{uptake were significantly enhanced in the dentate gyrus of kindled animals and reduced in CA1 relative to controls (Titulaer et al. 1994, 1995).}

In the present study, potential functional epilepsy-associated alterations in the physiological and pharmacological properties of GABA\textsubscript{A}R\textsubscript{s} were examined with the use of whole cell patch-clamp recordings from hippocampal CA1 and DG neurons isolated from rats in which a TLE condition was induced by pilocarpine injection an average of 6–7 wk before use.
METHODS

Injections

The protocol for pilocarpine injections used in this study was adapted from Mello et al. (1993). Adult rats, ~120 days postnatal, were kept in a climate-controlled (70°F) room on a 12-h light:dark cycle and provided with food and water ad libitum 1 wk before injection. Rats were injected first with scopolamine methyl nitrate (1 mg/kg ip) to minimize the peripheral effects of pilocarpine, and were subsequently injected 30 min later with pilocarpine (350 mg/kg ip). The pilocarpine injection usually triggered continuous seizures reminiscent of status epilepticus (SE): long-duration (>30 min) epileptic attacks within 10–30 min after pilocarpine injection. Rats that did not exhibit class 3 behavioral seizures after 1 h post pilocarpine injection were injected with a second dose of pilocarpine (175 mg/kg ip). Diazepam (4 mg/kg ip) in 50% propylene glycol was administered 1 h after the onset of SE to stop seizure activity. Additional diazepam was administered at 3 and 5 h post onset of SE as needed. Pilocarpine-induced SE usually took 3–5 h to terminate. To facilitate survival, weak rats were given an oral mixture of sucrose and powdered milk for several days after pilocarpine-induced SE. Control rats (n = 14) were treated in exactly the same way as pilocarpine-injected rats (n = 13), except that a subconvulsive dose of pilocarpine (35 mg/kg) was administered. Additionally, two-aged-matched naive animals without pilocarpine injections were also used as controls, and showed experimental results not different from subconvulsive treated rats. Therefore results from both subconvulsive and naive controls were pooled.

Epilepsy diagnosis

Beginning 2 wk after the pilocarpine injection, rats were video monitored for 8 h/day until at least one spontaneous seizure was observed. Seizures consisted at least of class 3 or higher activity on the scale of Racine (1972), i.e., clonic forelimb seizures. Most animals had generalized tonic clonic seizures (class 5). Animals with confirmed spontaneous seizures were then classified as epileptic. Before experimental use, epileptic animals were then subjected to 24 h of video monitoring to ensure that no seizures occurred during the 24 h before use. This ensured that acute effects of seizures on γ-aminobutyric acid (GABA) receptor properties were minimized. Animals usually required multiple 24-h video monitoring sessions, because most had at least one additional observed seizure during this later stage of intensive monitoring (range 1–9 seizures). The average number of class 3 or above behavioral seizures observed in the rat population employed in the present study was 2.2 (animals undoubtedly had many more seizures that were not observed, cf. Mello et al. 1993). From the 24-h video monitoring, the average frequency of spontaneous behavioral seizure occurrence was determined to be 0.8 seizures per day in the epileptic rat population employed in the present study. On average, animals were killed 6 wk and 5 days after pilocarpine injection (range 3–17 wk).

Staining

Hippocampal tissue immediately adjacent and dorsal to sections employed for cell isolations utilized in the patch recording experiments was placed in a 1.2% Na$_2$S solution in a sodium phosphate buffer for 20–30 min, and then transferred to a solution containing 1% paraformaldehyde and 1.25% glutaraldehyde in a sodium phosphate buffer and refrigerated overnight. The tissue was then placed in a 30% sucrose solution in a sodium phosphate buffer for 2–4 h, frozen in isopentane cooled with liquid nitrogen, and stored at −70°C until sectioning. Tissue was then cut on a cryostat into 25-μm sections containing ventral hippocampus, melted onto gelatin-coated slides, and stored at −70°C. Sections were then thawed, hydrated, and developed in Timm’s stain (Haug 1973). Slide-mounted sections were then dehydrated, placed in xylene, and coverslipped for histological examination.

Timm’s stain scoring

The scoring for the degree of mossy fiber sprouting into the supragranular layer of the dentate gyrus was determined blindly (i.e., by an investigator who did not know the seizure history of the animal) as described previously (Tauck and Nadler 1985). The Timm’s scores of 3 sections per animal were averaged for a final score for each animal. Mossy fibers were identified by black staining for zinc-containing synaptic terminals (e.g., Fig. 6). Sections with no or occasional supragranular dentate staining were given a score of 0. Sections with scattered Timm’s stain over all areas of the DG layer were scored 1. Sections that showed sparse staining interspersed with regions of heavier staining or a continuous band of intermediate staining in intensity between sections scored 1 and 3 were given a score of 2. Sections with a dense, continuous banded stain were scored 3 (Tauck and Nadler 1985). Significance was calculated by the Mann-Whitney rank sum test.

Acute isolation of neurons

Experiments were conducted on neurons acutely isolated from adult rat hippocampus with the use of methods that have been previously described (Gibbs et al. 1996a,c; Oh et al. 1995). The brain was dissected and placed in a 4°C chilled, oxygenated (95% O$_2$:5% CO$_2$) artificial cerebrospinal fluid solution composed of (in mM) 201 sucrose, 3 KCl, 1.25 NaHPO$_4$, 2 MgCl$_2$, 2 CaCl$_2$, 26 NaHCO$_3$, and 10 dextrose. Hippocampal slices (450 μm) were cut on a 12° agar ramp with a vibratome (Rafiq et al. 1993, 1995) and incubated for 1 h in an oxygenated medium containing (in mM) 120 NaCl, 5 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 25 glucose, and 20 pipеразин-2-етансульфонная кислота (PIPES), pH adjusted to 7.0 with NaOH at 32°C. Slices were enzymed in 3 mg/ml Sigma protease XXIII in PIPES, thoroughly rinsed, and incubated another 30 min in PIPES medium before dissociation. The dentate gyrus and CA1 of the hippocampus were visualized with the use of a dark-field microscope and dissected out. Chunks (1 mm$^3$) were then cut from each area, and cells were then mechanically dissociated by trituration of the chunks through a series of Pasteur pipettes of decreasing bore sizes. The resulting cell suspension was then plated on 35-mm culture dishes in a 2-hydroxyethylpiperazine-N,-2-ethane sulfonic acid (HEPES) medium composed of (in mM) 155 NaCl, 3 KCl, 1 MgCl$_2$, 3 CaCl$_2$, 0.0005 tetrodotoxin, and 10 HEPES-Na’, pH adjusted to 7.4 with NaOH.

Voltage-clamp recordings in isolated neurons

The intracellular (pipette) solution contained (in mM) 100 Trizma phosphate (dibasic), 28 Trizma base, 11 ethylene glycol bis-(β-aminoethylether)-N,N',N'-tetraacetic acid, 2 MgCl$_2$, and 0.5 CaCl$_2$, pH adjusted to 7.35 with NaOH. A 4-mV junction potential was measured between this solution and the bath solution. All voltages were corrected accordingly. Whole cell patch-clamp recording experiments were conducted on a Nikon inverted microscope equipped with Hoffman modulation contrast optics. Electrodes (4–8 MΩ) were pulled on a Narishige PP-83 microelectrode two-stage puller with the use of thin-walled borosilicate capillary glass (WPI, Sarasota, FL). The pipette solution also contained an intracellular ATP reconstitution consisting of 10 mM Mg$^{2+}$-ATP and 22 mM phosphocreatinine. The intracellular ATP maintenance solution was used to fill the shank of the electrode, but was omitted from the solution that was used to backfill the tip of the electrode.
Statistical analysis

The Drug concentrations and method of application

GABA was prepared as a 10 mM stock solution in HEPEs solution. Clonazepam (CNZ) and zolpidem were first dissolved in dimethyl sulfoxide (DMSO) at 100 and 10 mM, respectively, and then diluted to the final concentration in HEPEs medium. The maximum concentration of DMSO used in cellular perfusions was <0.001%, and this DMSO concentration has been shown not to alter GABA responses (Oh et al. 1995). The applied drug concentrations were as follows: GABA (Sigma, St. Louis, MO), 3–1,000 μM; CNZ (Sigma), 100 mM; zolpidem (RBI, Natick, MA), 100 mM; and zing (Sigma), 0.1–300 μM. Solution changes were accomplished with the use of a 13-barrel modified ‘‘sewer pipe’’ perfusion technique (Gibbs et al. 1996a,c), in which several solutions flowed out of parallel teflon tubes (0.2 mm ID) in a laminar fashion. Rapid (40–200 ms) and complete solution changes at a constant flow rate were then effected by moving the tube assembly laterally in relation to the neuron under study. No cross contamination was ever evident. After the seal was broken to institute whole cell recording mode, and 2–5 min were allowed to pass to establish stable leak currents (0 to 200 pA), GABA was applied for 4–6 s and washed out with control external solution for 30–40 s. The cell was pretreated with test drugs without GABA for 50–60 s and then test solutions were applied together with GABA. Drug effects with CNZ, zolpidem, and zing were expressed as percentage effects on GABA-evoked outward currents, recorded at a holding potential of −24 mM. Experiments were performed at room temperature (22–24°C).

Current density

Current density was calculated on the basis of the maximal response of a neuron to application of 1 mM GABA; this was then divided by the membrane capacitance of the cell, which was read directly off the capacitance compensation potentiometer on the patch amplifier. Capacitance readings in which this method is used are in good agreement with capacitance measurements derived from integrating the current response to a small depolarizing step. For example, membrane capacitance of postnatal day 5–25 thalamic neurons calculated by integration was 17.5 pF in the study by Huguenard et al. (1991), and 16–18 pF as assessed through transient cancellation by Oh et al. (1995). Current density was quantified assuming a capacitance/membrane area relationship of 1 μF/cm² (Gibbs et al. 1996a,c; Oh et al. 1995).

Statistical analysis

All values are expressed as means ± SE. Differences between means were tested with the use of Student’s t-test, or, for data that were not normally distributed, the Mann-Whitney rank sum test. Concentration-response curves were fitted by a Marquardt-Levenberg nonlinear least-squares routine, with the use of either ORIGIN (MicroCal Software, Northampton, MA) or ALLFIT (De Lean et al. 1987). The significance of differences in best-fit parameter values between curves was assessed with the use of constrained simultaneous curve fitting testing the equality of parameters, and ALLFIT, as described in De Lean et al. (1987). This method involves testing for equality of parameters by examining the statistical consequences (via an F test) of forcing them to be equal.

RESULTS

Selection of neurons

DG cells and CA1 neurons were selected for whole cell patch-clamp recording in acutely isolated cultures on the basis of anatomic criteria. These neurons were isolated from 1-mm³ chunks excised from the appropriate areas of hippocampal slices under stereomicroscopic examination, ensuring that neurons were obtained from the desired area. DG neurons recorded in the present study had rounded cell bodies 5–10 μM across and had unipolar dendrites. In DG culture plates, larger, pyramidal-shaped neurons were not recorded. CA1 neurons recorded in the present study were 10–25 μM across the soma, and were pyramidal, with obvious apical and basal dendrites. In CA1 cultures, smaller bipolar or fusiform cells were not recorded. There were no apparent gross differences in anatomy of cells between treatments within a given region. GABA responses were recorded and analyzed in a total of 106 DG neurons (50 control, 56 epileptic) and 72 CA1 neurons (45 control, 27 epileptic) from a total of 16 control and 13 spontaneously epileptic pilocarpine-treated animals in the present study. Subconvaluscin-dose pilocarpine controls (see METHODS) and age-matched naive controls were found not to differ significantly in their GABA receptor properties, so these two control populations were grouped together in the present analysis.

Epilepsy-associated alterations in postsynaptic GABA responses in DG neurons

Application of GABA to DG neurons voltage clamped at −24 mV elicited an outward current accompanied by a large conductance increase (Fig. 1). This response was due to activation of GABAₐRs because 1) the intracellular electrode solution contained no potassium, which would preclude outward movement of potassium ions; 2) this response was blocked in a concentration-dependent manner by the GABAₐR antagonists bicuculline (10–50 μM) and picrotoxin (1–100 μM) (Gibbs et al. 1996a, 1997; Oh et al. 1995); and 3) the reversal of the GABA-activated conductance was near to that predicted for a GABAₐR-evoked chloride conductance (Gibbs et al. 1996c; Oh et al. 1995). The theoretical reversal potential of GABA-evoked responses (Eₜ人生的) was calculated to be −70 mV with the use of the Goldman-Hodgkin-Katz equation for a chloride conductance (Goldman 1943; Hodgkin and Katz 1949), assuming a phosphate-to-chloride permeability ratio of 0.025 and an activity coefficient of 0.75 for the 166 mM external chloride solution (Bormann et al. 1987). The Eₜ人生的 in control DG neurons was −67.3 ± 3.3 (SE) mV (n = 3; all values are means ± SE here and below), compared with −63.9 ± 1.4 mV (n = 7) in pilocarpine DG neurons. These reversal potentials were not statistically different (P > 0.05, t-test).

To evaluate the possible occurrence of alterations in functional GABAₐR current density induced by epileptogenesis, GABA concentration-response curves were plotted for responses of DG cells to GABA applied in concentrations ranging from 3 to 1,000 μM. When plotted in a log GABA
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FIG. 1. γ-Aminobutyric acid (GABA) responses in dentate granule (DG) neurons isolated from control and epileptic (pilocarpine-treated) animals. A: traces of responses to varying concentrations of GABA in control and epileptic DG neurons illustrating the increase in the amplitude of GABA-evoked currents in pilocarpine DG neurons relative to controls. B: GABA concentration-response curves of control (n = 10) and pilocarpine (n = 8) DG neurons. Dentate cells of similar capacitance (range 5.8–13.2 pF) were chosen to normalize for potential membrane area differences. The concentration-response curve for the pilocarpine DG neurons showed a 109.2 and 88.8%, respectively, with no difference in the potency of GABA in between groups.

concentration vs. amplitude of GABA current graph, the GABA-evoked current was always found to increase in a sigmoidal manner as greater concentrations of GABA were applied to both control and epileptic DG cells (Fig. 1). The effect of application of increasing concentrations of GABA plateaued at 1 mM, as has been reported previously in acutely isolated rat (Gibbs et al. 1996a; Oh et al. 1995) and human cortical neurons (Gibbs et al. 1996c). GABA concentration-response curves were best fitted employing a nonlinear least-squares method assuming a monophasic sigmoidal GABA concentration-response relationship with the use of the equation

\[ I = I_{\text{max}} C^H \left( \frac{C}{C_{50}} + 1 \right)^{-H} \]

where \( C \) is the GABA concentration, \( I \) is the current elicited by a given GABA concentration, \( I_{\text{max}} \) is the maximal GABA current, \( C_{50} \) is the GABA concentration eliciting half-maximal current, and \( H \) is the Hill coefficient. Whole cell GABA-evoked currents of neurons with similar capacitance (6–13 pF) were analyzed and compared to try to control for the effects of any cellular size differences between treatment groups. A significant increase in the overall efficacy of GABA in evoking postsynaptic GABA_A_R responses was observed in DG neurons isolated from epileptic animals relative to controls (Fig. 1). The amplitude of GABA_A responses to 100 μM and 1 mM GABA were significantly increased by 109.2 and 88.8%, respectively, in DG pilocarpine neurons relative to controls (\( P < 0.003 \) and \( P < 0.004 \), respectively, t-test). This increase in GABA efficacy associated with epileptogenesis in DG neurons isolated from spontaneously epileptic pilocarpine-treated animals was not accompanied by any significant change in the apparent potency of GABA in activating GABA currents. The best-fit \( EC_{50} \) for the GABA concentration-response relationship were 39.2 ± 4.1 μM and
32.7 ± 4.0 μM in control and epileptic DG neurons, respectively (Fig. 3). These EC_{50} means were not significantly different (P = 0.46, not significant, F test). To more thoroughly characterize the GABA efficacy differences described above, GABA_{A,R} current densities (the maximal GABA current normalized to cellular capacitance) were quantified in control and epileptic DG neurons after application of a saturating concentration (1 mM) of GABA (see METHODS for specifics of this calculation). The capacitance-normalized GABA_{A,R} current density significantly increased in epileptic DG neurons (4.22 ± 0.48 pA/μm²; n = 14) in comparison with control neurons (2.37 ± 0.28 pA/μm²; n = 16). This reflects a 78% higher GABA current density in epileptic compared with control DG neurons (P < 0.002, t-test).

Epilepsy-associated alterations in postsynaptic GABA responses in CA1 neurons

As was seen in control and epileptic DG neurons (described above), E_{GABA} showed no significant differences in CA1 neurons of epileptic animals compared with controls. E_{GABA} was −66.0 ± 2.0 mV in control CA1 neurons (n = 5) and −62.4 ± 4.3 mV in pilocarpine CA1 neurons (n = 4), values that were not statistically different (P > 0.05, t-test). To quantify possible alterations in functional GABA_{A,R} current density in CA1 neurons induced by epileptogenesis, GABA concentration-response curves were plotted for data obtained in response to GABA application in concentrations ranging from 3 to 1,000 μM in a manner identical to that described for DG cells (above). GABA concentration-response curves were fitted as described above (Fig. 2).

Whole cell GABA-evoked currents in neurons with similar capacitance were analyzed and compared to normalize for possible cell size differences between CA1 groups. Unlike in DG neurons, where an increased efficacy of GABA was evident in epileptic neurons (Fig. 1), in CA1 neurons a significant decrease in the overall efficacy of GABA in evoking postsynaptic GABA_{A,R} responses was observed in neurons isolated from epileptic animals relative to controls (Fig. 2). GABA current amplitudes elicited by application of 100 μM and 1 mM GABA were significantly reduced by 43.0 and 45.4%, respectively (P < 0.02 and P < 0.01, respectively, t-test). To more accurately assess this GABA efficacy decrease in epileptic CA1 neurons relative to controls, GABA current densities (the maximal GABA current normalized to cellular capacitance) were quantified in control and epileptic DG neurons after application of a saturating concentration (1 mM) of GABA (as described above and in METHODS). Capacitance-normalized GABA current density decreased from 4.74 ± 0.92 pA/μm² (n = 18) to 2.32 ± 0.36 pA/μm² (n = 13) in control and epileptic CA1 neurons, respectively, a statistically significant 51% decrease (P < 0.05, t-test). The potency or EC_{50} of GABA_{A,R} responses varied significantly between control and epileptic neurons (Fig. 3). Control CA1 neurons had an EC_{50} of 35.8 ± 2 μM, and epileptic neurons had an EC_{50} of 24.5 ± 2.3 μM, in a population of cells with similar capacitances ranging between 12.5 and 20 pF (Fig. 3). These EC_{50} values were significantly different (P < 0.03, F test).

Epilepsy-associated alterations in zinc blockade of GABA-evoked currents in DG neurons

DG neurons were voltage clamped at −24 mV, and 10 μM GABA was applied alone and concurrently with varying concentrations of zinc to examine zinc blockade of GABA-evoked currents. This concentration of GABA was on the rising phase of the concentration-response curve for DG neurons and produced minimal desensitization (Fig. 1). Application of zinc in concentrations of 0.1–300 μM resulted in a concentration-dependent, sigmoidally increasing blockade of GABA-evoked currents in both control and epileptic DG neurons (Fig. 4). The data could be best fitted assuming a sigmoidal concentration-response relationship with the use of the equation

\[ \% \text{ Inhibition} = \frac{M_i(C^H + IC_{50}^H)}{C^H} \]

where M_i is the maximal zinc effect, C is the concentration of zinc, H is the Hill coefficient, and IC_{50} is the zinc concentration at which half-maximal effect was seen. The actual maximal level of GABA blockade inducible by zinc could not be determined because zinc concentrations >300 μM were neurotoxic to acutely isolated DG and CA1 neurons.

Zinc was found to block GABA-evoked currents to a much greater extent in epileptic DG neurons than in controls (Fig. 4). At 100 and 300 μM, zinc blocked GABA-evoked currents by 32.2 ± 2.9% and 31.3 ± 3.8% in control DG neurons, and by 76.3 ± 1.5% and 90.8 ± 0.6% in epileptic DG neurons, respectively. This corresponds to an epilepsy-associated statistically significant 136 and 190% increase in zinc block of DG GABA currents at 100 and 300 μM (P < 0.0001 for both sets of means, t-test).

Epilepsy-associated alterations in zinc blockade of GABA-currents in CA1 neurons

Zinc blockade of GABA-evoked currents was also studied in epileptic and control CA1 cells, with the use of identical methods to those employed in DG neurons (described above). Coapplication of zinc in a concentration range of 0.1–300 μM resulted in a concentration-dependent, sigmoidally increasing blockade of GABA-evoked currents, as was observed in DG neurons (Fig. 4). The data were best fitted with the use of the equation and methods described above. However, unlike the effects of epileptogenesis on zinc blockade of GABA currents evident in DG neurons, which was a significant enhancement of the efficacy of zinc (Fig. 4), little change in the efficacy of zinc in blocking GABA currents was evident in epileptic CA1 neurons relative to controls (Fig. 5). Zinc, at concentrations of 100 and 300 μM, blocked 61.1 ± 1.8% and 77.0 ± 1.5% of the GABA current in control neurons, and 52.4 ± 3.4% and 68.8 ± 2.5% of the GABA current in epileptic CA1 neurons (Fig. 5). This corresponds to modest 17 and 12% decreases in the level of block of GABA-evoked currents by 100 and 300 μM zinc accompanying epileptogenesis in CA1 neurons, which were nonsignificant and borderline significant differences, respectively (P > 0.05 for 100 μM mean; P < 0.04 for 300 μM mean; t-test).

Timm’s staining in control and epileptic hippocampi

One frequent pathological correlate of TLE is the presence of aberrantly located mossy fiber terminals, which
often sprout back into the molecular layer of the dentate gyrus in brains of epileptic patients or animals, in addition to innervating their normal targets in the hilus and CA3 (Babb et al. 1991; Mello et al. 1993; Sutula et al. 1989; Tauck and Nadler 1985; reviewed in McNamara 1994). This aberrant sprouting can be visualized, because the mossy fiber terminals contain high concentrations of zinc, a heavy metal that can be selectively stained for with the use of Timm’s stain (Haug 1973). Transverse slices of rat brain prepared from most of the animals immediately dorsal and adjacent to the sections employed in the acute isolation experiments described above were stained with the use of the Timm’s method to look for the presence and severity of epilepsy-associated aberrant zinc-containing terminals in the dentate supragranular layer (Fig. 6). Figure 6 shows photomicrographs of three sections from three different animals stained for the presence of zinc-containing synaptic terminals. No mossy fiber sprouting was present in the control section (Fig. 6, top), and significant sprouting was apparent in the pilocarpine-treated sections, with Fig. 6, middle, corresponding to a Tauck-Nadler score of 2 and Fig. 6, bottom, to a score of 3 (see METHODS for details) (Tauck and Nadler 1985). With the use of this scoring system, pilocarpine animals exhibited significant sprouting of mossy fibers into the molecular layer of the dentate gyrus (mean Timm’s score of 1.7 ± 0.2, n = 11) and controls showed little or no sprouting (mean score of 0.1 ± 0.1, n = 14) (P < 0.0001, Mann-Whitney rank sum test). This mean level of staining in the pilocarpine animals in the present study corresponds to patches of heavy molecular layer staining in the dentate gyrus, interspersed with regions of sparser staining, or a continuous band of intermediate staining in the molecular layer (e.g., Fig. 6, middle) (Tauck and Nadler 1985).
Figure 3. Normalized GABA concentration response curves of control and epileptic DG and CA1 neurons. A: GABA concentration-response curves of control \((n = 10)\) and epileptic \((n = 14)\) DG neurons with similar capacitances, normalized to the GABA response evoked by 1 mM GABA \((a saturating response)\) to better illustrate potential differences in the potency of GABA between groups. Note that there was no difference in the EC\(_{50}\) between the control and pilocarpine DG neurons. Control and pilocarpine DG neurons had best-fit EC\(_{50}\)s of \(39.2 \pm 4 \mu\text{M}\) and \(32.7 \pm 4 \mu\text{M}\), respectively \(\text{(not significantly different, } P > 0.05, F \text{ test)}\). B: GABA concentration-response curve in control \((n = 14)\) and pilocarpine \((n = 8)\) CA1 neurons with similar capacitances \((12.5 \pm 20 \text{pF})\), normalized as in A. Note the leftward shift or increase in potency of GABA in the epileptic CA1 neurons in relation to controls, corresponding to best-fit EC\(_{50}\)s of \(35.8 \pm 2 \mu\text{M}\) and \(24.5 \pm 2 \mu\text{M}\) in control and pilocarpine CA1 neurons, respectively \(\text{(significantly different, } P < 0.03, F \text{ test)}\).

Epilepsy-associated alterations in benzodiazepine augmentation of GABA-evoked currents in DG neurons

DG neurons were voltage clamped at \(-24 \text{ mV}\), and 10 \(\mu\text{M}\) GABA was applied alone and concurrently with 100 nM CNZ, a commonly prescribed anticonvulsant for the treatment of generalized and partial complex seizures \(\text{(Dreifuss \text{ et al.} 1975)}\), to examine CNZ augmentation of GABA\(_{\alpha}\)-evoked chloride currents. CNZ \((100 \text{ nM})\), which has been previously shown to produce maximal augmentation of the GABA currents in most neurons under the present recording conditions \(\text{(Gibbs \text{ et al.} 1996a; Oh \text{ et al.} 1995)}\), was employed to examine possible epilepsy-associated alterations in benzodiazepine modulatory effects on DG neurons. CNZ \((100 \text{ nM})\) augmented the GABA \((10 \mu\text{M})\) response by \(41.0 \pm 10.8\% \ (n = 11)\) in control DG neurons and by \(87.8 \pm 10.8\% \ (n = 20)\) in epileptic DG neurons, a statistically significant \(114\%\) increase in CNZ-evoked augmentation \(\text{(} P < 0.01, t\text{-test)}\) \(\text{(Fig. 7)}\).

CNZ is a broad-spectrum benzodiazepine, which acts at both benzodiazepine \(_1\) \((\text{BZ}_1)\) and \(_2\) \((\text{BZ}_2)\) receptors \(\text{(reviewed in Macdonald and Olsen 1994)}\). To more completely characterize epilepsy-associated alterations in the function of GABA\(_{\alpha}\)-Rs, the effects of zolpidem \((100 \text{ nM})\) were also examined and compared between control and epileptic DG neurons. Zolpidem is more specific agonist for \(_1\) receptors.
and has less effect on BZ₂ receptors (reviewed in Macdonald and Olsen 1994). When coapplied with GABA onto control DG neurons, zolpidem (100 nM) augmented the GABA-evoked current by 158.4 ± 26.1% \((n = 7)\), compared with a 54.2 ± 7.3% \((n = 10)\) zolpidem augmentation effect evident in epileptic DG neurons. This was a statistically significant 66% decrease in zolpidem efficacy accompanying epileptogenesis \((P < 0.02, t\text{-test})\) (Fig. 8).

**Epilepsy-associated alterations in benzodiazepine augmentation of GABA-evoked currents in CA1**

CNZ (100 nM) augmentation of CA1 neuron GABA currents was also studied, with the use of techniques identical to those employed in DG neurons (described above). In contrast to the epilepsy-associated enhanced in CNZ modulation of GABA-evoked currents evident in DG neurons, CNZ (100 nM) was much lower in efficacy in augmenting epileptic CA1 neuron GABA currents compared with controls. CNZ (100 nM) augmented GABA-evoked currents by 115.4 ± 9.3% in control CA1 neurons \((n = 11)\), and by 22.5 ± 6.1% in epileptic neurons \((n = 7)\) (Fig. 9), a statistically significant 81% decrease in CNZ efficacy \((P < 0.001, t\text{-test})\).

**DISCUSSION**

Significant alterations in GABA₂R function were evident in epileptic hippocampal neurons relative to controls. In epileptic DG neurons, GABA₂R current density increased 78% (Fig. 1), zinc blockade of GABA responses increased 190% (Fig. 4), and GABA₂R augmentation by 100 nM CNZ increased 114% (Fig. 7), whereas augmentation by the BZ₁ agonist zolpidem (100 nM) decreased 66% relative to con-
FIG. 5. Traces and concentration inhibition curves illustrating zinc blockade of GABA-evoked currents in CA1 neurons isolated from control and epileptic (pilocarpine-treated) rats. A: traces illustrating the blockade by zinc (0.1–300 μM) of currents evoked by GABA (10 μM). Increasing concentrations of zinc produced a similar increase in the levels of block of the GABA-evoked current in both control and epileptic neurons. B: zinc concentration % GABA blockade curves for control (n = 20) and epileptic (n = 12) CA1 neurons. Zinc, at concentrations of 100 and 300 μM, blocked 61.1 ± 1.8% and 77.0 ± 1.5% of the GABA current in control neurons, and 52.4 ± 3.4% and 68.8 ± 2.5% in pilocarpine CA1 neurons, corresponding to a modest 17% and 12% decrease in pilocarpine cells relative to controls. Note there was no significant decrease in potency of zinc in blocking GABA receptors in CA1 neurons isolated from epileptic animals, with best-fit zinc IC₅₀s of 28.3 ± 5.8 μM and 62.7 ± 24.7 μM (not significantly different, P > 0.05, F test). Best-fit maximal efficacy (M) of zinc was 90.1 and 98.3%, whereas Hill coefficients (H) were 0.7 and 0.5 for control and epileptic CA1 neurons, respectively.

In controls (Fig. 8). In contrast, in epileptic CA1 neurons, GABAₐR current density decreased 51% (Fig. 2). GABA was higher in potency (Fig. 3), zinc blockade of GABA currents was unchanged (Fig. 5), and CNZ (100 nM) augmentation of CA1 GABA currents decreased 81% (Fig. 9).

In physiological studies, Otis et al. (1994) described increased amplitude but not frequency of mIPSCs in DG neurons after kindling. Furthermore, Buhl et al. (1996) demonstrated enhancement in DG mIPSC zinc sensitivity after kindling. These functional alterations in DG neuron synaptic GABAₐRs are similar to findings in the present study. Whole cell DG GABAₐR maximal current responses were enhanced 78% after epilepsy development (Fig. 1), and synaptic GABAₐR responses were enhanced 75% after kindling (Otis et al. 1994). In the present study, zinc sensitivity of GABAₐR responses increased 190% in epileptic DG neurons, and synaptic GABAₐR responses were significantly blocked by zinc in kindled DG neurons, unlike in control cells (Buhl et al. 1996). The similarities between these two sets of findings implicate shared mechanisms contributing to epileptogenesis in these distinct animal models of chronic limbic epilepsy. However, Buhl et al. (1996) found no zinc sensitivity of control DG mIPSCs. This contrasts with the present study, in which control DG whole cell GABA-evoked currents were blocked by 30% at the 200 μM zinc concentrations employed in that study. This suggests that the control zinc sensitivity of DG GABAₐRs (Fig. 4), which has also been described by Kapur and Macdonald (1996), may be due to the contribution of extrasynaptic receptors to whole cell GABAₐR pharmacology. Furthermore, the enhanced zinc sensitivity of epileptic DG neuron GABAₐRs could be due to overexpression and incorporation of extrasynaptic GABAₐRs into the subsynaptic membrane. These hypotheses await more definitive support. The stochastic nature of synaptic function,
FIG. 6. Timm’s stain of 25-μm-thick brain sections containing the ventral hippocampus in control and pilocarpine specimens. Low-power (A, ×4) and higher-power (B, ×10) views of ventral hippocampal sections from control (top) and 2 different pilocarpine (middle and bottom) rats. Note the pronounced aberrant mossy fiber “sprouting” in the supragranular layer in the dentate gyrus of the pilocarpine hippocampal sections, with no supragranular staining apparent in the control section. Middle: section corresponding to a Tauck-Nadler Timm score of 2. Bottom: section corresponding to a Timm score of 3. Calibration bar: 250 μm (A), 100 μm (B).

particularly in mIPSC studies involving random activity in hundreds of synapses, makes identification and quantification of smaller amplitude changes difficult. A 30% block of GABA_ARs may not have been apparent in these studies. In addition, the nature of zinc blockade, which has been described as intermediate between competitive and noncompetitive (Celentano et al. 1991), makes it difficult to predict the exact effect the 30% block by 100–300 μM zinc of the current evoked by GABA (10 μM), described pharmacologically (Fig. 4), would have on a synapse, where millimolar GABA concentrations are achieved in the synaptic cleft.

Enhanced zinc sensitivity of epileptic DG GABA_ARs may be functionally important. Zinc localizes to mossy fiber terminals, and can be released in high concentrations (>100 μM) (Assaf and Chung 1984). Normally, mossy fibers do not “autoinnervate” DG neurons. With kindling, there are modest dentate gyrus circuit rearrangements, with fairly sparse levels of “sprouted” mossy fiber terminals in the supragranular layer (Cavazos et al. 1991; Sutula et al. 1989). Although GABA_ARs zinc block is enhanced in kindled dentate gyrus (Buhl et al. 1996), there is a low density of sprouted zinc-containing terminals. This contrasts with the pilocarpine model, in which robust mossy fiber sprouting is evident (Mello et al. 1993; Okazaki et al. 1995) (Fig. 6). Thus, in pilocarpine animals, enhanced zinc block of DG GABA_ARs and robust autoinnervation by zinc-containing
Fig. 7. Clonazepam (CNZ) modulation of GABA-evoked currents in DG neurons isolated from control and epileptic (pilocarpine-treated) animals. A: traces illustrating the modulation by CNZ (100 nM) of the current evoked by GABA (10 μM) in control and epileptic DG neurons. Note the increased efficacy of CNZ (100 nM) in the pilocarpine DG neurons relative to controls. B: histogram illustrating the increased efficacy of CNZ in augmenting GABA responses in pilocarpine DG neurons (n = 20) relative to controls (n = 11). CNZ (100 nM) augmented the GABA response by 41.0 ± 10.8% and 87.8 ± 10.8% in control and pilocarpine DG neurons, respectively, corresponding to a 114% increased efficacy of CNZ in epileptic DG neurons relative to controls. Asterisk: significantly different from control mean, P < 0.01, t-test.

Mossy fiber terminals coexist. Given that zinc can diffuse from its release site and interact with neighboring inhibitory receptors, the combined result of sprouting and enhanced zinc sensitivity could be dynamic disinhibition occurring during seizure discharges, or, as it was termed by Buhl et al. (1996), “a zinc-induced collapse of augmented inhibition” within the dentate gyrus.

Decreased functional GABA₆R current density (Fig. 2), enhanced potency of GABA (Fig. 3), and decreased benzodiazepine modulation of GABA₆Rs (Fig. 9) described in epileptic CA1 neurons in the present study constitute a “fingerprint” delineating a series of GABA₆R changes also seen in other studies in epileptic hippocampal and neocortical pyramidal neurons. In biochemical studies in kindled animals, [³H]muscimol binding and Cl⁻ influx decreased in CA1 compared with controls (Titulaer et al. 1994, 1995), consistent with the decreased GABA₆R current density in CA1 described in pilocarpine TLE rats (Fig. 2). In hippocampal cultures in which recurrent spontaneous seizure discharges can be induced by a one-time exposure to low-Mg²⁺ medium (Sombati and DeLorenzo 1995), this symptomatic fingerprint of GABA₆R alterations was evident accompanying develop-
FIG. 8. Zolpidem (ZOL) modulation of GABA-evoked currents in DG neurons isolated from control and epileptic (pilocarpine-treated) animals. A: traces illustrating the modulation by ZOL (100 nM) of the current evoked by GABA (10 μM) in control and epileptic DG neurons. Note the decreased efficacy of ZOL in the pilocarpine DG neurons relative to controls.

B: histogram illustrating the decreased efficacy of ZOL in augmenting GABA responses in pilocarpine DG neurons (n = 10) relative to controls (n = 7). ZOL (100 nM) augmented the GABA response by 158.1 ± 26.1% and 54.2 ± 7.3% in control and pilocarpine neurons, respectively. This was a 66% decrease in ZOL efficacy accompanying epileptogenesis. Asterisk: significantly different from control mean, P < 0.02, t-test.

ment of spontaneous epileptiform activity, and persisted for the life of the culture (Gibbs et al. 1997). GABA_A,R responses in human neocortical pyramidal neurons isolated from an epileptic focus exhibited similar decreased functional GABA_A,R density, increased GABA potency, and decreased benzodiazepine efficacy (Gibbs et al. 1996b) when compared with nonfocal pyramidal neuron GABA_A,R responses (Gibbs et al. 1996c). This consistent pattern of epilepsy-associated GABA_A,R alterations in pyramidal neurons in both epilepsy model systems and in humans suggests that these modifications may be important in seizure susceptibility, irrespective of the mechanisms eliciting this disorder.
Cloning/expression studies detailing functional properties of GABA\(_A\)Rs associated with specific subunit assemblies provide testable hypotheses concerning possible epilepsy-associated GABA\(_A\)R structural rearrangements underlying regionally distinct alterations in ensemble cellular inhibitory function evident in this study. GABA\(_A\)Rs are heteromers composed of several distinct protein classes, encoded by \(\alpha\)-, \(\beta\)-, \(\gamma\)-, \(\delta\)-, and \(\rho\)-families of mRNA subunits. The \(\gamma\)-subunit, when coexpressed with \(\alpha\)- and \(\beta\)-subunits, confers benzodiazepine sensitivity to GABA\(_A\)Rs, and influences zinc sensitivity (Draguhn et al. 1990; Saxena and Macdonald 1994; reviewed in Macdonald and Olsen 1994). GABA\(_A\)Rs consisting of \(\alpha\)- and \(\beta\)-subunits were potently blocked by zinc, whereas \(\gamma\)-subunit addition resulted in GABA\(_A\)Rs that were insensitive to zinc (Draguhn et al. 1990). Replacement of the \(\gamma\)- with a \(\delta\)-subunit in \(\alpha\),\(\beta\)-containing GABA\(_A\)Rs conferred zinc sensitivity (Saxena and Macdonald 1994). However, hippocampal neuronal (\(\gamma\)-containing) GABA\(_A\)Rs are zinc sensitive (Legendre and Westbrook 1990; Westbrook and Mayer 1987), and significantly reduced zinc sensitivity.

**Fig. 9.** CNZ modulation of GABA-evoked current in CA1 neurons isolated from control and epileptic (pilocarpine-treated) animals. *A:* traces illustrating the modulation by CNZ (100 nM) of the current evoked by GABA (10 \(\mu\)M) in control and pilocarpine CA1 neurons. Note the decreased efficacy of CNZ in the pilocarpine neurons relative to controls. *B:* histogram illustrating the decreased efficacy of CNZ in augmenting GABA responses in pilocarpine CA1 neurons (\(n = 7\)) relative to controls (\(n = 11\)). CNZ (100 nM) augmented the GABA response by 115.4 \(\pm\) 9.3\% and 22.5 \(\pm\) 6.1\% in control and pilocarpine CA1 neurons, respectively, corresponding to an 81\% decrease in CNZ efficacy relative to controls. Asterisk: significantly different from control mean, \(P < 0.001\), \(t\)-test.
ALTERED EPILEPTIC HIPPOCAMPAL GABA FUNCTION

TABLE 1. Summary of epilepsy-associated alterations in function of hippocampal neuron GABAA receptors

<table>
<thead>
<tr>
<th>Effect</th>
<th>Dentate</th>
<th>CA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA efficacy</td>
<td>↑↑</td>
<td>↓↓</td>
</tr>
<tr>
<td>GABA potency</td>
<td>↔</td>
<td>↑</td>
</tr>
<tr>
<td>Zinc blockade</td>
<td>↑↑</td>
<td>—</td>
</tr>
<tr>
<td>CNZ augmentation</td>
<td>↑↑</td>
<td>↓</td>
</tr>
<tr>
<td>ZOL augmentation</td>
<td>↑</td>
<td>ND</td>
</tr>
</tbody>
</table>

↑↑: >50% Elevation in response amplitude or potency. ↑: 25–50% Elevation in response amplitude or potency. ↓: >50% Decrease in response amplitude or potency. ↓↓: 25–50% decrease in response amplitude or potency. ↔: no effect. ND: not determined. GABA, γ-aminobutyric acid; CNZ, clonazepam; ZOL, zolpidem.

is evident in α1 compared with α2- and α3-containing GABAA Rs coexpressed with β- and γ-subunits (White and Gurley 1995). Epileptic DG GABAA R responses were 190% more sensitive to zinc blockade (Fig. 4). Furthermore, CNZ (100 nM) modulation of GABAA Rs was enhanced in epileptic DG neurons, whereas a BZ-receptor agonist was less effective (Figs. 7 and 8). This altered GABAA R pharmacology in DG neurons accompanying epileptogenesis could possibly be explained by an α-subunit switch in existing receptors, or incorporation of a new population of GABAA Rs containing α2 or α3 subunits, and exhibiting higher levels of BZ sensitivity. Epilepsy-associated enhanced incorporation of α2 subunits into GABAA Rs in DG (Kamphius et al. 1995), associated with overall decreased contribution of α1 and α2 subunits due to simple stoichiometry, would confer increased zinc sensitivity (White and Gurley 1995) and decreased efficacy of zolpidem relative to CNZ in augmenting GABA responses, resulting in a shift from BZ1 to BZ2 GABAA R pharmacology of these neurons. Epilepsy-associated enhanced CNZ augmentation in DG neurons could result from enhanced expression of the γ2 subunit (Kamphius et al. 1995), incorporation of new α2- or α3-containing receptors, or an α-subunit switch, because both α- and γ-subunits determine benzodiazepine modulation of GABAA Rs (reviewed in Macdonald and Olsen 1994). Epilepsy-associated decreased benzodiazepine sensitivity (Fig. 9) and enhanced GABA potency (Fig. 3) evident in CA1 neurons could be explained parsimoniously by reduced γ2 subunit expression in the residual receptors. GABA more potently activates GABAA Rs lacking a γ-subunit, and these receptors would not be modulated by benzodiazepines.

Table 1 summarizes the findings of the present study, illustrating the overall effects of induction of an epileptic state on functional GABA receptor properties in hippocampal DG and CA1 neurons. What is most effectively illustrated are the contrasting epilepsy-associated changes evident in these two cell types. GABA efficacy was significantly elevated in epileptic DG neurons, and reduced in epileptic CA1 neurons, with little change in GABA potency evident in DG and a significantly enhanced potency of GABA evident in CA1. Zinc blockade of GABA currents was significantly enhanced in epileptic DG neurons, and unaffected in epileptic CA1 neurons, whereas CNZ augmentation of GABA responses was enhanced in DG neurons and reduced in CA1 neurons after development of an epileptic condition. These novel differential alterations in functional postsynaptic GABA responses in these distinct populations of hippocampal neurons could translate into significant and distinct alterations in circuit properties accompanying epileptogenesis in these areas of the hippocampus.

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