Pharmacological plasticity of GABA<sub>A</sub> receptors at dentate gyrus synapses in a rat model of temporal lobe epilepsy

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In the lithium–pilocarpine model (Li-pilocarpine) of temporal lobe epilepsy, GABA<sub>A</sub> receptor-mediated inhibitory postsynaptic currents (GABA<sub>A</sub> IPSCs) were recorded in dentate gyrus granule cells (GCs) from adult rat hippocampal slices. The properties of GABA<sub>A</sub> IPSCs were compared before and after superfusion of modulators in control conditions (Li-saline rats) and in Li-pilocarpine rats 24–48 h and 3–5 months (epileptic rats) after status epilepticus (SE). The mean peak amplitude of GABA<sub>A</sub> IPSCs increased by about 40% over Li-saline values in GCs 24–48 h after SE and remained higher in epileptic rats. In Li-pilocarpine rats, studied at 24–48 h after SE, diazepam (1 µm) lost 65% of its effectiveness at increasing the half-decay time ($T_{50\%}$) of GABA<sub>A</sub> miniature IPSCs (mIPSCs). Diazepam had no effects on mIPSC $T_{50\%}$ in epileptic rats.

The benzodiazepine ligand flumazenil (1 µM), acting as an antagonist in Li-saline rats, exhibited a potent inverse agonistic effect on GABA<sub>A</sub> mIPSCs of GCs from Li-pilocarpine rats 24–48 h and 3–5 months after SE. The neurosteroid allopregnanolone (100 nM), which considerably prolonged GABA<sub>A</sub> mIPSCs in Li-saline rats, totally lost its effect in rats studied 24–48 h after SE. However, this decrease in effectiveness was transient and was totally restored in epileptic rats. In addition to the up-regulation in the number of receptors at individual GC synapses, we propose that these ‘epileptic’ GABA<sub>A</sub> receptors possess benzodiazepine binding sites with altered allosteric properties. The failure of benzodiazepine and neurosteroid to potentiate inhibition early after SE may be a critical factor in the development of epileptogenesis and occurrence of seizures.

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Mesial temporal lobe epilepsy (MTLE) is a common form of drug-refractory epilepsy (Engel, 1998). Hippocampal sclerosis, the main neuropathological feature of MTLE, is characterized by massive neuronal loss and gliosis in CA1, CA3 and the hilus, while most granule cells of the dentate gyrus are preserved. It is associated with axon and synaptic reorganization of surviving neurons. Reorganized axons include sprouting of excitatory mossy fibres, inhibitory GABAergic neurones, and fibres with other neurotransmitters such as neuropeptide Y and somatostatin (Mathern et al. 1997). Occurrence of epileptic seizures is the consequence of an imbalance in the neurotransmission systems in favour of neuronal hyperexcitation. The plasticity of inhibitory GABA<sub>A</sub> receptor-mediated synaptic inhibition is considered to contribute to hyperexcitability in the dentate gyrus granule cells layer in MTLE patients. Indeed, the dentate gyrus critically regulates seizures by making synchronous activity from entorhinal cortex less able to invade the hippocampus. However, a failure in the gating function of the dentate gyrus can generate robust paroxysmal discharges (Lothman et al. 1992; Behr et al. 1998).

Alterations in GABA neurotransmission associated with MTLE in dentate gyrus granule cells have been observed pre- and postsynaptically. At the neuronal level, strong up-regulations of the GABA neuronal marker glutamate decarboxylase (GAD) have been observed during the latent and chronic phases of lithium–pilocarpine and pilocarpine models of MTLE (Brooks-Kayal et al. 1998; André et al. 2001). At the postsynaptic level, an increased density of GABA<sub>A</sub> receptors has been noted in the dentate gyrus of human MTLE patients (Brooks-Kayal et al. 1998; Loup et al. 2000) and in the kindling (Otis et al. 1994; Nusser

Claire Leroy and Pierrick Poisbeau contributed equally to the work reported here.
alterations of GABA-mediated inhibitory transmission in the dentate gyrus granule cells of epileptic rats than in controls enhances GABA-evoked currents less potently in dentate gyrus granule cells of epileptic rats than in controls. Recent studies reported that allopregnanolone convulsant effects (Kokate et al. 1995, 1996). Physiological nanomolar concentrations of this steroid have anti-convulsant effects (Kokate et al. 1995, 1996; Frye & Scalize, 2000). Recent studies reported that allopregnanolone enhances GABA-evoked currents less potently in dentate gyrus granule cells of epileptic rats than in controls (Mtchedlishvili et al. 2001).

Thus, the objective of the present study was to evaluate alterations of GABA_A-mediated inhibitory transmission in dentate gyrus granule cells occurring at different times in the epileptogenic process, i.e. during early epileptogenesis (24–48 h after SE) and after the occurrence of numerous spontaneous recurrent seizures (3–5 months after SE) in the lithium–pilocarpine model of MTLE. This model reproduces the temporal development of the disease, i.e. an initial event – here SE – followed by a latent seizure-free phase characterized by the genesis of neuronal damage and the development of a hyperexcitable circuit underlying the occurrence of spontaneous recurrent seizures (Cavalheiro, 1995). As in humans, the neuro-pathology includes hippocampal sclerosis (André et al. 2001; Roch et al. 2002). In this model, we characterized the properties of GABA_A-receptor-generated IPSCs and their changes in sensitivity to benzodiazepines and neurosteroids by whole-cell patch-clamp recording in granule cells from hippocampal slices of Li-pilocarpine- and Li-saline-treated rats.

Methods

Animals and status epilepticus induction

Adult male Sprague–Dawley rats from Janvier Breeding Center (Le Genest-St-Isle, France) were maintained under standard laboratory conditions on a 12:12 h light–dark cycle (lights on at 07.00 h). All experiments were conducted in conformity with the rules set by the EC Council Directive (86/69/EEC) of November 24, 1986 and French Department of Agriculture (License no. 67-97 issued to A.N. and 67-116 to P.P.).

Status epilepticus (SE) was induced in rats weighing 250–300 g. Lithium chloride (3 mequiv kg⁻¹, Sigma, St Louis, MO, USA) was administrated i.p. to all rats 18–20 h before a s.c. injection of pilocarpine (25 mg kg⁻¹, Sigma) in the experimental groups or saline in the control group. All animals were given a subcutaneous injection of methylscopolamine (1 mg kg⁻¹, Sigma) 30 min before pilocarpine or saline. Methylscopolamine is used to reduce the peripheral consequences of the convulsant, i.e. the pilocarpine. Lithium chloride potentiates the convulsive effect of pilocarpine reducing the amount of pilocarpine that has to be injected.

The behavioural characteristics of SE were similar to those observed in previous studies (Roch et al. 2002). Within 5 min of pilocarpine injection, rats developed diarrhoea, pilorection and other signs of cholinergic stimulation. Over the following 15–20 min, they exhibited head bobbing, scratching, masticatory automatisms, wet-dog shakes and exploratory behaviour. Episodes of head and bilateral forelimb myoclonic movements with rearing and falling started at around 20–25 min and progressed to SE at about 50 min after pilocarpine administration. SE was characterized by long-lasting, sustained clonic seizure activity accompanied by episodes of rearing and falling. A
total of 75 rats, 25 Li-saline and 50 Li-pilocarpine rats were used for this study. Within the Li-pilocarpine-injected rats, three rats did not develop SE. These rats survived but were excluded from this study. Moreover, nine rats that developed SE died during the course of SE because of cardio-respiratory complications mainly caused by pulmonary hypersecretion during the sustained seizures. After the SE phase, the 38 surviving rats experienced a latent seizure-free phase of a mean duration of 11 ± 4 days, after which all animals exhibited two to three spontaneous recurrent seizures per week until kill (Roch et al. 2002). Occurrence of seizures was video-recorded for 10 h per day. Li-pilocarpine-treated rats were used either early, i.e. 24 or 48 h after the onset of SE or late, i.e. about 3–5 months after SE after they had developed repeated spontaneous recurrent seizures for several months.

Thus, 63 rats were available for electrophysiological recordings. Only three epileptic rats were excluded from the study after slice preparation because the hippocampus was too slightly damaged or extremely damaged, both conditions being considered outside the range of usual hippocampal sclerosis. Within the remaining 60 rats, we were unable to monitor electrophysiological activity from 19 rats (13 Li-saline and 6 Li-pilocarpine) because cell recordings were too poor, unstable or unsuccessful. Finally, a total of 41 rats could be used for electrophysiological recordings: 12 Li-saline rats, 11 rats studied 24–48 h after Li-pilocarpine-induced SE and 18 epileptic rats examined 3–5 months after SE. Rats used at both 24–48 h and 3–5 months after Li-saline injection composed the Li-saline group.

Electrophysiological study

Brain dissection and slice preparation. Rats were deeply anaesthetized by intraperitoneal injection of a mixture of ketamine (100 mg kg−1, Merial, Lyon, France) and xylazine (5 mg kg−1, Rompun, Bayer, Germany). Animals were decapitated and brains were rapidly removed from the skull while being continuously refreshed with oxygenated ice-cold (−4°C) sucrose–artificial CSF (S-aCSF). S-aCSF contained 248 mM sucrose, 11 mM glucose, 2 mM NaHCO3, 2 mM KCl, 1.25 mM KH2PO4, 2 mM CaCl2, 2 mM MgSO4 (pH 7.35 ± 0.05). S-aCSF was bubbled continuously with 95% O2–5% CO2. Meningeas were rapidly and delicately removed from the brain immersed in S-aCSF. The brain region containing the hippocampus was isolated and glued using cyanoacrylate cement applied to the dorsal side of the brain (enabling horizontal slicing) to the platform of a Vibratome chamber filled with oxygenated ice-cold S-aCSF. Slices (300–350 µm thick) were cut in the horizontal plane and were incubated in normal aCSF at room temperature (23–28°C) for at least 1 h before electrophysiological recordings. They were continuously perfused with oxygenated normal aCSF in which 125 mM NaCl was substituted for sucrose. Lastly, they were transferred to a recording chamber under a Zeiss Axioscope equipped with infrared differential interference contrast (IR-DIC) and water immersion objectives capable of visualizing neurones in thick living tissue.

Electrophysiological recordings and data acquisition. All recordings were performed at room temperature. Patch pipettes were obtained by pulling borosilicate glass capillaries with inner filament using a horizontal laser puller (P-2000, Sutter Instruments, Novato, CA, USA). The pipettes were filled with a solution containing 130 mM CsCl, 2 mM MgCl2, 10 mM Hepes (pH 7.3, adjusted using CsOH). Biocytine was added to the intracellular solution for neuronal labelling. A typical granule cell labelled using biocytine is shown in Fig. 1.

Whole-cell patch-clamp recordings were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with > 80% series resistance compensation. Recordings were low-pass filtered (5–10 kHz), digitized, and stored on videotape. Off-line, recordings were filtered at 2 kHz and digitized at 4–10 kHz on an Intel Pentium-based computer. Data were analysed using the Strathclyde electrophysiology software (Dr J. Dempster, University of

Figure 1. Illustration of a typical dentate gyrus granule cell recorded from a Li-saline rat

The cellular morphology was reconstructed with confocal microscopy using biocytine immunocytochemistry. Scale bar: 0–100 µm.
Strathclyde, UK) and in-house software kindly supplied by Dr Y. De Koninck. Detection of individual mIPSCs was performed using a software trigger described in detail in previous studies (Poisbeau et al. 1997, 1999). Over 95% of events satisfying the trigger criteria were detected, even during compound mIPSCs. For each experiment, all detected events were examined and any noises whose compliance with trigger specification was spurious were rejected.

Statistical analysis. The mean amplitudes, total rise times, half-decay times \((T_{50\%})\), and frequency of occurrence of mIPSCs were compared between groups using one-way (saline/pilocarpine/epileptic) or two-way ANOVA with repeated measures (with or without drug), followed by Tukey’s post hoc multiple comparison test (Sigmastat 2.0). Statistical significance was considered as \(P < 0.05\). For each condition analysis, 4–12 rats and 7–32 cells were used. In none of the experimental group was there any statistically significant difference between rats.

In the figures, decay time constants of mIPSCs were fitted using non-linear least square methods, and goodness of fit was evaluated by fitting subsets of points drawn from the whole set of data points, the assessment of the reduced \(\chi^2\) values, and the change in the \(F\) values calculated from the sum of squared differences from the fitted line. The half-decay time values \((T_{50\%})\) of mIPSCs are also represented graphically in cumulative probability plots. The Kolmogorov-Smirnov (KS) test was used to compare two cumulative probability distributions to each other and statistical significance was set at \(P < 0.01\). All other numerical data are expressed as means ± s.e.m.

Drug application

For all IPSC recordings, slices were continually perfused with oxygenated aCSF containing 2 mm kynurenic acid (Fluka, Neu-Ulm, Germany). For mIPSC recordings, 0.5 \(\mu\)m tetrodotoxin (TTX, Sigma) was added. Modulations of GABA\(_A\) receptors were tested using 1 \(\mu\)m diazepam (Sigma), 1 and 10 \(\mu\)m flumazenil (gift of Roche, Basel, Switzerland) and 100 \(\mu\)m allopregnanolone (Sigma). All drugs were prepared as 1000 times concentrated frozen stock solution aliquots. Diazepam and allopregnanolone were diluted in 96% ethanol whereas all other drugs were prepared in distilled water.

Results

In the present study, the development of hippocampal sclerosis was very progressive. At 24 h after SE, there was already some neuronal loss, which was marked in the hilus and more moderate in Ammon’s horn, as has been shown previously (Roch et al. 2002). At 3–5 months after SE, neuronal loss had worsened and there was a major atrophy of the hippocampus. By this time, massive neuronal loss had occurred in CA3, CA1 and the hilus of the dentate gyrus, while CA2 pyramidal cells survived. The great majority of cells were also preserved in the granule cell layer of the dentate gyrus which was subject to a degree of dispersion.

Characteristics of GABA\(_A\) receptor-mediated sIPSCs and mIPSCs recorded from dentate gyrus granule cells in lithium–saline- and lithium–pilocarpine-treated rats

We began by analysing the spontaneous and miniature GABA\(_A\) receptor-mediated synaptic currents (GABA\(_A\) sIPSCs and GABA\(_A\) mIPSCs, respectively) recorded from the dentate gyrus granule cells (GCs) of Li-saline- and Li-pilocarpine-treated rats. Recordings were performed at a holding potential of −60 mV and in the presence of 2 mm kynurenic acid, an antagonist of ionotropic glutamate receptors. To record GABA\(_A\) mIPSCs, steady-state concentrations of tetrodotoxin (0.5 \(\mu\)m) were added to the perfusion medium.

Table 1 summarizes IPSC characteristics obtained in the three animal groups, i.e. Li-saline and Li-pilocarpine rats at 24–48 h and 3–5 months after SE. For both sIPSCs and mIPSCs, the total rise time and half-decay time were similar in all groups of rats (Table 1). However, as early as 24–48 h after SE, IPSCs increased significantly in amplitude compared to the Li-saline condition (+44\% and +25\% for sIPSCs and mIPSCs, respectively). There was no further change in sIPSC and mIPSC amplitudes in the epileptic Li-pilocarpine group (+59\% and +33\% for sIPSCs and mIPSCs, respectively, compared to Li-saline rats). Conversely, at 24–48 h after SE, the frequency of GC sIPSCs and mIPSCs was unchanged compared to Li-saline rats. At 3–5 months after SE, the frequency of mIPSCs was still similar to that recorded in Li-saline and Li-pilocarpine animals studied at 24–48 h after SE, while the frequency of sIPSCs was increased by a factor of 2–2.5 compared to Li-saline and Li-pilocarpine animals studied at 24–48 h after SE (Table 1). Representative GC recordings obtained from Li-saline and epileptic Li-pilocarpine animals at 3–5 months after SE shown in Fig. 2, together with an averaged trace obtained from 10 isolated GABA\(_A\) sIPSCs, illustrate the increase in frequency and amplitude of sIPSCs in epileptic Li-pilocarpine compared to Li-saline rats.
Changes of pharmacological properties of synaptic GABA<sub>A</sub> receptors after lithium–pilocarpine-induced SE

To test the change in pharmacological properties of synaptic GABA<sub>A</sub> receptors, their sensitivity to diazepam, flumazenil and allopregnanolone on mIPSCs was studied in Li-saline and Li-pilocarpine rats, early and late after SE. Table 2 summarizes the amplitude, total rise time, half-decay time (T<sub>50%</sub>) and frequency of mIPSCs obtained before and after drug perfusion in the three groups, i.e. Li-saline and Li-pilocarpine rats at 24–48 h and 3–5 months after SE.

Modulation of GABA<sub>A</sub> receptor-mediated mIPSCs by benzodiazepines. Perfusion of diazepam (1 µM), a classical benzodiazepine agonist used clinically to block epileptic seizures, had no significant effects on the mean peak amplitude and frequencies of mIPSCs in Li-saline or Li-pilocarpine rats (Table 2). Conversely, the effect of diazepam on the total rise time and the mean half-decay time (T<sub>50%</sub>) of mIPSCs could be observed. In Li-saline rats, diazepam increased the T<sub>50%</sub> of all GABA<sub>A</sub> mIPSCs. The increase in T<sub>50%</sub> appears as a rightward shift of cumulative probability (Fig. 3A), with a prolongation of the mean T<sub>50%</sub> by 46% (Fig. 3D) and an increase in total rise time of 21% (Fig. 3E). Washout experiments indicated that the effect of diazepam perfusion on mIPSCs is reversible (Fig. 3F). In Li-pilocarpine rats, 24–48 h after SE, diazepam was already significantly (62%) less effective at potentiating T<sub>50%</sub> compared to Li-saline rats (Fig. 3D, Table 2). This decrease in the effectiveness of diazepam was found throughout the whole mIPSC population and led to a diminished shift of the cumulative probability (Fig. 3B). In epileptic Li-pilocarpine animals, no significant potentiating effect of diazepam was observed (Table 2 and Fig. 3D). This led to a strong match between the cumulative probability of the T<sub>50%</sub> of mIPSCs before and after diazepam perfusion (Fig. 3B), illustrating the GCS’ progressive loss of sensitivity to diazepam over the course of the epileptogenic process. The effects of diazepam on GABA<sub>A</sub> sIPSCs in GCs from Li-saline (data not shown, n = 3) and epileptic Li-pilocarpine animals (data not shown, n = 7) were similar to those reported here for mIPSCs.

To find out more about the changes in the properties of the GABA<sub>A</sub> receptor benzodiazepine binding site, we perfused flumazenil, a benzodiazepine site antagonist, onto the GABA<sub>A</sub> receptors. During our preliminary investigations, we tested two concentrations of flumazenil, 1 µM and 10 µM. Since the effect of flumazenil was similar at both concentrations, all the data presented here were obtained using 1 µM of flumazenil. As previously reported, flumazenil had no effect on GABA<sub>A</sub> mIPSCs in Li-saline animals (Table 2). Neither the mean rise time nor T<sub>50%</sub> of the mIPSCs was affected during perfusion of flumazenil (Fig. 4A, D and E). Conversely, this was not the case for recordings performed in Li-pilocarpine GCs. Flumazenil reduced the GABA<sub>A</sub> mIPSC T<sub>50%</sub> significantly, by 17–18%, in all GCs recorded from Li-pilocarpine animals 24–48 h or 3–5 months after SE (Fig. 4D). This decrease in T<sub>50%</sub> led to a leftward shift of the curves in both groups of Li-pilocarpine rats (Fig. 4B and C). In contrast to diazepam, flumazenil did not produce any potentiation of the total rise time of mIPSCs recorded either from Li-saline or Li-pilocarpine rats (Fig. 4E). Washout experiments indicated that the effect of flumazenil perfusion on Li-pilocarpine rats is reversible (Fig. 4F).

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Table 1. Characteristics of spontaneous and miniature GABA<sub>A</sub> receptor-mediated IPSCs recorded from the hippocampal GCs of Li-saline and Li-pilocarpine rats examined 24–48 h and 3–5 months after SE

<table>
<thead>
<tr>
<th></th>
<th>Amplitude (pA)</th>
<th>RT (ms)</th>
<th>T&lt;sub&gt;50%&lt;/sub&gt; (ms)</th>
<th>Frequency (Hz)</th>
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<tr>
<td><strong>Spontaneous GABA&lt;sub&gt;A&lt;/sub&gt; receptor-mediated IPSCs</strong></td>
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<tr>
<td>Li-saline</td>
<td>-40.3 ± 3.4</td>
<td>1.21 ± 0.09</td>
<td>10.8 ± 0.6</td>
<td>0.75 ± 0.21</td>
<td>11</td>
</tr>
<tr>
<td>Li-Pilo 24–48 h</td>
<td>-58.0 ± 4.8**</td>
<td>1.36 ± 0.09</td>
<td>11.1 ± 0.6</td>
<td>0.60 ± 0.11</td>
<td>11</td>
</tr>
<tr>
<td>Li-Pilo 3–5 months</td>
<td>-63.9 ± 3.5**</td>
<td>1.30 ± 0.09</td>
<td>10.9 ± 0.7</td>
<td>1.59 ± 0.25**</td>
<td>18</td>
</tr>
<tr>
<td><strong>Miniature GABA&lt;sub&gt;A&lt;/sub&gt; receptor-mediated IPSCs</strong></td>
<td></td>
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<tr>
<td>Li-saline</td>
<td>-35.6 ± 1.2</td>
<td>1.31 ± 0.04</td>
<td>8.9 ± 0.3</td>
<td>0.57 ± 0.06</td>
<td>32</td>
</tr>
<tr>
<td>Li-Pilo 24–48 h</td>
<td>-44.5 ± 1.9**</td>
<td>1.23 ± 0.06</td>
<td>9.2 ± 0.2</td>
<td>0.59 ± 0.07</td>
<td>22</td>
</tr>
<tr>
<td>Li-Pilo 3–5 months</td>
<td>-47.3 ± 2.2**</td>
<td>1.38 ± 0.05</td>
<td>9.5 ± 0.2</td>
<td>0.55 ± 0.04</td>
<td>29</td>
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</table>

Mean peak amplitudes, total rise time (RT), half-decay time (T<sub>50%</sub>) and frequency of occurrence were analysed. Values are expressed as means ± S.E.M. of the number of cells (n). *P < 0.05, **P < 0.005, statistically significant differences from Li-saline rats; †P < 0.05, statistically significant difference from Li-pilo rats, 24–48 h.
Modulation of GABA_A receptor-mediated mIPSCs by allopregnanolone

In GCs from Li-saline rats, perfusion of allopregnanolone (AP, 100 nM) significantly increased the GABA_A mIPSC mean $T_{50\%}$ and rise time, while the amplitude and frequency of the mIPSCs were not affected (Table 2). The effect of AP on $T_{50\%}$ corresponded to a significant prolongation of GABA_A mIPSCs (Fig. 5A and D). However, in Li-pilocarpine rats, the sensitivity of GABA_A mIPSCs to AP changed according to the time that had elapsed between SE and GC recordings. Surprisingly, at 24–48 h after SE, perfusion of 100 nM AP did not produce any significant modulation of GABA_A mIPSCs (Table 2). The lack of effect on $T_{50\%}$ can be seen by superimposing both cumulative curves, before and after AP perfusion (Fig. 5B). This insensitivity was only transient, as AP strongly potentiated the effect on both the total rise time and mean $T_{50\%}$ in epileptic Li-pilocarpine rats (Fig. 5D and E). The prolonging effect of $T_{50\%}$ is illustrated by a rightward shift of the cumulative curves (Fig. 5C). The difference between Li-saline and epileptic Li-pilocarpine rats in terms of the extent to which AP modulated $T_{50\%}$ was not statistically significant ($P = 0.17$). As with benzodiazepine modulation, washout experiments indicated that the effects of AP perfusion on Li-saline and epileptic Li-pilocarpine rats are reversible (Fig. 5F).

Discussion

The present data reflect drastic changes in GABA_A-mediated inhibition within dentate gyrus GCs in the Li-pilocarpine model of MTLE. Changes occur in both the biophysical properties and sensitivity of GABA_A receptors to allosteric modulators. We show that synaptic GABA_A receptors become progressively insensitive to diazepam. Conversely, flumazenil has no effect in Li-saline rats and displays inverse agonist properties in Li-pilocarpine rats as early as 24 h after SE. Likewise, we report for the first time that synaptic GABA_A receptors temporarily loose their sensitivity to neurosteroids early after SE.

Enhanced GABA_A-mediated inhibition in lithium-pilocarpine-treated rats

In accordance with previous findings in human and various models of TLE (Otis et al. 1994; Buhl et al. 1996; Gibbs et al. 1997; Shumate et al. 1998; Cohen et al. 2003), the present data demonstrate an increase in GABA_A-mediated inhibition in the dentate gyrus of epileptic Li-pilocarpine rats. From the recordings of sIPSCs and mIPSCs, this potentiation appears to be both pre- and postsynaptic.

At the postsynaptic level, our data show an increase in the mean peak amplitude of mIPSCs and sIPSCs in Li-pilocarpine rats studied at 24–48 h and 3–5 months after SE compared to Li-saline rats. This most probably reflects an increased number of GABA_A receptor channels at individual synapses, reflecting an adaptive process aimed at the reinforcement of inhibitory neurotransmission occurring early during epileptogenesis and persisting in chronically epileptic rats. This type of up-regulation of
et al. receptors from rat granule cells (see for example Cohen GABAA receptor density in the dentate gyrus has already been reported both in human MTLE (Loup et al. 2000) and in the kindling (Otis et al. 1994; Nuesser et al. 1998), pilocarpine (Fritschy et al. 1999) and kainate (Schwarzer et al. 1997) models. However, a recent study reported a reduced amplitude of mIPSCs in pilocarpine-treated rats at 7 days after SE (Cohen et al. 2003). Thus, the potentiation observed in the present study shortly after SE is, rather, a postictal consequence of SE and hence an adaptive process reinforcing inhibitory neurotransmission in response to the hyperexcitability triggered by seizures, as observed in epileptic Li-pilocarpine rats at 3–5 months after SE.

In addition, the frequency of sIPSCs undergoes a two-fold increase only in Li-pilocarpine rats studied 3–5 months after SE. The absence of an increase in the frequency of mIPSCs indicates that the inhibitory hyperactivity is due to increased presynaptic neuronal activity. This adaptation is quite delayed compared to the change in amplitude, which was already recorded 24–48 h after SE.

The mean amplitudes and kinetics of both sIPSCs and mIPSCs recorded in the present study are in the same range as those from previous studies performed on GABA_A receptors from rat granule cells (see for example Cohen et al. 2003). Conversely, the frequency values of IPSCs recorded here are lower than those reported in most studies (Otis et al. 1994; Buhl et al. 1996; Poisbeau et al. 1997, 1999; Cohen et al. 2003). These differences may be linked to the preparation of the slices, which were relatively thin (250–300 µm) and were not cut at an angle, making it possible to preserve all entorhino-hippocampal connections. Recordings were also performed at room temperature (20–22°C), while most studies adopted a higher temperature – usually 33–35°C – increasing the probability of neurotransmitter release.

### Altered pharmacology of GABA_A-mediated inhibition in lithium–pilocarpine-treated rats

Many studies have characterized the basal pharmacological properties of synaptic GABA_A receptors expressed at GC synapses. They have revealed a high responsiveness to benzodiazepines and neurosteroid agonists and only a weak or absent sensitivity to zinc in both human (Shumate et al. 1998) and rat GCs (Gibbs et al. 1997; Mitchevishvili et al. 2001; Cohen et al. 2003). Accordingly, in the present study, diazepam and allopregnanolone increased the half-decay and rise times of mIPSCs in Li-saline rats. The increases in the half-decay time probably result from an increase in the apparent affinity of GABA for the GABA_A receptor. Conversely, increases in the mean rise time induced by diazepam or allopregnanolone perfusion were observed in this study. However, both drugs had no effect on the mean peak amplitudes or frequencies of mIPSCs. The absence of an effect on amplitude could be explained

### Table 2. Main characteristics of GABA_A receptor-mediated miniature IPSCs recorded before and after drug perfusion from hippocampal GCs of Li-saline and Li-pilocarpine rats examined 24–48 h and 3–5 months after SE

<table>
<thead>
<tr>
<th>Drug</th>
<th>Condition</th>
<th>Amplitude (pA)</th>
<th>RT (ms)</th>
<th>T50% (ms)</th>
<th>Frequency (Hz)</th>
<th>n</th>
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<tbody>
<tr>
<td>Diazepam (DZP)</td>
<td>Li-saline Before DZP</td>
<td>−35.5 ± 2.4</td>
<td>1.36 ± 0.081</td>
<td>8.0 ± 0.51</td>
<td>0.50 ± 0.0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>After DZP</td>
<td>−35.2 ± 2.5</td>
<td>1.64 ± 0.09**</td>
<td>11.5 ± 0.6**</td>
<td>0.48 ± 0.06</td>
<td>7</td>
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<tr>
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<td>Li-Pilo 24–48 h</td>
<td>−46.7 ± 3.9</td>
<td>1.13 ± 0.03</td>
<td>8.7 ± 0.2</td>
<td>0.56 ± 0.1</td>
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<td>After DZP</td>
<td>−46.1 ± 4.2</td>
<td>1.30 ± 0.04</td>
<td>10.2 ± 0.4**</td>
<td>0.55 ± 0.17</td>
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<td>Li-Pilo 3–5 months</td>
<td>−43.3 ± 2.0</td>
<td>1.38 ± 0.09</td>
<td>9.9 ± 0.4</td>
<td>0.54 ± 0.07</td>
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<tr>
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<td>After DZP</td>
<td>−43.8 ± 2.6</td>
<td>1.43 ± 0.08</td>
<td>10.2 ± 0.4</td>
<td>0.54 ± 0.08</td>
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<td>Flumazenil (FLU)</td>
<td>Li-saline Before FLU</td>
<td>−35.2 ± 1.6</td>
<td>1.26 ± 0.1</td>
<td>9.7 ± 0.5</td>
<td>0.50 ± 0.07</td>
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<td>After FLU</td>
<td>−35.6 ± 1.6</td>
<td>1.31 ± 0.1</td>
<td>9.9 ± 0.7</td>
<td>0.48 ± 0.06</td>
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<td>Li-Pilo 24–48 h</td>
<td>−43.4 ± 3.8</td>
<td>1.32 ± 0.11</td>
<td>10.1 ± 0.4</td>
<td>0.56 ± 0.18</td>
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<td>After FLU</td>
<td>−42.0 ± 3.8</td>
<td>1.29 ± 0.10</td>
<td>8.2 ± 0.3*</td>
<td>0.55 ± 0.17</td>
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<td>Li-Pilo 3–5 months</td>
<td>−45.4 ± 3.2</td>
<td>1.47 ± 0.08</td>
<td>9.3 ± 0.3</td>
<td>0.55 ± 0.05</td>
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<tr>
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<td>After FLU</td>
<td>−46.8 ± 3.2</td>
<td>1.42 ± 0.08</td>
<td>7.7 ± 0.3*</td>
<td>0.53 ± 0.05</td>
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<td>Allopregnanolone (AP)</td>
<td>Li-saline Before AP</td>
<td>−36.0 ± 2.1</td>
<td>1.28 ± 0.07</td>
<td>9.2 ± 0.2</td>
<td>0.65 ± 0.13</td>
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<td>After AP</td>
<td>−36.5 ± 2.4</td>
<td>1.58 ± 0.11**</td>
<td>12.5 ± 0.4**</td>
<td>0.58 ± 0.12</td>
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<td>Li-Pilo 24–48 h</td>
<td>−43.5 ± 2.8</td>
<td>1.22 ± 0.1</td>
<td>8.8 ± 0.3</td>
<td>0.59 ± 0.09</td>
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<td>After AP</td>
<td>−41.5 ± 3.1</td>
<td>1.26 ± 0.1</td>
<td>8.8 ± 0.5</td>
<td>0.57 ± 0.10</td>
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<tr>
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<td>Li-Pilo 3–5 months</td>
<td>−56.5 ± 5.8</td>
<td>1.24 ± 0.07</td>
<td>9.0 ± 0.71</td>
<td>0.54 ± 0.12</td>
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<tr>
<td></td>
<td>After AP</td>
<td>−55.8 ± 5.5</td>
<td>1.44 ± 0.08*</td>
<td>11.0 ± 0.7*</td>
<td>0.55 ± 0.13</td>
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</table>

Mean peak amplitudes, total rise time (RT), half-decay time (T50%) and frequency of occurrence were analysed. Values are expressed as means ± S.E.M of the number of cells (n). *P < 0.005, **P < 0.001, statistically significant difference from Li-saline rats.
Figure 3. Effects of 1 µM diazepam (DZP) on GABA<sub>A</sub> mIPSC kinetics in Li-saline rats and both groups of Li-pilocarpine rats (24–28 h and 3–5 months after SE)

Left graphs represent cumulative probability plots of all mIPSC half-decay times (T<sub>50%</sub>) for a single GC before (thin line) and after perfusion of 1 µM DZP (thick line) in Li-saline rats (A) and Li-pilocarpine rats 24–48 h after SE (B) and 3–5 months after SE (C). Although diazepam considerably prolonged mIPSCs in Li-saline rats (A, rightward shift of the T<sub>50%</sub> distribution significant at P < 0.01, KS test), it showed a low efficacy (but still significant at P < 0.01, KS test) in modulating mIPSCs shortly after SE (B). This decreased sensitivity of synaptic GABA<sub>A</sub> receptors to diazepam was aggravated in epileptic rats. At this time, diazepam was totally unable to modulate these receptors and this resulted in the superimposition of the cumulative probability curves of mIPSCs recorded in epileptic GCs (C). This is confirmed by the progressive decrease in the percentage potentiation of mIPSC T<sub>50%</sub> (D) and in the total rise time induced by diazepam (E). The effect of diazepam perfusion on T<sub>50%</sub> mIPSCs is reversible, as illustrated in the washout experiment performed in a Li-saline rat (F). * P < 0.005, ** P < 0.001, statistically significant differences from the recording before diazepam perfusion; +++ P < 0.01, statistically significant differences between groups.

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Figure 4. Effects of flumazenil (FLU, μM) on GABA_A mIPSCs kinetics recorded in GCs from Li-saline and both groups of Li-pilocarpine rats (24–48 h and 3–5 months after SE)

Left graphs represent cumulative probability plots of all mIPSC half-decay times (T_{50%}) for a single GC before (thin line) and after the perfusion of flumazenil (thick line) in Li-saline rats (A) and Li-pilocarpine rats 24–48 h after SE (B) and 3–5 months after SE (C). While flumazenil had no effect on T_{50%} in Li-saline rats, it decreased T_{50%} in Li-pilocarpine rats as early as 24–48 h after SE and the effect did not evolve during the course of epileptogenesis. This is confirmed by the strong decrease in T_{50%} (P < 0.01, KS test) induced by flumazenil in both groups of Li-pilocarpine rats (D). Conversely, flumazenil perfusion did not influence the total rise time in either Li-saline or Li-pilocarpine rats (E). The effect of flumazenil perfusion on T_{50%} mIPSCs was reversible, as illustrated in the washout experiment performed in a Li-pilocarpine rat (F). ∗P < 0.005, **P < 0.001, statistically significant differences from the recording before flumazenil perfusion; ++P < 0.01, statistically significant differences between groups.
by a saturating quantity of GABA in the synaptic cleft in relation to the number of synaptic receptors, as reported previously (see review and references in Mody et al. 1994).

Loss of sensitivity to diazepam potentiation of mIPSCs in epileptic GCs. Our data show the appearance of refractoriness to diazepam modulation of synaptic GABA_A receptors. At 24–48 h after SE, there is already a large decrease in the effect of diazepam on potentiation of mIPSC deactivation time, which demonstrates the rapid loss of sensitivity of GABA_A receptors to diazepam. These data are in accordance with a previous study reporting pharmacological alterations of the total GC GABA_A receptor population as early as 45 min after the onset of pilocarpine SE (Kapur & Macdonald, 1997). We confirm here the same alterations of GABA_A receptors at individual synapses. Moreover, the loss of sensitivity of GABA_A receptors to diazepam is a progressive process during epileptogenesis, since it is more marked 3–5 months than 24–48 h after SE. Our data are in accordance with previous reports in the literature suggesting that GABA_A receptors in epileptic GCs have a decreased sensitivity to benzodiazepines (Gibbs et al. 1997; Kapur & Macdonald, 1997; Shumate et al. 1998; Mtchedlishvili et al. 2001; Jones et al. 2002; Cohen et al. 2003).

Many hypotheses have been put forward to explain the refractoriness to benzodiazepine agonists after prolonged limbic seizures. Firstly, the benzodiazepine binding site may lose its functionality as a result of uncoupling linked to an alteration in the conformation of GABA_A receptors in MTLE (Klein et al. 1995; Lyons et al. 2000). Secondly, many studies have reported numerous changes in the mRNA and protein expression of GABA_A receptor subunits in human MTLE (Lou et al. 2000) and various animal models (Rice et al. 1996; Schwarzer et al. 1997; Brooks-Kayal et al. 1998; Fritschy et al. 1999). In general, these studies report a down-regulation of α1 and γ2 mRNA subunits, both of which confer a high sensitivity to benzodiazepines and low sensitivity to zinc when incorporated (Jones-Davis & Macdonald, 2003). Simultaneously, there is an up-regulation of α3, α4, α5 and δ subunits (Rice et al. 1996; Brooks-Kayal et al. 1998; Fritschy et al. 1999). GABA_A receptors containing α3, α4, α5 and δ subunits are highly sensitive to zinc and those containing α4 and δ subunits have no or low affinity to benzodiazepines, respectively (Barnard et al. 1998; Mody, 2001). Therefore, the majority of changes in subunit expression reported in the literature—mainly the up-regulation of α4 and δ subunits—induced by pilocarpine SE, are in accordance with the loss of diazepam sensitivity observed in the present study (Brooks-Kayal et al. 1998). GABA_A receptors containing α3 or α5 subunits have a high affinity for agonists binding at type II benzodiazepine sites and therefore can be modulated by agonists with a ‘broad spectrum’ action (Jones-Davis & Macdonald, 2003) such as diazepam, which was used here. Consequently, an up-regulation of α3 and α5 subunits within epileptic GCs is not consistent with a loss of effectiveness of diazepam on GABA_A receptor currents. Thus, the many subunit alterations of GABA_A receptors described in MTLE do not fully explain why synaptic GABA_A receptors of epileptic GCs have lost their sensitivity to benzodiazepines. Thirdly, most of the subunits forming synaptic GABA_A receptors contain consensus sequences for various kinases and phosphatases (Kittler & Moss, 2003). Seizures are known to modulate the activities of kinases (Jope et al. 1992) but it is still not known whether post-translational modification can alter the benzodiazepine sensitivity of GABA_A receptors (Jones et al. 2002).

Paradoxical effect of flumazenil on mIPSCs of epileptic GCs. Flumazenil is currently described as a selective competitive benzodiazepine antagonist with a high affinity for the benzodiazepine binding site of GABA_A receptors (Barnard et al. 1998). Therefore, flumazenil can inhibit the effects of both agonists and inverse agonists by displacing the binding but is devoid of intrinsic activity on GABA transmission (Li et al. 2001). Moreover, at low doses, flumazenil has no detectable electrophysiological effect but at high doses, it displays an ‘agonist-like’ effect as shown in acutely dissociated CA1 pyramidal cells (Buldakova & Weiss, 1997). Its low efficacy may explain why this intrinsic activity goes undetected in most studies and why flumazenil is currently regarded as an antagonist (Weiss et al. 2002). Finally, flumazenil displays different effects depending on the subunit composition of the GABA_A receptor. Flumazenil blocks the effects of benzodiazepine at receptors containing α1, α2, α3 or α5 subunits but acts as an agonist at receptors containing α4 or α6 subunits (Whittemore et al. 1996; Hauser et al. 1997; Thomson et al. 2000).

Under our experimental conditions, we never detected an agonist-like effect of flumazenil on GCs in Li-saline rats (data not shown). On the other hand, the synaptic GABA_A receptors of GCs in Li-pilocarpine rats, while insensitive to diazepam, develop a strong response to flumazenil perfusion. In fact, flumazenil displayed an inverse agonist-like effect in reducing the half-decay time of mIPSCs at both 24–48 h and 3–5 months after SE. An inverse agonist effect of flumazenil was similarly shown

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Figure 5. Effects of allopregnanolone (AP, 100 nM) on GABA_A mIPSCs kinetics in GCs from Li-saline and both groups of Li-pilocarpine rats (24–28 h and 3–5 months after SE).

Left graphs represent cumulative probability plots of all mIPSC half-decay times (T_{50\%}) for a single GC before (thin line) and after the perfusion of AP (thick line) in Li-saline rats (A) and Li-pilocarpine rats 24–48 h after SE (B) and 3–5 months after SE (C). AP induced a prolongation of GABA_A mIPSCs in both Li-saline and epileptic Li-pilocarpine rats, as illustrated by a rightward shift of T_{50\%} for most mIPSCs (A and C, *P < 0.01 for both, KS test). Conversely, AP temporarily lost its effect on T_{50\%} over the first 2 days after SE, as illustrated by the fact that the plots before and after AP totally matched one another (B). This is confirmed by the transient loss of the T_{50\%} potentiation (D) and the rise time (E) induced by AP in Li-pilocarpine rats examined 24–48 h after SE. The effect of AP perfusion on T_{50\%} mIPSCs is reversible, as illustrated in the washout experiment performed in a Li-saline rat (F). *P < 0.005, **P < 0.001, statistically significant differences from the recording before AP perfusion; +++P < 0.01, statistically significant differences between groups.
in CA1 pyramidal neurones from control animals (King et al. 1985). The effect of flumazenil is not consistent solely with the up-regulation of α4 and δ subunits because receptors incorporating these subunits are insensitive to benzodiazepines (Barnard et al. 1998; Mody, 2001). Variability of the effects of flumazenil has been reported in numerous pathophysiological conditions (Sand et al. 2000). For example, flumazenil can reduce epileptic activity in both untreated patients and patients acutely treated with benzodiazepines (Hart et al. 1991; Sharief et al. 1993) or who have developed a tolerance to benzodiazepine agonists Polc et al. 1995; Reisner & Pham, 1995; Tietz et al. (1999).

The paradoxical effects of flumazenil have led many authors to suggest that there may be endogenous ligands for benzodiazepine receptors and that flumazenil may act by competing with or displacing these substances from the benzodiazepine binding site (Rothstein et al. 1992; Polc et al. 1995; Lugaresi et al. 1998; Wallace et al. 2001). These endogenous compounds have been shown in both human and rat hippocampus (Alho et al. 1989; Ball et al. 1989) and their plasma and CSF concentration is increased in epileptic patients (Ferrarese et al. 1998) and during idiopathic recurrent stupor episodes (Rothstein et al. 1992; Lugaresi et al. 1998). The present data are not inconsistent with the presence of endogenous benzodiazepine ligands but would need further exploration to determine whether or not changes in the concentration and properties of these ligands may have occurred in Li-pilocarpine rats.

Finally, despite the extensive reorganization and neuronal loss in Li-pilocarpine rats, the accessibility of flumazenil to GABA_A receptors at GC synapses does not seem to be facilitated or reflect increased diffusion leading to higher local concentrations, since the effects of 1 and 10 µM flumazenil were identical in both control and epileptic rats. Moreover, at higher concentrations, flumazenil would, rather, act as a positive agonist (Buldakova & Weiss, 1997; Hauser et al. 1997; Thomson et al. 2000).

**Preservation of neurosteroid sensitivity in mIPSCs of epileptic GCs.** In our study, the effects of allopregnanolone on potentiation of GABA_A-mediated currents recorded in Li-saline GCs were preserved in epileptic GCs. Conversely, Mtchedlishvili et al. (2001) have shown that GABA_A receptors from GCs lose sensitivity to neurosteroids in rats rendered epileptic by self-sustained SE. This difference may be linked to model characteristics, the extent of neuronal loss and tissue reorganization or the type of GABA_A receptor population studied, i.e. the whole population in their study and the synaptic one in the present study. Allopregnanolone is one of the most potent endogenous positive modulators of GABA_A receptors (Lambert et al. 1996). In addition, allopregnanolone, like pentobarbital, increases the apparent affinity for GABA at GABA_A receptors (Lambert et al. 1995). This confers strong anticonvulsant properties to allopregnanolone that have been described in rats exposed to GABA_A receptor antagonists, kainic acid or perforant path stimulation (Lambert et al. 1995; Frye & Scalize, 2000). Thus, the fact that allopregnanolone preserves its potent effect on mIPSCs in epileptic rats indicates that a large proportion of synaptic GABA_A receptors remain sensitive to neurosteroids in these animals. Neurosteroids affect GABA_A receptors containing most subunits, except those comprising the α4 subunit (Smith et al. 1998a,b). Conversely, the α4β3δ subunit composition seems highly sensitive to neurosteroids in both control and epileptic rats studied 24–48 h after SE. Refractoriness to neurosteroid modulation is only transient since the effect of allopregnanolone is recovered at 3–5 months after SE. Although changes in subunit composition of the GABA_A receptor have been reported for the most part in chronically epileptic animals, a transient subunit change could render the receptor insensitive to the neurosteroid ligands. This phenomenon occurs during postnatal development (Cooper et al. 1999; Mtchedlishvili et al. 2003). In the dentate gyrus, GABA_A receptors are sensitive to neurosteroids at postnatal day 10 (P10), insensitive at P20 and sensitive again in adult rats (Cooper et al. 1999; Mtchedlishvili et al. 2001, 2003). Concurrently, from P12, there is a down-regulation of α4 and β1 subunits and the δ subunit becomes detectable (Laurie et al. 1992) – a result which correlates well with the relative insensitivity of granule cells of the P20 rats to neurosteroids (Cooper et al. 1999). The early and transient loss of GABA_A receptor sensitivity to neurosteroids recorded in the present study is possibly the result of changes in the brain metabolism of neurosteroids. Indeed, the sensitivity of dentate GC GABA_A synapses to neurosteroids varies according to the compound and reflects local metabolism (Belelli & Herd, 2003; Lambert et al. 2003), which could be transiently disturbed by all the events characterizing SE. Finally, SE may lead to an overproduction of peripheral and/or endogenous neuroactive steroids inducing acute alterations...
in GABA<sub>A</sub> receptor properties and decreasing sensitivity to neurosteroids. These compounds contribute to homeostasis during acute stress (Barbaccia et al. 2001), in which situation their plasma and brain concentrations increase markedly (Purdy et al. 1991). This is also the case for postictal circulating levels of allopregnanolone in epileptic children (Grosso et al. 2003).

**Conclusion**

This study reveals chronic alterations of synaptic GABA<sub>A</sub> receptors that appear very early after SE and lead to altered GABA<sub>A</sub> receptor function in GCs of Li-pilocarpine rats. Thus, despite the enhancement of GABA<sub>A</sub> receptor currents in epileptic GCs, changes in their modulation may compromise the gatekeeper function of the dentate gyrus and facilitate seizure generation and propagation. Such plasticity with regard to endogenous modulators such as zinc and neurosteroids favours increased hyperexcitability and susceptibility to seizures. Understanding the refractoriness of epileptic GCs to benzodiazepine excitability and susceptibility to seizures. Understanding such plasticity with regard to endogenous modulators might lead to new therapeutic approaches and their clinical use as an alternative therapy to benzodiazepines.

**References**


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