Diminished allopregnanolone enhancement of $GABA_A$ receptor currents in a rat model of chronic temporal lobe epilepsy

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(Resubmitted 24 May 2001; accepted after revision 26 July 2001)

1. Neurosteroid modulation of $GABA_A$ receptors present on dentate granule cells (DGCs) acutely isolated from epileptic (epileptic DGCs) or control rats (control DGCs) was studied by application of GABA with or without the modulators and by measuring the amplitude of peak whole-cell currents.

2. In epileptic DGCs, GABA efficacy (1394 ± 277 pA) was greater than in control DGCs (765 ± 38 pA).

3. Allopregnanolone enhanced GABA-evoked currents less potently in epileptic DGCs ($EC_{50} = 92.7 \pm 13.4$ nM) than in control DGCs ($EC_{50} = 12.9 \pm 2.3$ nM).

4. Pregnenolone sulfate inhibited GABA-evoked currents with similar potency and efficacy in control and epileptic DGCs.

5. Diazepam enhanced GABA-evoked currents less potently in epileptic ($EC_{50} = 69 \pm 14$ nM) compared to the control DGCs ($EC_{50} = 29.9 \pm 5.7$ nM).

6. There were two different patterns of zolpidem modulation of $GABA_A$ receptor currents in the epileptic DGCs. In one group, zolpidem enhanced $GABA_A$ receptor currents but with reduced potency compared to the control DGCs ($EC_{50} = 134 \pm 20$ nM *vs.* $EC_{50} = 52 \pm 13$ nM). In the second group of epileptic DGCs zolpidem inhibited $GABA_A$ receptor currents, an effect not observed in control DGCs.

7. Epileptic DGCs were more sensitive to Zn^{2+} inhibition of $GABA_A$ receptor currents ($IC_{50} = 19 \pm 6$ μM) compared to control ($IC_{50} = 94.7 \pm 7.9$ μM).

8. This study demonstrates significant differences between epileptic and control DGCs. We conclude that (1) diminished sensitivity of $GABA_A$ receptors of epileptic DGCs to allopregnanolone can increase susceptibility to seizures; (2) reduced sensitivity to diazepam and zolpidem, and increased sensitivity to Zn^{2+} indicate that loss of allopregnanolone sensitivity is likely to be due to altered subunit expression of postsynaptic $GABA_A$ receptors present on epileptic DGCs; and (3) an inverse effect of zolpidem in some epileptic DGCs demonstrates the heterogeneity of $GABA_A$ receptors present on epileptic DGCs.

Temporal lobe epilepsy (TLE) is a common form of drug-refractory epilepsy in which resection of the hippocampus can render a majority of patients seizure-free (Spencer & Spencer, 1994). TLE is frequently accompanied by hippocampal sclerosis, which refers to neuronal loss and gliosis in CA1, CA3 and the hilus, while granule cells in the dentate gyrus are relatively preserved (Sloviter, 1994). It is believed that the principal cells of the dentate gyrus, the dentate granule cells (DGCs), gate propagation of paroxysmal activity through a tri-synaptic circuit in the hippocampus: (1) enthorinal cortex to dentate gyrus, (2) dentate gyrus to CA3 pyramidal cells via mossy fibres, and (3) CA3 to CA1 via Schafer collaterals (Walther *et al.* 1986; Lambert & Jones, 1989; Jones & Lambert, 1990; Lothman *et al.* 1992). However, the gating function of dentate gyrus can collapse (Behr *et al.* 1998) and it can generate robust paroxysmal discharges (Lothman *et al.* 1991; Lothman *et al.* 1992; Stringer & Lothman, 1989).

The epileptic dentate gyrus undergoes multiple changes in excitatory and inhibitory neurotransmission that can allow the breakdown of its function. For example, in the synaptic reorganization of the dentate gyrus, axons of DGCs sprout collaterals to the inner molecular layer of the dentate gyrus (Tauck & Nadler, 1985; Sutula *et al.* 1989; Houser, 1992), and excitatory synapses form between

DGCs and sprouted mossy fibres resulting in recurrent excitatory circuits (Wuarin & Dudek, 1996). The potency of NMDA is increased, as are the amplitude and duration of NMDA receptor-mediated EPSPs (Mody & Heinemann, 1987; Mody *et al.* 1988; Kohr & Mody, 1994).

Plasticity of γ-aminobutyric acid (GABA)-mediated inhibition may also contribute to the altered function of DGCs in TLE. Early studies on animal models of TLE demonstrated increased benzodiazepine binding in the dentate gyrus of kindled rats (Fanelli & McNamara, 1983; Shin *et al.* 1985) and increased Cl^- influx in synaptosomes isolated from dentate gyri of kindled rats (Titulaer *et al.* 1995*a*). In electrophysiological studies, increased inhibition in the dentate gyrus was demonstrated by increased paired pulse depression in kindling (de Jonge & Racine, 1987), which persisted for periods of up to 8 weeks following the last seizure (Oliver & Miller, 1985). More recent studies showed that the amplitude of $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) is increased in DGCs after kindling (Otis *et al.* 1994) and was associated with an increased number of $GABA_A$ receptors in DGCs in kindled rats (Nusser *et al.* 1998*a*). An increased density of $GABA_A$ receptors was found in DGCs in the pilocarpine model of TLE in rats (Gibbs *et al.* 1997), and in neurons isolated from hippocampi of patients with intractable chronic TLE (Brooks-Kayal *et al.* 1998). Despite enhancement of $GABA_A$ receptor currents in DGCs, the gating function of these neurons breaks down during seizures. A proposed mechanism for this breakdown is abnormal modulation of $GABA_A$ receptors by zinc (Zn^{2+}). Zn^{2+} decreased rise time, peak amplitude, and the decay time constant of mIPSCs in DGCs of kindled rats (Buhl *et al.* 1996). Similarly, in rats with lithium/pilocarpine-induced TLE, Zn^{2+} inhibits whole-cell $GABA_A$ receptor currents in DGCs (Gibbs *et al.* 1997; Brooks-Kayal *et al.* 1998).

In addition to Zn^{2+}, the $GABA_A$ receptor is modulated by neuroactive steroids, such as allopregnanolone and pregnenolone sulfate (PS) (Macdonald & Olsen, 1994). Neuroactive steroids are a group of compounds synthesized in the brain by glial cells from circulating steroid hormones (progesterone, oestrogen, testosterone, and cortisone) or *de novo* from cholesterol (Compagnone & Mellon, 2000). One of the most potent endogenous modulators of the $GABA_A$ receptor function is the tertahydro-derivative of progesterone, 3α-hydroxy-5α-pregnane-20-one (3α,5α-THP, or allopregnanolone) (Lambert *et al.* 1996). Physiological, nanomolar concentrations of allopregnanolone have an anti-convulsant effect (Belelli *et al.* 1989; Kokate *et al.* 1999*a*). PS is a natural sulfated derivative of pregnenolone. In contrast to allopregnanolone, PS potently inhibits $GABA_A$ receptors (Majewska *et al.* 1988). The potential physiological significance of PS was demonstrated in studies that assessed the spatial memory performance of cognitively impaired, aged rats. In these studies, cognitive impairment was correlated by bilateral intra-hippocampal injection of PS (Vallee *et al.* 1997). The neurosteroid modulation of $GABA_A$ receptors on dentate granule cells of naive and epileptic rats has not yet been studied and is the focus of the current study.

METHODS

Induction of TLE

All experimental procedures on rats were performed according to the protocol approved by the University of Virginia Animal Use and Care Committee. The method for inducing TLE in rats has been described in detail in the past (Lothman *et al.* 1989). Briefly, rats were anaesthetized with ketamine (50 mg kg^{-1}) and xylazine (40 mg kg^{-1}) and implanted with a pair of bipolar stimulating electrodes in the left posterior ventral hippocampus (AP 3.6, ML 4.0, DV 5.0 from dura; incisor bar at +5.0). After 1 week of recovery, the left hippocampus was stimulated with 10 s trains of 50 Hz, 1 ms, 400 mA biphasic square wave current pulses delivered every 13 s for 90 min to induce status epilepticus (SE) (Lothman *et al.* 1989). Approximately 4–6 weeks after the stimulation, the rats developed spontaneous limbic seizures with the motor component. For this study, seizures were documented either by continuous EEG recording or by direct observation of the spontaneous seizure (Bertram *et al.* 1997). The epileptic animals were killed at least 24 h after the last seizure, and DGCs were isolated from these rats. The responses from stimulated but non-epileptic rats were no different from age-matched naive rats so the data from these animals were pooled. All animals were housed individually under conditions of a 12 h:12 h light–dark cycle and had free access to food and water.

Acute isolation of DGCs

DGCs were isolated according to the protocol of Kay & Wong (1986), later modified (Kapur & Coulter, 1995). Rats were decapitated under halothane anaesthesia, and the brains were dissected free and chilled to 4 °C for 1 min in a piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (Pipes) buffer solution composed of (mM): NaCl 120, KCL 5, $CaCl_2$ 1.5, $MgCl_2$ 1, D-glucose 25, Pipes 20 (pH 7.0) (all chemicals from Sigma, St Louis, MO, USA, unless noted otherwise). The region containing the hippocampus was blocked. The brain was blot-dried, mounted on a vibratome (Camden Instruments, UK) stage with the caudal part facing up, and 400–500 μm coronal slices containing hippocampus were cut. The slices were allowed to recover for at least 1 h in Pipes solution before being transferred to an enzyme solution. Slices were incubated in continuously oxygenated Pipes solution, containing 3 mg ml^{-1} type XXIII protease enzyme from *Aspergillus oryzae*, at 32 °C for 45 min. The enzyme incubation was terminated by transferring the slice into enzyme-free Pipes solution, and after a minimum of 30 min of recovery, DGCs were isolated from caudal parts of both left and right ventral hippocampi. The dentate gyrus was dissected from the rest of the slice under a microscope and cut into 0.5 mm^3 chunks. These chunks were triturated through 0.5 mm and 0.3 mm diameter fire-polished glass pipettes and the isolated neurons were plated on 35 mm plastic Petri dishes. DGCs were identified by their typical oval shape and single process. DGCs acutely isolated from epileptic rats were referred to as 'epileptic DGCs' and those isolated from control animals were referred to as 'control DGCs'.

Whole-cell patch clamp recordings of $GABA_A$ receptor currents

Thin-walled borosilicate patch electrodes of 1.5 mm external diameter (Sutter Instruments, Novato, CA, USA) were pulled on a horizontal Flaming Brown P-97 model puller (Sutter Instruments) using a 2-stage pull to a final resistance of 6–8 MΩ. Patch electrodes were filled with recording solution containing (mM): Trizma

Table 1. Potency of allosteric modulators of $GABA_A$ receptor in control and epileptic DGCs

Drug	Epileptic DGCs	Control DGCs
GABA	32.6 ± 6.1 μM	29.2 ± 4.3 μM
Allopregnanolone	92.7 ± 13.4 nM*	12.9 ± 2.3 nM
PS	17.4 ± 2 μM	13.1 ± 1.6 μM
Diazepam	69 ± 14 nM*	29.9 ± 5.7 nM
Zolpidem	93.7 ± 6.5 nM*	44.2 ± 8.6 nM
Zn^{2+}	19 ± 6 μM*	94.7 ± 7.9 μM

Values are means ± S.E.M. * $P < 0.05$.

Table 2. Hill coefficients derived from the equation that best fitted the data

Drug	Epileptic DGCs	Control DGCs
GABA	0.8 ± 0.57	1.05 ± 0.17
Allopregnanolone	1.39 ± 0.24	1.33 ± 0.35
PS	0.64 ± 0.07	0.93 ± 0.23
Diazepam	1.2 ± 0.2	1.4 ± 0.24
Zolpidem	0.99 ± 0.55	1.4 ± 0.6
Zn^{2+}	−0.9 ± 0.35	−0.7 ± 0.22

Values are means ± S.E.M. The Hill slopes of the fits for GABA and various allosteric modulators of $GABA_A$ receptor were not significantly different.

phosphate (dibasic) 110, Trizma base 28, EGTA 11, $MgCl_2$ 2, $CaCl_2$ 0.5; pH 7.40; the osmolarity was 270–275 mosmol l^{-1}. ATP disodium salt, in a final concentration of 3 mM, was included in the intracellular solution before recording. The extracellular medium contained (mM): NaCl 155, KCl 3, $MgCl_2$ 1, $CaCl_2$ 3 and Hepes-Na^+ 10; pH 7.4. The osmolarity was 314–322 mosmol l^{-1}. Whole-cell currents were recorded at room temperature with an Axopatch 200A amplifier (Axon Instruments) and low-pass filtered at 2 kHz with an 8 pole Bessel filter prior to digitization, storage and display using standard patch clamp techniques (Hamill *et al.* 1981). Acutely isolated neurons were studied on the stage of an inverted Nikon microscope. Cell capacitance was compensated and the capacitance value was obtained from the potentiometer on the amplifier. Currents were displayed on-line on a Gould Viper TA11 digital chart recorder. Additionally, currents were recorded and stored on a Pentium II personal computer using the Axoscope 7.0 program (Axon Instruments) and digitized at 400 Hz. Peak currents were measured manually from the computer recordings and confirmed by chart paper recording.

Allopregnanolone (Tocris, Ballwin, MO, USA), PS, zolpidem (Research Biochemicals, Natick, MA, USA), and diazepam were dissolved in dimethysulphoxide (DMSO) and stock solutions were stored at −20°C. On the day of the experiment, stock solutions of drugs were diluted in extracellular solution. $ZnCl_2$ (Fluka, Buchs, Switzerland) was first dissolved in distilled water at a concentration of 50 mM and then diluted in the extracellular solution to a final concentration ranging from 1 μM to 1 mM. GABA was dissolved in the extracellular solution.

Drugs were applied to the DGCs with a modified U-tube 'multipuffer' application system (Bormann, 1992; Greenfield & Macdonald, 1996) with the tip of the application pipette placed 100–200 μm from the neuron. Data from each DGC were fitted to a four-parameter logistic equation (equation for a sigmoid curve):

$$I = I_{max}/(1 + 10^{\log EC_{50} - \log[drug]} \times \text{Hill slope}),$$

where I is the peak GABAR current at a given GABA concentration. The Hill slope, EC_{50}, and I_{max} (maximal current) were derived from the equation that best fitted the observed data by the least square fit method using a curve fitting program (Graphpad Prism 3.0) on an IBM PC-compatible computer. All values are reported as means ± S.E.M.

RESULTS

Increased efficacy of GABA in the epileptic DGCs

Whole-cell GABA-evoked currents were recorded from control and epileptic DGCs. GABA at 10 μM was applied to the DGC repeatedly, 3–5 times, and an increase in peak amplitude (run-up) occurred during the first several minutes of recordings, possibly due to the adjustment of the cell to the ATP load. To avoid the confounding effect of run-up, data collection was started after stable responses to GABA were achieved. Control and epileptic DGCs had similar capacitances (12–15 pF). GABA at 30 μM elicited significantly larger currents in the epileptic DGCs (838 ± 179 pA, $n = 5$,) than in control DGCs (443 ± 27 pA, $n = 6$, $P = 0.015$, Student's unpaired t test). In order to understand the mechanism of larger currents in epileptic DGCs, the GABA concentration response relationship was studied by applying multiple concentrations of GABA (1 μM to 1 mM) to epileptic and control DGCs. The amplitudes of peak currents recorded in response to each concentration of GABA were recorded, averaged and fitted to an equation for sigmoidal function (see Methods). The potency (EC_{50}) and efficacy (maximum current elicited) were derived from the equation with the best fit to the sigmoidal function in control and epileptic DGCs. EC_{50} of GABA in the epileptic DGCs was 32.6 ± 6 μM ($n = 5$), similar to that in the control DGCs −29.2 ± 4 μM, ($n = 6$, $P = 0.72$, unpaired t test; Fig. 1 and Table 1). However, the maximal $GABA_A$ receptor current recorded in epileptic DGCs, 1394 ± 277 pA, was larger than in control DGCs, 765 ± 38 pA (Fig. 1 and Table 1). In epileptic DGCs, the Hill coefficient was 0.8 ± 0.57 ($n = 5$), and in control DGCs it was 1.05 ± 0.17 ($n = 6$). The difference was not statistically significant ($P = 0.04$, unpaired t test; Table 2). This increased GABA efficacy in epileptic DGCs is most likely to reflect an increased density of $GABA_A$ receptors on epileptic DGCs, since the capacitance (and hence cell surface area) of control and epileptic DGCs was similar (12–15 pF).

Allopregnanolone enhancement of $GABA_A$ receptor currents

Allopregnanolone enhancement of $GABA_A$ receptor currents in control and epileptic DGCs was studied. In studies of augmentation of GABA responses by positive allosteric modulators such as allopregnanolone, 10 μM GABA was used to elicit control responses, since this concentration was 0.5 log units less than the EC_{50} in control and epileptic DGCs, and allowed discrimination of peak current enhancement. Allopregnanolone (10 nM)

enhancement of 10 μM GABA-evoked currents in epileptic DGCs was significantly diminished ($12.3 \pm 3\%$, $n = 5$), compared to that in control DGCs, ($52.3 \pm 6\%$, $n = 6$, $P = 0.001$, unpaired t test; Fig. 2*A* and *B*, and Table 1).

A detailed characterization of allopregnanolone enhancement of $GABA_A$ receptor-mediated currents was studied in control and epileptic DGCs by co-applying multiple concentrations (300 pM to 1 μM) of allopregnanolone with 10 μM GABA. The peak amplitude of currents elicited by application of 10 μM GABA alone was the control response and augmentation of current was measured as the percentage increase above control response. In order to avoid the effects of possible run-down on the actual modulation of GABA currents, GABA was applied before and after the application of each concentration of allopregnanolone. The amplitude of the current elicited by co-application of a given concentration of allopregnenolone and 10 μM GABA was compared to the mean current amplitude of those two GABA-elicited currents. When the amplitude of a current elicited by the second application of GABA was smaller than 90% of that elicited by the first application, the recording was excluded from further analysis.

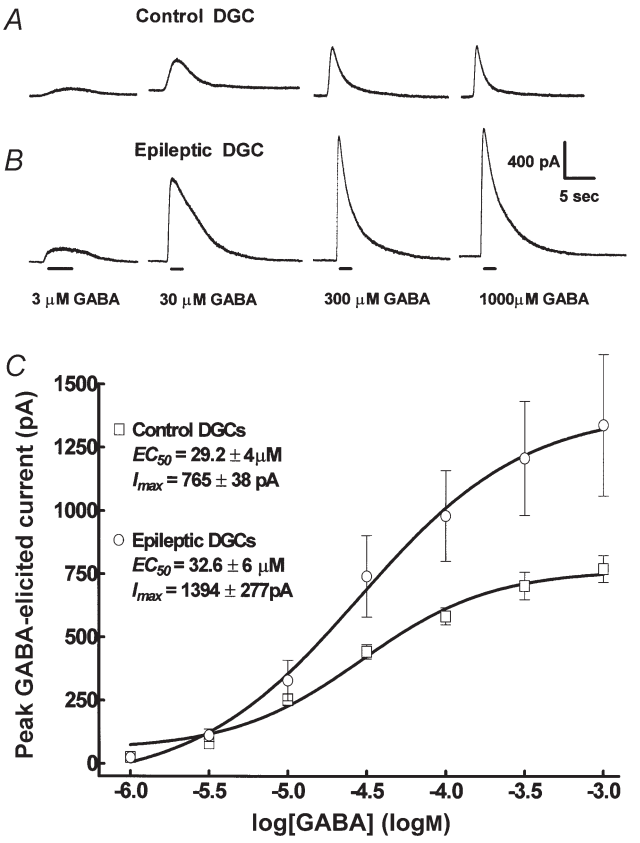

Figure 1. GABA-elicited currents in control and epileptic DGCs

A, in a control DGC, increasing concentrations of GABA (1 μM to 1 mM) elicited currents with increasing amplitude of peak current. *B*, the same concentrations of GABA evoked larger currents in an epileptic DGC then in control. In this and all subsequent figures concentration of the drug is indicated at the bottom of the traces. Duration of the drug application (bars) is indicated below the traces. *C*, concentration of GABA (log[GABA]) and GABA-elicited peak current (pA) relationships were plotted for control (□, $n = 6$) and epileptic (○, $n = 5$) DGCs. In this and all subsequent figures, each point represents the mean of the amplitude of peak current for a given concentration of GABA, and error bars show S.E.M. The data were fitted to the equation of a sigmoid curve (see Methods) and the lines indicate the best fit to the data. EC_{50} and the Hill coefficients were derived from the equation of the sigmoidal function that best fitted the data.

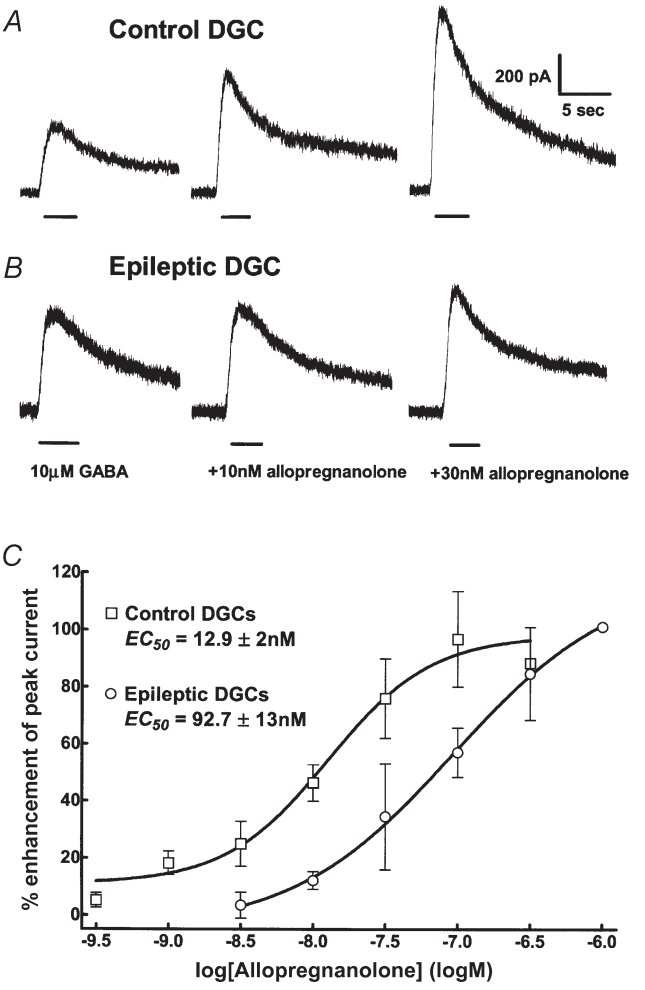

Figure 2. Allopregnanolone enhancement of GABA-elicited currents

A, in a control DGC, current elicited by 10 μM GABA compared to that elicited by co-application of 10 and 30 nM allopregnanolone with GABA. The peak current elicited by GABA was robustly enhanced by 10 and 30 nM allopregnanolone. *B*, in contrast, the same concentrations of allopregnanolone poorly enhanced $GABA_A$ receptor currents in an epileptic DGC. *C*, allopregnanolone concentration–response curves were obtained for control (□, $n = 6$) and epileptic DGCs (○, $n = 5$). Note that in epileptic DGCs the curve shifted to the right.

In epileptic DGCs, there was a significant right shift of the allopregnanolone concentration–response curve. The potency (EC_{50}) of allopregnanolone enhancement of GABA-evoked currents was markedly diminished in epileptic DGCs (92.7 ± 13 nM, $n = 5$) compared to that in control DGCs (12.9 ± 2 nM, $n = 6$, $P = 0.002$, *t* test; Fig. 2 and Table 1). In the epileptic DGCs, the Hill coefficient was 1.39 ± 0.24, similar to that in control DGCs, 1.33 ± 0.35 (Table 2). Allopregnanolone efficacy was similar in control and epileptic DGCs; the maximal augmentation of GABA current by allopregnanolone was 96 ± 13.4% in epileptic DGCs and 106.5 ± 10.8% in control DGCs ($n = 5$. $P = 0.56$, unpaired *t* test).

PS inhibition of $GABA_A$ receptor currents

PS modulation of $GABA_A$ receptor currents was compared in epileptic and control DGCs. In epileptic DGCs, PS (30 μM) inhibited 30 μM GABA-evoked currents to 24.4 ± 5% of that evoked by 30 μM GABA alone ($n = 10$). In control DGCs, peak current amplitude evoked by co-application of 30 μM PS and 30 μM GABA was 27.4 ± 4% ($n = 9$) of that evoked by 30 μM GABA alone. There was no difference in inhibition of GABA current by 30 μM PS in epileptic and control DGCs ($P = 0.64$, unpaired *t* test). The PS concentration (300 nM to 300 μM)–$GABA_A$ receptor current inhibition relationship was studied further in epileptic and control DGCs. The potency and efficacy of PS inhibition of $GABA_A$ receptor currents were also similar in epileptic and control DGCs. In epileptic DGCs, the IC_{50} for PS was 17.4 ± 2 μM ($n = 10$) and in control DGCs it was 13.1 ± 1 μM ($n = 14$; Fig. 3 and Table 1). The maximal inhibition of GABA currents by PS was similar in epileptic DGCs (7.2 ± 0.7%, $n = 7$) and control DGCs (7.6 ± 1.9%, $n = 6$, $P = 0.82$, unpaired *t* test; Fig. 3). The Hill coefficient of the fit was 0.93 ± 0.23 in control DGCs, and 0.64 ± 0.07 ($n = 10$, $P = 0.72$; Table 2) in epileptic DGCs. Therefore, neither the potency nor the efficacy of PS inhibition of $GABA_A$ receptor-mediated currents is altered in DGCs in chronic temporal lobe epilepsy.

Diazepam enhancement of $GABA_A$ receptor currents

One possible mechanism of diminished allopregnanolone sensitivity of $GABA_A$ receptors in epileptic DGCs could be the altered subunit composition of the receptor. Allosteric modulation of GABA receptor function by allopregnanolone is markedly decreased when the α4 (Smith *et al.* 1998*a*) or δ subunits are expressed (Zhu *et al.* 1996). Both α4 and δ subunits are known to reduce diazepam sensitivity of $GABA_A$ receptors (Saxena & Macdonald, 1994). Diazepam (30 nM) enhanced 10 μM GABA-evoked currents by 19.9 ± 3.9% ($n = 8$) in epileptic DGCs (Fig. 4) and by 32 ± 2.4% ($n = 8$, $P = 0.014$, *t* test) in control DGCs. A detailed diazepam concentration (3 nM to 1 μM)–response relationship was studied to further characterize this difference in diazepam effect in control and epileptic DGCs. The EC_{50} for diazepam enhancement of GABA-evoked currents in epileptic DGCs was 69 ± 14 nM ($n = 9$) and 29.9 ± 5.7 nM in control DGCs ($n = 8$, $P = 0.04$, *t* test; Fig. 4 and Table 1). The Hill coefficient remained unchanged, 1.4 ± 0.24 *versus* 1.2 ± 0.2 (Table 2). The maximal diazepam-induced enhancement of 10 μM GABA-elicited currents was similar in epileptic (61.2 ± 7%, $n = 9$) and control DGCs (63.6 ± 3%, $n = 8$, $P = 0.77$, paired *t* test). Therefore, the diazepam concentration response–curve is shifted to the right in epileptic DGCs compared to controls.

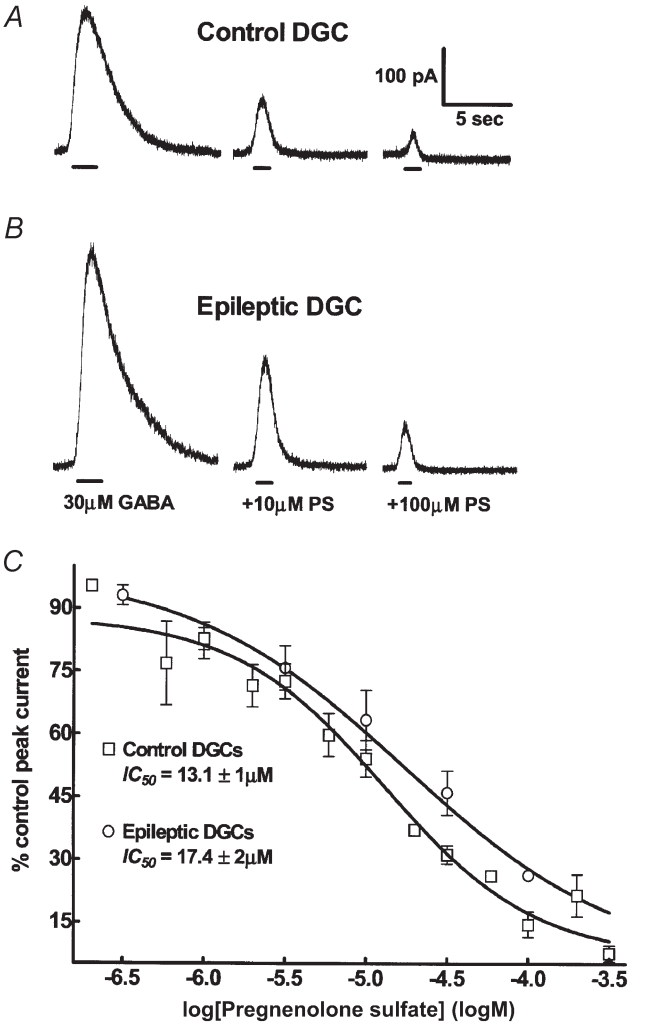

Figure 3. Inhibition of GABA-elicited currents by PS

A, in a control DGC, current elicited by 30 μM GABA compared to that elicited by co-application of 10 and 100 μM pregnenolone sulfate (PS) and GABA. The peak current elicited by GABA was strongly inhibited by 30 and 100 μM PS. *B*, in an epileptic DGC, inhibition of GABA currents by the same concentrations of PS was not different from the control cell. *C*, PS concentration–DGC $GABA_A$ receptor current relationships. PS concentration–response curves were obtained for control (□, $n = 14$) and epileptic DGCs (○, $n = 10$).

Zolpidem modulation of $GABA_A$ receptor function in the epileptic DGCs

Zolpidem is a subunit-selective benzodiazepine type I receptor agonist with high affinity for α1-subunit-containing $GABA_A$ receptors. The α1 subunit-containing $GABA_A$ receptors are robustly enhanced by allopregnanolone (Shingai *et al.* 1991), but suppression of the subunit diminishes allopregnanolone sensitivity (Brussaard *et al.* 1997). In this study, we identified two different patterns of zolpidem modulation of $GABA_A$ receptor function in epileptic DGCs. In one group of epileptic DGCs, zolpidem (100 nM) enhanced 10 μM GABA-evoked currents by 16.2 ± 10% ($n = 5$), while in control DGCs, the enhancement was 54.1 ± 9.8%, ($n = 5$, $P = 0.02$, t test). In the second group of epileptic DGCs ($n = 3$), 100 nM zolpidem inhibited 10 μM GABA-evoked currents by 8.1 ± 3.3%. It is important to note that epileptic DGCs, in which zolpidem inhibited $GABA_A$ receptor currents, were isolated from the same animals in which zolpidem enhanced GABA-evoked currents. Zolpidem did

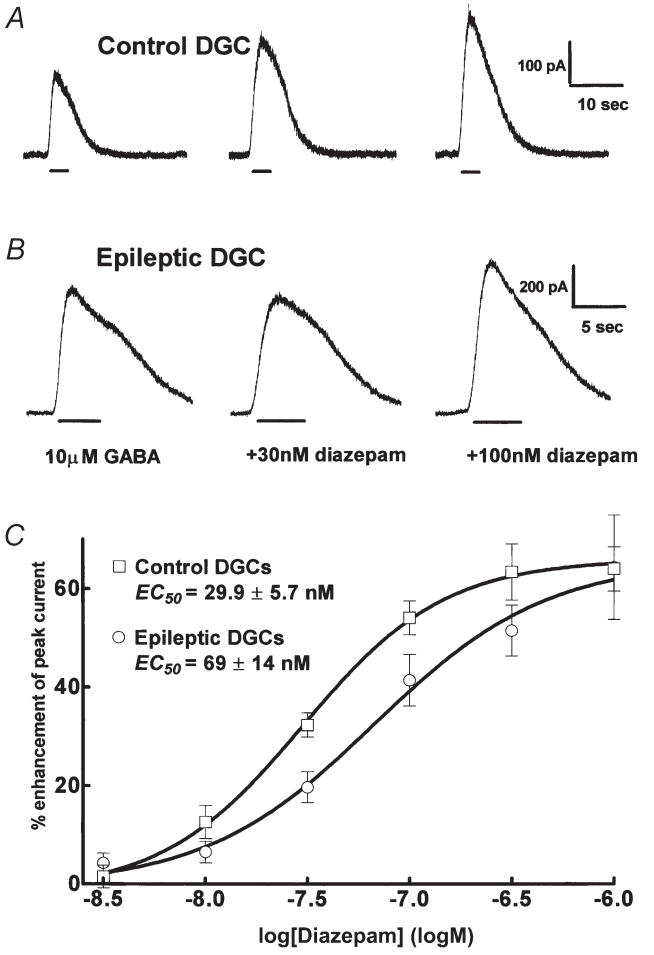

Figure 4. Diazepam enhancement of 10 μM GABA-elicited currents

A, in a control DGC co-application of 30 and 100 nM diazepam with 10 μM GABA strongly enhanced 10 μM GABA-elicited currents. *B*, in an epileptic DGC, 30 nM diazepam did not enhance GABA-elicited current, and 100 nM diazepam was less effective than in the control cell. *C*, diazepam concentration–DGC $GABA_A$ receptor current relationships. Diazepam concentration–response curves were obtained for control (□, $n = 8$) and epileptic DGCs (○, $n = 9$). The maximal enhancement of $GABA_A$ receptor currents remained similar for control and epileptic cells.

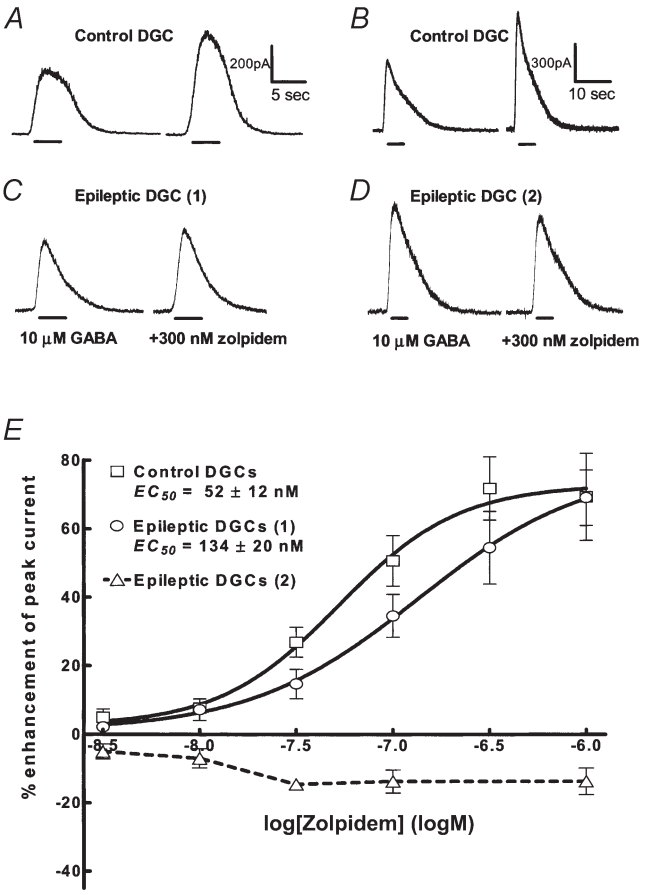

Figure 5. Heterogeneity of zolpidem responses in epileptic DGCs

A and *B*, in the two control DGCs co-application of 10 μM GABA with 300 nM zolpidem robustly enhanced 10 μM GABA-elicited current. *C*, in one group of epileptic DGCs 300 nM zolpidem enhancement of 10 μM GABA-elicited current was reduced compared to control DGCs. *D*, in a group of epileptic DGCs 300 nM zolpidem inhibited 10 μM GABA-elicited current, as demonstrated by the current traces. *E*, zolpidem concentration–DGC $GABA_A$ receptor current relationships. Zolpidem concentration–response curves were obtained for control (□, $n = 7$) and epileptic DGCs. Note that in one population of epileptic DGCs (○, $n = 7$) zolpidem enhanced $GABA_A$ receptor currents, whereas in the other population (△, dashed line, $n = 8$) zolpidem inhibited GABA currents.

not inhibit GABA-evoked currents in any control DGCs tested. Each group of epileptic DGCs was evaluated in detail.

In a group of eight epileptic DGCs, the relationship between zolpidem concentration (3 nM to 1 μM) and the enhancement of $GABA_A$ receptor currents was evaluated. In these epileptic DGCs, zolpidem enhanced GABA currents with lower potency ($EC_{50} = 134 \pm 20$ nM) than in control DGCs ($EC_{50} = 52.2 \pm 12.6$ nM, $n = 6$, $P = 0.007$, t test; Fig. 5 and Table 1). The Hill coefficient was 0.99 ± 0.55 in epileptic DGCs and it was 1.4 ± 0.6 ($n = 8$) in control DGCs (Table 2). In these epileptic DGCs, maximal enhancement of 10 μM GABA-evoked currents by zolpidem was 61.3 ± 12.4%, similar to that in the control DGCs (66.9 ± 9%, $n = 6$, $P = 0.64$, t test). In this group of epileptic DGCs, the zolpidem concentration–response curve was shifted to the right compared to control DGCs.

Zolpidem inhibition of GABA currents in the second group of epileptic DGCs was also characterized further ($n = 8$). Zolpidem inhibition occurred at 10 nM (9.4 ± 1.7%) and increased insignificantly at 1 μM (14.6 ± 2.1%, $P = 0.69$). These results indicated that zolpidem inhibited $GABA_A$ receptor currents in the second group of epileptic DGCs. This pattern of zolpidem modulation is qualitatively different from that found in control and other epileptic DGCs since zolpidem consistently enhanced $GABA_A$ receptor currents in these neurons.

Zn^{2+} modulation of $GABA_A$ receptor currents in epileptic DGCs

Increased Zn^{2+} inhibition of GABA-evoked currents in DGCs was reported in the kindling model (Buhl *et al.* 1996), in the lithium/pilocarpine model of epilepsy (Gibbs *et al.* 1997) and in DGCs isolated from hippocampi of patients with intractable TLE (Brooks-Kayal *et al.* 1998; Shumate *et al.* 1998). Zn^{2+} (30 μM) inhibited peak amplitudes of 30 μM GABA-elicited currents more profoundly in epileptic DGCs (44.2 ± 3%, $n = 6$) compared to control DGCs (32.2 ± 3.3%, $n = 7$, $P = 0.024$, t test; Fig. 6). The Zn^{2+} concentration (1 μM to 1 mM)–$GABA_A$ receptor current inhibition relationship was studied in epileptic and control DGCs. In control, the IC_{50} was 94.7 ± 7.9 μM, and the Hill coefficient was -0.7 ± 0.2 ($n = 11$; Table 2); in epileptic DGCs, the IC_{50} was 19 ± 6 μM, and the Hill coefficient was -0.9 ± 0.35 ($n = 9$, $P = 0.02$, t test). These results indicated that the potency of Zn^{2+} in inhibiting $GABA_A$ receptor currents was increased in epileptic DGCs. Maximal inhibition of $GABA_A$ receptor currents by $ZnCl_2$ tended to be higher in epileptic DGCs compared to control DGCs. However, the difference did not reach a statistically significant value. In control DGCs, 300 μM $ZnCl_2$ inhibited GABA currents by 67.7 ± 2.6% ($n = 5$). In epileptic DGCs, the same concentration of $ZnCl_2$ inhibited GABA currents by 77.5 ± 2.6% ($n = 5$, $P = 0.08$, t test).

DISCUSSION

This study demonstrated that (1) the efficacy of GABA is increased in epileptic DGCs compared to the control DGCs; (2) the sensitivity of $GABA_A$ receptors to allopregnanolone enhancement is markedly reduced in epileptic DGCs, whereas PS-mediated inhibition of $GABA_A$ receptor currents remains unchanged; (3) the sensitivity to diazepam enhancement of $GABA_A$ receptor currents is diminished in epileptic DGCs compared to controls; (4) there are two groups of epileptic DGCs with

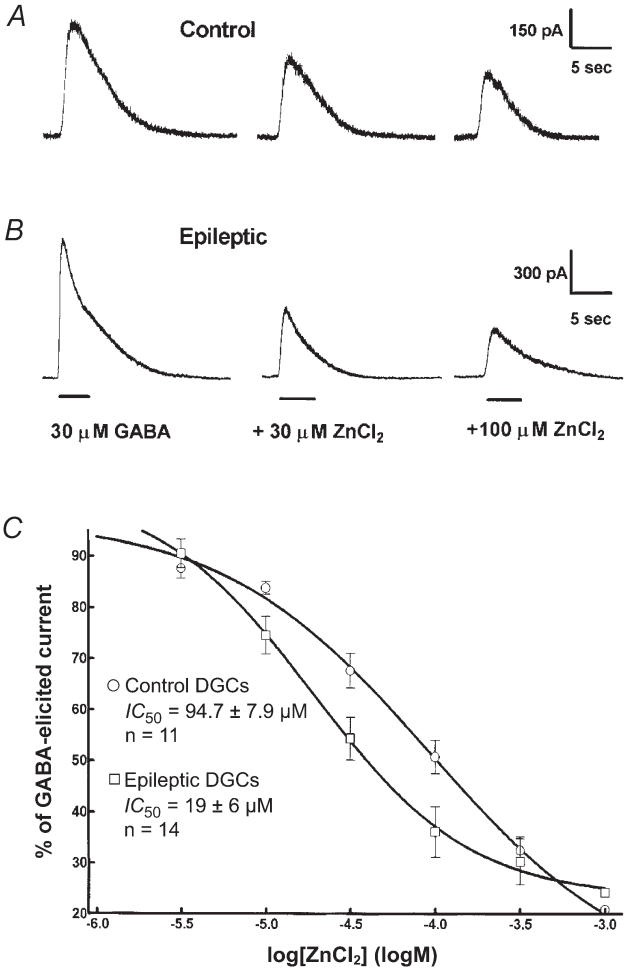

Figure 6. Zn^{2+} inhibition of GABA-elicited currents

A, co-application of 30 and 100 μM Zn^{2+} with 30 μM GABA caused inhibition of 30 μM GABA-elicited currents in a control DGC, *B*, inhibition was more profound in an epileptic DGC. *C*, Zn^{2+} concentration–DGC $GABA_A$ receptor current relationships. Concentration–response curves were obtained for control (○, $n = 11$) and epileptic DGCs (□, $n = 14$). Note the increased potency of Zn^{2+} in inhibiting of $GABA_A$ receptor currents, evoked in epileptic DGCs. The maximal enhancement of $GABA_A$ receptor currents remained similar for control and epileptic cells.

respect to modulation of $GABA_A$ receptors by zolpidem: in one there was decreased augmentation of GABA currents by zolpidem compared to the control, whereas in the other zolpidem inhibited $GABA_A$ receptor currents; and (5) Zn^{2+} inhibition of $GABA_A$ receptors is increased in epileptic DGCs.

We studied the properties of $GABA_A$ receptors on epileptic DGCs studied in acutely isolated neurons. Acute isolation of neurons has certain advantages and limitations. A clear benefit is that detailed concentration–response relationships and the potency and efficacy of various drugs can be studied in individual neurons. A drawback is that enzymatic treatment followed by mechanical isolation of differentiated neurons may damage synaptic elements, which are better preserved in slice preparations. However, some key findings made in slice preparations, such as increased $GABA_A$ receptor currents (Nusser *et al.* 1998*a*) and increased Zn^{2+} sensitivity of $GABA_A$ receptor-mediated currents in the dentate gyrus in kindling (Buhl *et al.* 1996) have been replicated in acutely isolated neurons (Gibbs *et al.* 1997; Shumate *et al.* 1998) It is therefore likely that the findings regarding neurosteroids would be valid in a more intact slice preparation.

Acute isolation of neurons may shift the ratio of extrasynaptic to synaptic receptors present on an acutely isolated cell towards more extrasynaptic receptors. In cerebellar granule cells, extrasynaptic receptors, which mainly mediate tonic inhibition, contain the δ subunit. (Nusser *et al.* 1998*b*). Since the δ subunit may alter sensitivity of $GABA_A$ receptors to neuroactive steroids (Zhu *et al.* 1996), it is possible that the diminished allopregnanolone sensitivity observed in the current study is due to a shift in the ratio of extrasynaptic to synaptic receptors. However, since the protocol for the cell isolation was identical for both control and epileptic dentate gyri, it appears unlikely that the procedure of isolation would have resulted in a group-specific altered proportion of $GABA_A$ receptors. Clearly, it is important to localize specific receptor subunits in epileptic DGCs and that is the subject of a separate ongoing study.

The augmentation and inhibition of $GABA_A$ receptor currents were studied by co-application of drugs with 10 and 30 μM GABA, respectively, because we intended to detect differences in the affinity and potency of the drug between control and epileptic cells. These concentrations are useful for studying the pharmacological modulation of $GABA_A$ receptors but do not mimic the GABA concentration in the synaptic cleft.

Loss of allopregnanolone sensitivity may increase susceptibility to seizures

This study demonstrated that $GABA_A$ receptor currents are less sensitive to allopregnanolone enhancement in epileptic DGCs compared to control DGCs. Allopregnanolone is an endogenous anticonvulsant (Belelli *et al.* 1989; Kokate *et al.* 1999*a*) that potentiates the antiseizure effect of flurazepam in mice (Deutsch *et al.* 1996) and its intraperitoneal injection evoked sleep in a manner similar to that of benzodiazepines (Lancel & Faulhaber, 1996). Intraperitoneal injections of allopregnanolone significantly increased the dose of NMDA necessary to induce seizures in rats (Budziszewska *et al.* 1998) and subcutaneous injections of allopregnanolone increased the latency of kainic acid-induced seizures (Frye & Scalise, 2000). This study also found that the potency and efficacy of PS remained unchanged. PS is a physiologically active sulfated derivative of pregnenolone. Endogenous PS in the hippocampus is believed to enhance memory, and age-related decline in memory function of rats correlates well with decline in hippocampal PS levels. A number of studies have shown that the physiological action of PS is reciprocal to that of allopregnanolone. PS has convulsant properties (Kokate *et al.* 1999*b*), inhibits $GABA_A$ receptor currents (Majewska *et al.* 1988), enhances NMDA currents (Bowlby, 1993), and exacerbates NMDA-induced death of cultured hippocampal neurons (Weaver *et al.* 1998). Sustained inhibition of GABA currents by PS, in parallel with its ability to enhance NMDA-mediated currents, could shift an excitatory/inhibitory balance toward increased excitation. When augmentation of $GABA_A$ receptor-mediated currents by allopregnanolone is diminished in epileptic DGCs, this increased excitation can trigger a seizure.

Neurosteroid modulation of $GABA_A$ receptors may be of particular relevance in the catamenial exacerbation of seizures. Catamenial exacerbation of epilepsy is characterized by recurrent increased frequency of seizures at specific times during the menstrual cycle in association with rising and falling levels of progesterone and oestrogen. Oestrogen is known to be proconvulsant; progesterone is anticonvulsant (for review, see Herzog, 1999). Progesterone exerts its anticonvulsant effects largely by its conversion to allopregnanolone. A blockade of the enzymatic conversion of progesterone to allopregnanolone by finasteride resulted in a loss of the anticonvulsant properties of progesterone against pentilentetrazolium-induced seizures in mice (Kokate *et al.* 1999*a*). It was suggested that the catamenial exacerbation of seizures was due to rapid withdrawal from allopregnanolone, which was the result of the rapid decline of serum levels of progesterone prior to menses (Reddy & Rogawski, 2000). Treatment of pseudopregnant rats with the 5α reductase inhibitor finateride, which blocks synthesis of allopregnanolone from progesterone, resulted in increased susceptibility to pentylentetrazol-induced seizures (Reddy *et al.* 2001). The findings of the current study suggest that, in addition to changes in sex hormone concentrations during the menstrual cycle, loss of allopregnanolone enhancement of $GABA_A$ receptor function may contribute to the catamenial exacerbation of seizures.

Diazepam modulation

The present study found that the potency of diazepam enhancement of $GABA_A$ receptor currents was diminished in epileptic DGCs compared to that in controls. The efficacy remained unchanged. Diazepam and clonazepam are 'broad spectrum' benzodiazepines. That is, they bind to both BZ type 1 and BZ type 2 benzodiazepine receptors. In contrast to the present study, Gibbs *et al.* (1997) found that 100 nM clonazepam enhanced 10 μM GABA-evoked currents more robustly in epileptic DGCs compared to control DGCs. It is difficult to compare the results of the two studies further because it is unclear whether the potency or efficacy of clonazepam is increased in the lithium/pilocarpine model. In addition to chronic TLE, there are several other conditions where the brain is more susceptible to seizures, commonly referred to as hyper-excitable states. Withdrawal from alcohol is known to increase seizure susceptibility in humans and in experimental animals. A specific diazepam-insensitive $GABA_A$ receptor is expressed in hippocampal neurons during alcohol withdrawal (Mhatre *et al.* 1988; Mhatre & Ticku, 1989). Increased seizure activity occurs in epileptic women during the premenstrual period presumably due to physiological withdrawal of progesterone (Smith *et al.* 1998*a*) and hippocampal $GABA_A$ receptors are rendered diazepam insensitive during progesterone withdrawal (Smith *et al.* 1998*b*). Immature brains are more susceptible to seizures than adult brains and $GABA_A$ receptors present on DGCs of neonatal rats are insensitive to diazepam (Kapur & Macdonald, 1999). Finally, during prolonged seizures, $GABA_A$ receptors expressed by DGCs rapidly rendered diazepam insensitive (Kapur & Macdonald, 1997). This benzodiazepine insensitivity may lower seizure threshold since there is some evidence for the existence of an endogenous benzodiazepine agonist in the brain (Rothstein *et al.* 1992*a*, *b*).

Zolpidem modulation

There were two populations of epileptic DGCs. In one, zolpidem augmented GABA-evoked currents, but less than in control DGCs, whereas in the other zolpidem inhibited $GABA_A$ receptor currents. Zolpidem never inhibited $GABA_A$ receptor currents in control DGCs. The findings in the first group of DGCs were similar to those of Gibbs *et al.* (1997) and Brooks-Kayal *et al.* (1998) in lithium/pilocarpine-induced TLE. In epileptic DGCs, augmentation of $GABA_A$ receptor currents by 100 nM zolpidem was significantly diminished compared to the controls (Gibbs *et al.* 1997).

Zolpidem inhibited $GABA_A$ receptor currents in one population of epileptic DGCs, whereas it uniformly augmented GABA currents in control cells. While zolpidem-insensitive receptors occur in CA1 pyramidal neurons, such receptors have not been described in DGCs in the past. This finding suggested a functional heterogeneity of $GABA_A$ receptors present on epileptic DGCs. One of the factors mediating this functional heterogeneity of $GABA_A$ receptors may be increased neurogenesis in DGCs in epilepsy. Compared with other hippocampal areas, the unique property of DGCs is that they undergo prolonged postnatal neurogenesis (Altman & Das, 1966). Seizures in the pilocarpine model of TLE, as well as in perforant path stimulation, produce a marked increase in DGC proliferation and subsequent migration (Parent *et al.* 1997). This phenomenon might contribute to an increased number of immature DGCs in epilepsy associated with the functional properties of $GABA_A$ receptors found in DGCs in neonatal animals. Neonatal DGCs have pharmacological properties similar to those in adult epilepsy including insensitivity to diazepam (Kapur & Macdonald, 1999).

Increased number of $GABA_A$ receptors

The finding that GABA elicited larger currents in DGCs isolated from epileptic animals, whereas its potency remained the same as in the control cells, was likely to be due to the increased number of $GABA_A$ receptors on epileptic DGCs. In the present study, it is conceivable that due to the relatively slow rate of drug application by the U tube device (10–90 rise time of 50 ms; Greenfield & Macdonald, 1996) a certain fraction of $GABA_A$ receptors was desensitized before the current peaked. Low concentrations of GABA cause pronounced desensitization of $GABA_A$ receptors (Celentano & Wong, 1994) and reduce availability of $GABA_A$ receptors (Overstreet *et al.* 2000) thereby reducing the total number of receptors contributing to the whole-cell response. However, since the same application system was used in control and epileptic DGCs, peak $GABA_A$ receptor currents would be underestimated with similar ratios in both preparations.

Several previous studies using other experimental models of epilepsy have demonstrated increased GABA-mediated inhibition in the dentate gyrus. Paired pulse inhibition, a measure of recurrent inhibition, increasesd in dentate gyrus in the kindling model (Tuff *et al.* 1983; de Jonge & Racine, 1987) and persisted for periods of up to 8 weeks following the last seizure (Oliver & Miller, 1985). Receptor binding studies demonstrated increased benzodiazepine binding to synaptosomes isolated from the dentate gyrus of kindled animals (McNamara *et al.* 1980; Fanelli & McNamara, 1983; Shin *et al.* 1985). Binding of [^{3}H]muscimol was significantly increased in dentate gyrus, whereas it was decreased in the CA1 area of kindled rats (Titulaer *et al.* 1994). Muscimol induced increased uptake of labelled Cl^- in synaptosomes isolated from the dentate gyri of kindled rats (Titulaer *et al.* 1995*a*). A similar increase of [^{3}H]flunitrazepam (Valdes *et al.* 1982; Shin *et al.* 1985; Titulaer *et al.* 1995*c*) and [^{3}H]*t*-butylbicyclophosphorothionate (Titulaer *et al.* 1995*b*) binding was found in DGCs of kindled rats. In Lothman's model of TLE, polysynaptic IPSPs are reduced or eliminated in the CA1 area, whereas they are unchanged in the dentate gyrus (Mangan *et al.* 1995)

indicating that GABA-mediated inhibition is preserved in the dentate gyrus in this model of TLE.

More directly comparable to the present study are published whole-cell voltage clamp recordings from DGCs in rats made epileptic by the lithium/pilocarpine injection. $GABA_A$ receptor currents were increased in DGCs acutely isolated from rats in the lithium/pilocarpine model of TLE (Gibbs *et al.* 1997) and from the dentate gyri of patients who underwent temporal lobectomy (Gibbs *et al.* 1996). An increased number of functionally active $GABA_A$ receptors had been proposed as a main factor contributing to the increased amplitude of mIPSCs in the dentate gyrus of kindled rats (Otis *et al.* 1994). An increased number of $GABA_A$ receptors on DGCs after kindling was confirmed by immunogold staining (Nusser *et al.* 1998*a*).

Increased zinc sensitivity

The present study confirms the previous findings of Buhl *et al.* (1996) and Gibbs *et al.* (1997) that $GABA_A$ receptors present on epileptic DGCs have enhanced susceptibility to Zn^{2+} inhibition. It has been demonstrated that 200 μM Zn^{2+} diminished the frequency and enhanced the decay of mIPSCs recorded from DGCs of kindled rats whereas it does not alter the decay kinetics of mIPSCs in control slices. In addition, Zn^{2+} inhibition of $GABA_A$ receptor currents is markedly increased in DGCs acutely isolated from rats with pilocarpine-induced TLE compared to controls (Gibbs *et al.* 1997). These seminal studies have led to the formulation of the zinc hypothesis (Buhl *et al.* 1996; Coulter, 1999), which proposes a mechanism to explain the collapse of inhibition in epileptic DGCs. Furthermore, Zn^{2+}-sensitive $GABA_A$ receptors are found on DGCs isolated from hippocampi of humans with temporal lobe epilepsy (Gibbs *et al.* 1996). In animal models with TLE, zinc-containing mossy fibres sprout new recurrent connections onto granule cells. Based on the proximity of zinc-containing mossy fibres and zinc-sensitive postsynaptic $GABA_A$ receptors mediating IPSCs, Buhl *et al.* (1996) proposed that during intense activity, zinc released from sprouted mossy fibres spills into inhibitory synapses and inhibits action of GABA on its receptor, leading to a collapse of inhibition in the epileptic dentate gyrus. The present study confirms that in another model of temporal lobe epilepsy, $GABA_A$ receptors on DGCs have increased sensitivity to Zn^{2+}.

Mechanisms of altered $GABA_A$ receptor properties

Studies on recombinant as well as native $GABA_A$ receptors have shown that expression of α subunits is necessary to maintain allopregnanolone sensitivity which varies depending on the type of expressed α subunit. In recombinant $GABA_A$ receptors, allopregnanolone has maximal effect in the presence of an α1 subunit; and the presence of the γ2 subunit increases allopregnanolone potency (Shingai *et al.* 1991) A dramatic decline of $GABA_A$ receptor sensitivity to allopregnanolone occurred in hippocampal CA1 pyramidal neurons when an α4 subunit was over-expressed; normal sensitivity is restored after intrahippocampal infusion of an α4 subunit antisense deoxynucleotide (Smith *et al.* 1998*a*). β subunits do not alter the sensitivity of recombinant $GABA_A$ receptors to allopregnanolone (Maitra & Reynolds, 1998). The effect of the δ subunit expression on neurosteroid sensitivity remains uncertain. Expression of the δ subunit in recombinant $GABA_A$ receptors results in the loss of sensitivity to the analogue of allopregnanolone – 3α,21-dihydroxy-5α-pregnan-20-1 (THDOC; Zhu *et al.* 1996). In contrast, in mice lacking the δ subunit, there is a marked reduction of sensitivity to the sedative and anxiolytic effects of neurosteroids (Mihalek *et al.* 1999). This effect was the opposite of that predicted by recombinant receptor studies.

The altered expression of α1, α4 or δ subunits might explain the reduced sensitivity of $GABA_A$ receptors to allopregnanolone on epileptic DGCs. These subunits are also known to alter the diazepam, zolpidem and Zn^{2+} sensitivity of $GABA_A$ receptors (Macdonald & Olsen, 1994) and changes in the combination of the subunits may explain concurrent changes in the sensitivity to these drugs in epileptic DGCs. In naive rats, DGCs expressed α1, α2, α4, and δ subunit mRNA (Wisden *et al.* 1992; Sperk *et al.* 1997) and immunoreactivity for these subunits has been documented in the rat dentate gyrus (Sperk *et al.* 1997). Diminished α1 subunit mRNA expression and increased α4 and δ subunit mRNA expression in DGCs of epileptic rats has been reported in the lithium/pilocarpine model (Brooks-Kayal *et al.* 1998).

In conclusion, diminished sensitivity of DGCs to allopregnanolone and sustained sensitivity to PS in TLE, in combination with other well-described pathological changes, are likely to increase susceptibility to seizures. A detailed analysis of the functional properties of $GABA_A$ receptors on epileptic DGCs revealed a complex picture. Past studies have emphasized two properties of these receptors, namely, increased GABA efficacy and enhanced zinc sensitivity. In addition to these changes, the present study demonstrated diminished sensitivity of epileptic DGC $GABA_A$ receptors to allopregnanolone and diazepam and demonstrated heterogeneity of $GABA_A$ receptor sensitivity to zolpidem in epileptic DGCs.

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Acknowledgements

This project was sponsored by the American Epilepsy Society with support from the Milken Family Foundation to Z.M. and NINDS grants KO2-NS 02081 and RO1 NS40337 to J.K. We thank Dr Kevin Kelly and Dr Patrick Mangan for editing the manuscript and John Williamson for producing and monitoring epileptic rats.

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