Development/Plasticity/Repair

Plasticity of $GABA_B$ Receptor-Mediated Heterosynaptic Interactions at Mossy Fibers After Status Epilepticus

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Several neurotransmitters, including GABA acting at presynaptic $GABA_B$ receptors, modulate glutamate release at synapses between hippocampal mossy fibers and CA3 pyramidal neurons. This phenomenon gates excitation of the hippocampus and may therefore prevent limbic seizure propagation. Here we report that status epilepticus, triggered by either perforant path stimulation or pilocarpine administration, was followed 24 hr later by a loss of $GABA_B$ receptor-mediated heterosynaptic depression among populations of mossy fibers. This was accompanied by a decrease in the sensitivity of mossy fiber transmission to the exogenous $GABA_B$ receptor agonist baclofen. Autoradiography revealed a reduction in $GABA_B$ receptor binding in the stratum lucidum after status epilepticus. Failure of $GABA_B$ receptor-mediated modulation of mossy fiber transmission at mossy fibers may contribute to the development of spontaneous seizures after status epilepticus.

Key words: **epilepsy; $GABA_B$ receptor; status epilepticus; mossy fibers; CA3; seizures**

Introduction

Hippocampal mossy fibers represent a major input from dentate granule cells to the hippocampal CA3 field. They exhibit several forms of presynaptic modulation of transmitter release, including marked short-term (Salin et al., 1996) and long-term (Harris and Cotman, 1986) use-dependent plasticity. They are sensitive to several neurotransmitters that depress transmitter release, including glutamate (Kamiya et al., 1996), GABA (Min et al., 1998; Vogt and Nicoll, 1999), and peptides (Weisskopf et al., 1993) acting at metabotropic receptors. Mossy fiber transmission may be under such profound modulation because hippocampal principal cells are highly vulnerable to excitotoxicity (Meldrum, 1993). Nevertheless, these modulatory mechanisms can break down: excessive activity in the dentate gyrus can spread into the hippocampus and can result in neuronal loss that resembles that seen with kainate administration (Sloviter, 1991).

Failure of modulation of mossy fiber transmission may also contribute to the delayed development of spontaneous seizures (epileptogenesis) after an insult. An important example of such a phenomenon is the occurrence of spontaneous seizures after an episode of status epilepticus (SE), defined as seizure activity lasting >30 min (Shorvon, 1994). SE results in acute changes in transmission in the dentate gyrus that could contribute to the maintenance of ongoing seizure activity and the later occurrence of spontaneous seizures (Lothman et al., 1990). Much attention has been given to reductions in inhibition in the dentate gyrus after SE (Sloviter, 1987; Hellier et al., 1999; Doherty and Dingledine, 2001; Kobayashi and Buckmaster, 2003). However, changes also occur in the modulation of excitatory transmission at mossy fiber → CA3 synapses (Goussakov et al., 2000).

We have focused on a form of modulation of mossy fiber → CA3 transmission that reflects the release of endogenous neurotransmitters from neighboring mossy fibers. In acute slices from guinea pigs, a train of stimuli delivered to one group of mossy fibers depresses transmission mediated by another population of mossy fibers. This "heterosynaptic depression" can be mediated both by group II metabotropic glutamate receptors and by $GABA_B$ receptors (Min et al., 1998; Vogt and Nicoll, 1999). The source of GABA that contributes to heterosynaptic depression is unclear. It could originate from interneurons, but another possible source is mossy fibers themselves, which, in addition to glutamate, contain GABA (Sloviter et al., 1996). Indirect evidence for mossy fiber GABAergic transmission has been reported in guinea pigs (Walker et al., 2001) and after seizure-like activity in rats (Gutierrez and Heinemann, 2001).

Here we show that $GABA_B$ receptor-mediated heterosynaptic depression is present at the normal rat mossy fiber synapse, but it is significantly reduced 24 hr after SE in two rodent models. The loss of heterosynaptic depression was seen despite an increase in immunogold staining for mossy fiber GABA. However, it was accompanied by a reduction in the sensitivity of mossy fiber transmission to a $GABA_B$ receptor agonist and by a loss of $GABA_B$ receptor binding revealed by autoradiography. Downregulation of presynaptic $GABA_B$ receptors may play an important role in

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the maintenance of seizure activity during SE and in the development of epilepsy.

Materials and Methods

Epilepsy models

All animal procedures followed the Animal (Scientific Procedures) Act of 1986.

Pilocarpine model. Limbic status epilepticus was induced in adult male Sprague Dawley rats (270–330 gm) by injection of the muscarinic agonist pilocarpine (310–340 mg/kg, i.p.) (Turski et al., 1989). To lessen peripheral cholinergic effects, scopolamine methyl nitrate (1 mg/kg, i.p.) was administered 30 min before and 30 min after pilocarpine. The onset of SE was defined as the appearance of stage 3 (Racine, 1972) seizures followed by continuous clinically detectable seizure activity. Clinically overt SE was terminated after 90–120 min by injection of diazepam (10 mg/kg, i.p.).

Perforant path stimulation model. This method has been described in detail previously (Walker et al., 1999). In brief, male Sprague Dawley rats (270–330 gm) were anesthetized with 1–2% halothane in O_2. An earth electrode was positioned subcutaneously, and a monopolar recording electrode was implanted stereotaxically into the right hippocampus (coordinates, 2.5 mm lateral and 4 mm caudal from bregma). A bipolar stimulating electrode was implanted in the right hemisphere and advanced into the angular bundle (coordinates, 4.4 mm lateral and 8.1 mm caudal from bregma) to stimulate the perforant path. The depths of the electrodes were adjusted to maximize the slope of the dentate granule cell field potential (Walker et al., 1999). The electrodes were held in place with dental acrylic and skull screws. The animals were allowed to recover from anesthesia. Seven days later, the perforant path was electrically stimulated with 2–3 mA 50–150 μsec monopolar pulses at 20 Hz for 2 hr; this induced self-sustaining SE that was terminated after 3 hr with propofol (50 mg/kg, i.p.).

Animals were killed 24 hr or 3 weeks after SE with an overdose of pentobarbitone (500 mg/kg, i.p).

Electrophysiology

Transverse hippocampal slices (400 μm thick) were obtained from control rats and rats after SE and were stored in an interface chamber for at least 1 hr before transfer to a submersion recording chamber. The storage and perfusion solution contained (in mM): 119 NaCl, 2.5 KCl, 4 $MgSO_4$, 4 $CaCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose and was gassed with 95% O_2 and 5% CO_2 (23–25°C). Field EPSPs (fEPSPs) were recorded using glass microelectrodes (resistance, ~1 MΩ) filled with the perfusion solution, positioned in the stratum lucidum, the mossy fiber termination zone. Two bipolar stainless steel stimulating electrodes were positioned in the dentate granule cell layer, and 0.5–1 mA pulses (80 μsec duration) were applied with constant current stimulators. The electrode positions and stimulus intensities were adjusted until mossy fiber fEPSPs of maximal amplitude were recorded. Slices were discarded if mossy fiber fEPSPs could not be elicited (see Results), probably reflecting noncongruence of the slicing plane and the plane containing the mossy fibers, which run in a lamellar pattern from the dentate gyrus. This occurred with similar frequency in control and post-SE animals.

Naloxone (10 μM) was present in all two-pathway experiments to avoid interference from opioid receptor-mediated heterosynaptic depression (Weisskopf et al., 1993). Sensitivity to the group II metabotropic glutamate receptor agonist (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG-IV, 1 μM) was used in all experiments to verify that fEPSPs were mediated by mossy fiber synapses (Kamiya et al., 1996). The following drugs were used to block $GABA_B$, AMPA/kainate, and group II metabotropic glutamate receptors (mGluRs); CGP52432 (5 μM), SCH50911 (20 μM), 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX) (50 μM), and LY341495 (500 nM). NO711 (20 μM) was used to block GABA uptake. The $GABA_B$ receptor agonist baclofen (0.1–10 μM) was used to determine $GABA_B$ receptor sensitivity. Drugs were obtained from (Tocris Cookson, Bristol, UK), except for DCG-IV and NO711 (Sigma, St. Louis, MO).

Immunogold labeling for GABA

For electron microscopic postembedding immunogold labeling, ultrathin brain sections were obtained from adult male Sprague Dawley rats (three controls and two after SE, induced by pilocarpine as described above). Ultrathin sections (50 nm) were cut with a Reichert (Depew, NY) Ultracut and collected on pioloform-coated single-slot nickel grids. Grids were then mounted in a grid support plate, soaked in phosphate buffer (PB) for 30 min, and preincubated in an incubation medium (IM) consisting of PB with 1% bovine serum albumin (A4503; Sigma) and 5% fetal calf serum for 30 min at room temperature. Sections were then incubated with a rabbit anti-GABA antibody (1:4000 in IM; A2052; Sigma) overnight at 4°C. After thorough washing (four times for 10 min in PB) and preincubation in IM (30 min), the secondary antibody (goat anti-rabbit IgG coupled to 10 nm gold particles; Sigma) was applied at a dilution of 1:100 in IM for 4 hr at 37°C. Preparations were washed subsequently in PB (five times for 10 min) before final rinsing in double-distilled water. The sections were contrasted with uranyl acetate (4 min) and Reynold's lead citrate (50 sec) according to standard electron microscopic methods. Preparations were examined using a Philips (Eindhoven, The Netherlands) 201C electron microscope. A control preparation from which the primary antibody was omitted showed no immunolabeling.

Autoradiography

Brains removed from control or post-SE rats were rapidly frozen in isopentane cooled in liquid nitrogen and stored at −70°C. Sections (12 μm thick) were cut on a cryostat (2800 Frigocut; Reichert), thaw-mounted onto Superfrost Plus slides (75 × 25 × 1.0 mm; BDH Chemicals, Poole, UK), and then stored at −20°C until used. On the day of binding experiments, sections were left to equilibrate to room temperature. Slides were then incubated in 50 mM Tris buffer containing 2.5 mM $CaCl_2$, pH 7.4, for 20 min before incubating in fresh buffer for 60 min. They were then dried in room air. Each section was then incubated for 60 min and subsequently dried in [^{3}H]CGP62349 (at concentrations of 0.5, 0.75, 1, 2, 4, and 8 nM) to determine total binding. [^{3}H]CGP62349 at the same concentrations plus CGP54626A (10 μM) was used for incubation and then dried to determine nonspecific binding. The slides were then washed in buffer two times for 1 min and briefly rinsed in distilled water to remove buffer salts. The slides were dried in room air and then apposed to film (Hyperfilm-3H; Amersham Biosciences, Arlington Heights, IL). The films were developed 28 d after exposure to the slides. Quantification of receptor autoradiography was achieved by film densitometry using an image analysis system (Microcomputer Imaging Device; Imaging Research Inc., St. Catharines, Ontario, Canada), and optical density was converted to femtomoles per milligram of bound ligand. Total binding in the stratum lucidum was assessed in four to eight sections per concentration of [^{3}H]CGP62349 for each animal. Binding parameters, receptor density (B_{max}), and affinity (K_D) were determined by the use of the Langmuir equation in Prism PC software (Graph Pad, San Diego, CA). [^{3}H]CGP62349 was a gift from Dr. Wolfgang Froestl (Novartis, Basel, Switzerland).

Data are expressed as mean ± SEM. The baclofen data were analyzed using a best least squares fit to $I_{max}/(1 + IC_{50}/[\text{baclofen}])$, where I_{max} is the maximal inhibition, and the IC_{50} is the concentration of baclofen that results in half-maximal inhibition (KyPlot; KyensLab Inc., Tokyo, Japan). Results were compared using two-tailed paired or unpaired Student's *t* test, as appropriate.

Results

$GABA_B$ receptors mediate heterosynaptic depression in rats

Because the role of $GABA_B$ receptors in heterosynaptic depression has hitherto only been studied in juvenile guinea pigs, we first asked whether it could be induced at the mossy fiber → CA3 synapse in acute hippocampal slices from adult control rats. All data were obtained with the opioid receptor antagonist naloxone (10 μM) in the perfusion solution to remove any confounding effect of presynaptic opioid receptors (Weisskopf et al., 1993). The recording solution normally contained 4 mM Ca^{2+} and 4 mM Mg^{2+} to reduce the likelihood of epileptiform bursting. We first verified that the two stimulating electrodes in stratum granulosum evoked fEPSPs recorded in the stratum lucidum with prop-

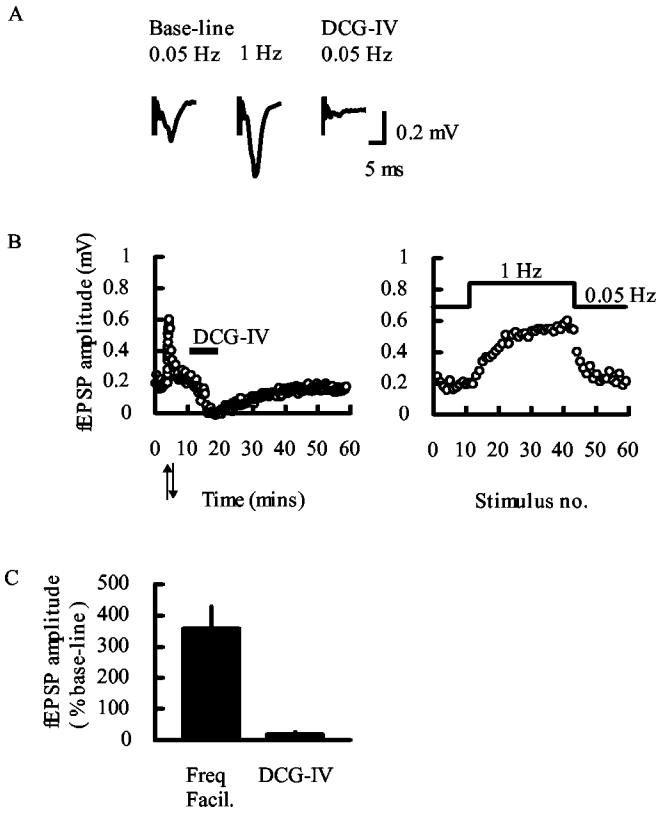

Figure 1. Mossy fiber fEPSPs were identified by demonstrating marked frequency facilitation (Freq Facil.) and sensitivity to the group II metabotropic glutamate receptor agonist DCG-IV. *A*, fEPSPs (traces are averages of 5 trials, taken from experiment shown in *B*) recorded in the stratum lucidum while stimulating the stratum granulosum of the dentate gyrus at baseline frequencies of 0.05, 1, and 0.05 Hz in the presence of the group II metabotropic glutamate receptor DCG-IV (1 μM). *B*, Left graph, Example of an experiment demonstrating the identification of mossy fiber fEPSPs by frequency facilitation (arrows, change in stimulation frequency from 0.05 to 1 Hz) and DCG-IV sensitivity. Right graph, Same experiment showing the effect of an increase in frequency plotted against stimulus number. *C*, Summary data ($n = 8$). The fEPSP amplitude increased to at least 200% of baseline (20th/1st response) when the stimulus frequency was increased from 0.05 to 1 Hz, and the mGluR agonist DCG-IV (1 μM) decreased fEPSP amplitude to <30% of baseline.

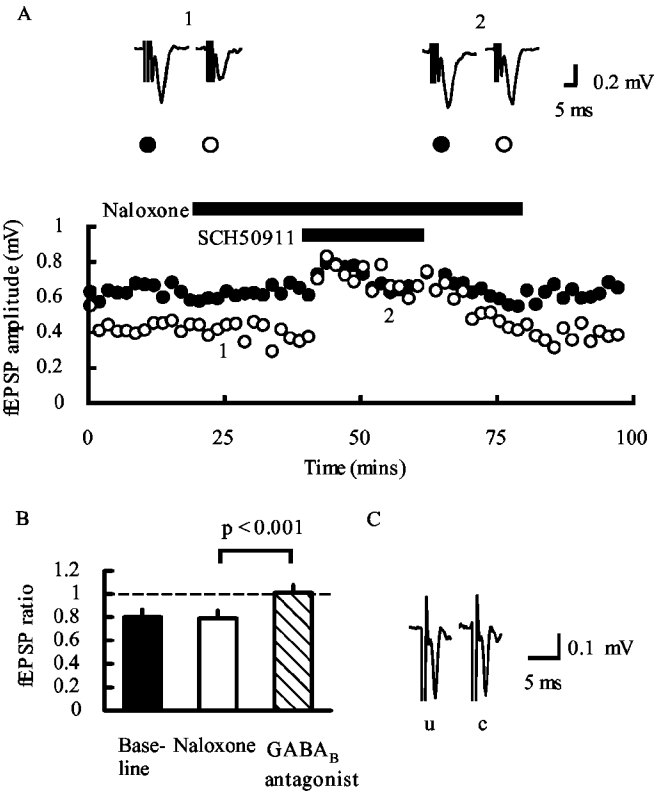

Figure 2. Heterosynaptic depression evoked in control slices is mediated by $GABA_B$ receptors. A conditioning train was applied to the conditioning pathway preceding every 10th stimulus delivered to the test pathway. *A*, Example of one experiment from a control slice. Heterosynaptic depression was observed as a reduction in amplitude of the fEPSPs that were preceded by a train (open circles) compared with fEPSPs not preceded by a train (each filled circle represents the average of the 9 unconditioned fEPSPs in each cycle). The opioid antagonist naloxone (10 μM) had no effect on the magnitude of depression. The $GABA_B$ receptor antagonist SCH50911 (20 μM) abolished the depression. fEPSPs (averages of 5 trials each) are shown in the absence (1) and presence (2) of SCH50911. *B*, Summary of the ratio of conditioned/unconditioned fEPSP amplitudes in 8 control slices showing the abolition of heterosynaptic depression by blocking $GABA_B$ receptors. *C*, Example of presynaptic fiber volleys from one experiment showing that the conditioned presynaptic fiber volley [preceded by a conditioning train (c)] is not different in amplitude or shape from the unconditioned fiber volley [not preceded by a train (u)]. Traces are averages of responses in the presence of 50 μM NBQX from two cycles of nine unconditioned stimuli and one conditioned stimulus.

erties consistent with mossy fibers. fEPSPs showed marked short-term frequency-dependent facilitation (increase in fEPSP amplitude more than twice baseline) when the stimulation frequency was increased from 0.05 to 1 Hz (Fig. 1*A–C*). In all experiments, we confirmed that they showed high sensitivity to the group II mGluR agonist DCG-IV (1 μM), characteristic of mossy fiber synapses (Kamiya et al., 1996) (Fig. 1A,B,C).

In addition, we verified that the two pathways were separate by confirming that the fEPSP elicited in one pathway was not facilitated by a preceding stimulus to the other pathway (cross-facilitation was 4 ± 3%; $n = 6$).

At the end of each experiment, we applied the AMPA/kainate receptor antagonist NBQX (50 μM) to record the remaining presynaptic fiber volley, which was then subtracted from the fEPSP before analysis of the results. A high concentration of NBQX was used to ensure that all AMPA and kainate receptors were blocked (Bortolotto et al., 1999).

Once two separate mossy fiber pathways had been identified, we used the following stimulation protocol to induce heterosynaptic depression. Single stimuli were applied to the "test" pathway at a frequency of 0.05 Hz. Three hundred milliseconds before every 10th stimulus, a train of five stimuli at 50 Hz was applied to the "conditioning" pathway. The magnitude of heterosynaptic depression was calculated by comparing the amplitude of every 10th fEPSP recorded [conditioned fEPSP ($fEPSP_C$)] to the average amplitude of the nine preceding fEPSPs [unconditioned fEPSP ($fEPSP_U$)]. The fEPSP ratio was calculated as $fEPSP_C/fEPSP_U$.

Heterosynaptic depression, expressed as (1 − fEPSP ratio) × 100%, was observed in all slices from adult control rats (21 ± 7% depression; $n = 8$ slices from different animals) (Fig. 2*A,B*). Bath perfusion of $GABA_B$ receptor antagonists (20 μM SCH50911; $n = 6$; or 5 μM CGP52432; $n = 2$) completely abolished heterosynaptic depression in all cases (0 ± 6% depression; $p < 0.001$ for comparison with baseline) (Fig. 2*A,B*). Thus, in slices from control adult rats, heterosynaptic depression evoked with this protocol was entirely mediated by $GABA_B$ receptors, with no detectable contribution from mGluRs (Min et al., 1998; Vogt and Nicoll, 1999).

Blocking $GABA_B$ receptors also caused a small increase in the size of the unconditioned fEPSPs in two of eight control slices (Fig. 2*A*). This did not reach significance in either control or

post-SE slices when all experiments were included ($p = 0.45$; paired t test). Because heterosynaptic interactions mediated by spillover of the transmitter are temperature-dependent (Asztely et al., 1997; Mitchell and Silver, 2000), we also measured heterosynaptic depression at 34°C, using the same stimulation protocol. $GABA_B$ receptor-mediated heterosynaptic depression was identical to that recorded at room temperature (21 ± 8% depression; $n = 3$ slices from different animals; $p = 0.9$; unpaired t test, when compared with depression at room temperature).

To verify that this phenomenon is present at more physiological concentrations of divalent cations, we repeated the experiments with a perfusion solution containing 2.5 mM Ca^{2+} and 1.3 mM Mg^{2+}. In two slices, this yielded heterosynaptic depression of 33 ± 4%, which was reduced to 4 ± 2% with $GABA_B$ receptor antagonists.

Although $GABA_B$ receptors profoundly depress transmitter release, a potentially confounding effect is efflux of K^+ from neurons, which could alter axon recruitment, action potential propagation, or both. We therefore compared the presynaptic fiber volleys (recorded in the presence of NBQX to abolish the fEPSP) with and without the 50 Hz train in the conditioning pathway. Neither the amplitude ($p = 0.69$; paired t test; $n = 8$) nor the shape of the fiber volley was significantly affected (Fig. 2*D*), implying that heterosynaptic depression is not mediated by an increase in extracellular K^+.

SE abolishes $GABA_B$ receptor-mediated heterosynaptic depression

We interleaved the experiments described above with identical experiments performed on hippocampal slices taken from rats 24 hr after pilocarpine-induced SE. We used the same criteria to identify two separate mossy fiber pathways (cross-facilitation, 0 ± 6%; $n = 11$).

The degree of frequency-dependent facilitation was not significantly different from that measured in control slices (post-SE, 232 ± 25% of baseline, $n = 12$; control, 358 ± 67%, $n = 8$; measured as 20th fEPSP/1st fEPSP amplitude when stimulation was increased from 0.05 to 1Hz; $p = 0.1$) (Fig. 3*A*). The nonsignificant trend for less frequency facilitation in the post-SE animals is consistent with the findings of Goussakov et al. (2000), who showed a significant loss of facilitation after kainic acid-induced SE. The more marked changes observed by Goussakov et al. (2000) may be attributable to differences in stimuli to induce SE (kainic acid vs pilocarpine), stimulation frequency (100 instead of 1 Hz), a longer interval from SE to killing of the animals (several weeks instead of 24 hr), or a combination thereof.

In post-SE slices, DCG-IV perfusion reduced the fEPSPs to 22 ± 5% of baseline. This depression was also indistinguishable from that seen in control slices (19 ± 8%; $p = 0.8$) (Fig. 3*A*).

In contrast to control animals, heterosynaptic depression was not detected after pilocarpine-induced SE (1 ± 4% depression; $n = 12$; $p = 0.75$; paired t test comparing conditioned with unconditioned fEPSPs) (Fig. 3*B*,*C*). Thus, despite the apparently normal short-term plasticity and mGluR sensitivity of mossy fiber synapses, they were unaffected by trains of stimuli delivered to neighboring axons.

These results do not distinguish between a generic effect of limbic seizures and a specific result of the chemoconvulsant used to evoke SE. We therefore asked whether a different model of SE, which does not involve the use of an exogenous convulsant, also interfered with $GABA_B$ receptor-mediated modulation of mossy fiber transmission. We examined hippocampal slices taken from six animals 24 hr after SE evoked by perforant path stimulation

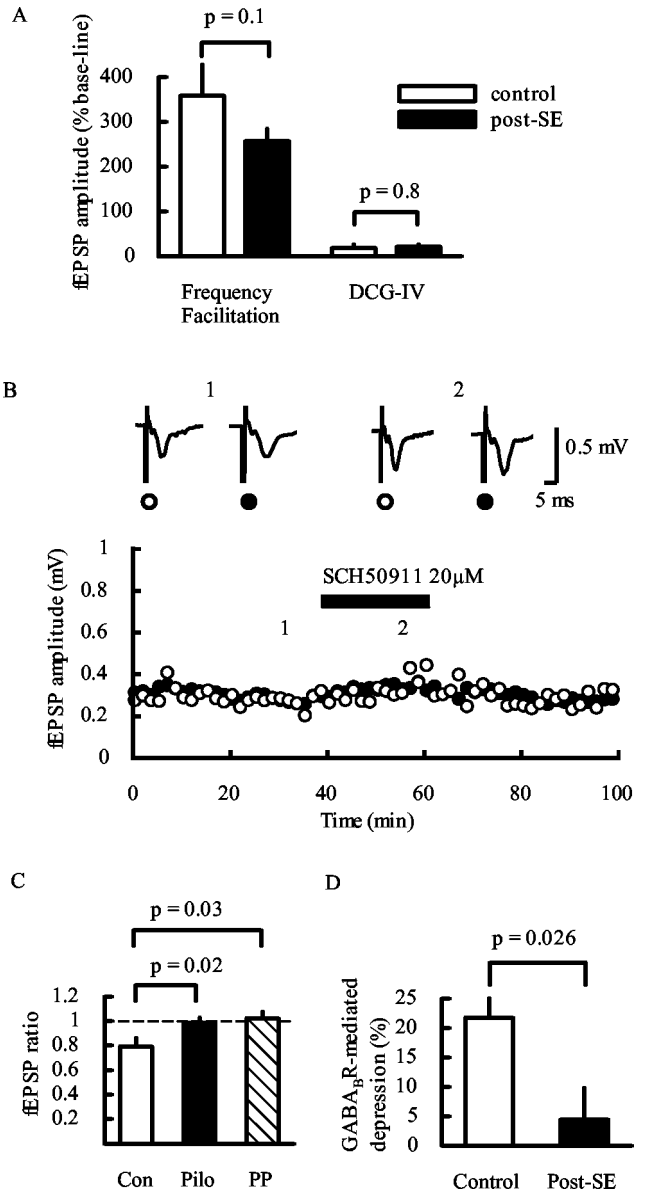

Figure 3. $GABA_B$ receptor-mediated heterosynaptic depression was absent after SE. *A*, Mossy fiber fEPSPs in slices after pilocarpine-induced SE ($n = 12$) showed frequency facilitation and DCG-IV sensitivity, which was not significantly different from that of control slices. *B*, Heterosynaptic depression was absent after pilocarpine-induced SE: example of one experiment. fEPSPs are shown in the absence and presence of SCH50911 (each trace is the average of 5 trials; filled circles, conditioned; open circles, unconditioned). The $GABA_B$ receptor antagonist SCH50911 had no significant effect. *C*, There was no significant heterosynaptic depression in animals after pilocarpine-induced (Pilo) or perforant path (PP) stimulation-induced SE in contrast to the marked depression seen in control (Con) animals. *D*, Size of $GABA_B$ receptor-mediated heterosynaptic depression, estimated from the effect of the $GABA_B$ antagonists SCH50911 and CGP52432 on the fEPSP ratio (SE: both models combined; $n = 18$).

(see Materials and Methods). Heterosynaptic depression was again absent in slices from all six animals (−2 ± 5% depression; $n = 6$) (Fig. 3*C*). We also examined slices from two animals treated with pilocarpine that had not developed SE. Both animals received diazepam injections identical to those administered to the animals that experienced SE. Heterosynaptic depression was observed in slices from these animals (45 ± 4% depression). Thus, we conclude that loss of heterosynaptic depression is related to SE per se rather than to the stimulus used to evoke it.

To test whether the loss of heterosynaptic depression after SE

was attributable to the emergence of a compensatory heterosynaptic facilitation, we applied the $GABA_B$ receptor antagonists SCH50911 (20 μM) and CGP52432 (5μM) during the two-pathway protocol in slices after SE. This had no significant effect on the fEPSP ratio in slices from the post-SE animals (5 ± 4% change in the fEPSP ratio; $n = 18$). This was significantly different from that of controls (22 ± 3% change in the fEPSP ratio; $n = 8$) when $GABA_B$ receptor antagonists were applied ($p = 0.026$; unpaired t test for difference between SE and control animals). This result implies that loss of heterosynaptic depression after SE was not attributable to the emergence of a compensatory heterosynaptic facilitation but because of failure of $GABA_B$ receptor-mediated depression.

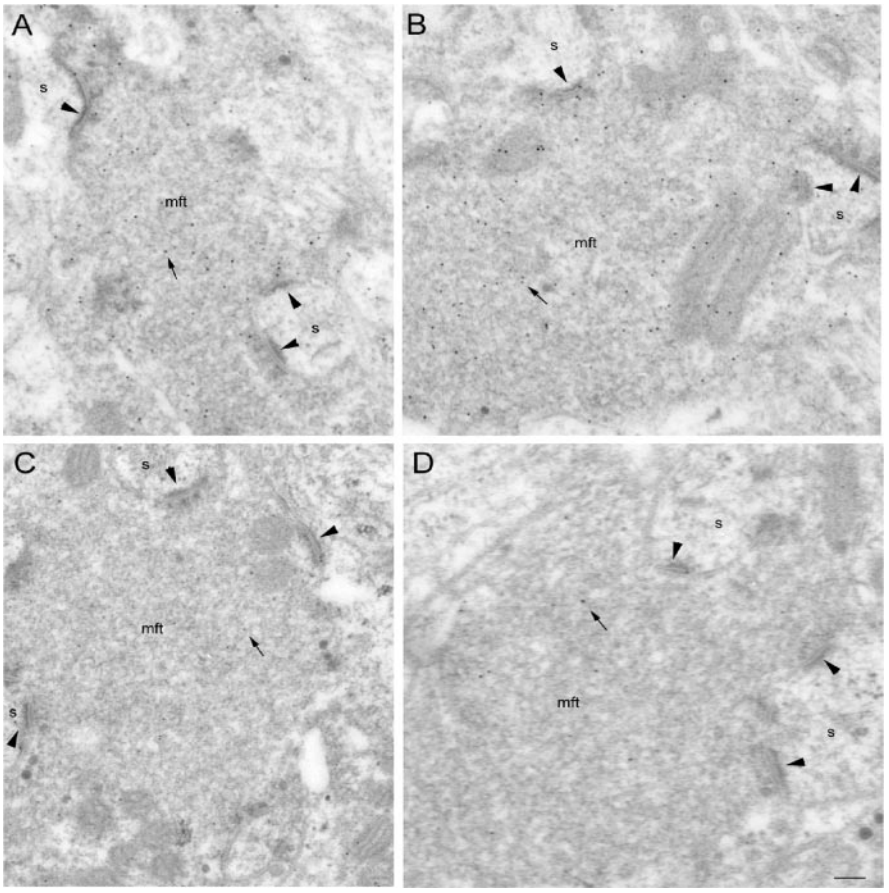

Figure 4. Sections through GABA immunogold-labeled mossy fiber terminals in SE (*A, B*) and control (*C, D*) animals. Tissue from animals after SE have significantly higher density of immunogold particles (arrows). Arrowheads, Synaptic sites; mft, mossy fiber terminal; s, spine. Scale bar: *A, B*, 100 nm; *C*, 120 nm; *D*, 60 nm.

SE is accompanied by an increase in mossy fiber GABA

The loss of heterosynaptic depression could contribute to the persistence of seizure activity during SE, to the subsequent development of epilepsy, or to both. The precise relationship of the failure of $GABA_B$ receptor-mediated heterosynaptic depression to these phenomena is beyond the scope of the present study. Instead, we asked what cellular alterations could underlie this result.

There are two main sources of GABA that could mediate this heterosynaptic depression. First, the GABA could originate from mossy fibers because there is circumstantial evidence that mossy fibers themselves can release GABA (see Introduction). Second, interneurons recruited by the mossy fibers could release GABA that spills into the extracellular space. From the first hypothesis, a possible explanation for loss of $GABA_B$ receptor-mediated heterosynaptic depression is that there is a reduction in the releasable GABA in mossy fibers after SE. This is, however, difficult to reconcile with reports that immunolabeling for GABA and glutamic acid decarboxylase increases after seizure-like activity (Sloviter et al., 1996) and that this is accompanied by the emergence of a monosynaptic GABAergic signal (Gutierrez and Heinemann, 2001). Nevertheless, much of the evidence for increased mossy fiber GABA is difficult to relate directly to the present study because of different seizure models or because different criteria were used to identify mossy fibers. Indeed, most reports have relied on light microscopy, which does not ensure that the epitope is localized to mossy fiber terminals. We therefore applied immunogold electron microscopy to detect GABA-like immunoreactivity in mossy fiber terminals.

Figure 4 shows examples of electron micrographs taken from the stratum lucidum. Mossy fiber terminals were identified by their large size, multiple active zones, and numerous vesicle profiles. In addition, we also used a modified Timm's reaction in one control animal and one post-SE animal to confirm the identity of the mossy fiber terminals on the basis of their high zinc content (Seress and Gallyas, 2000). We did not use these animals for quantitative analysis because a different fixation method was used. Immunogold particles in mossy terminals of control animals (Fig. 4*C,D*) were present at a density of 12 ± 1/μm^2 (56 terminals from two animals). This was significantly greater than the background density of 4 ± 1/μm^2 ($p < 0.001$). We repeated these measurements in two post-SE animals (Fig. 4*A,B*). The GABA immunogold particle density in mossy fiber terminals was doubled 24 hr after SE induced by pilocarpine to 24 ± 2/μm^2 (45 terminals from two animals; $p < 0.001$ compared with controls). Thus, the immunolabeling, although confirming more indirect evidence for GABA in mossy fibers, lends no support to the hypothesis that the amount available to be released is decreased after SE. On the contrary, there is more GABA in mossy fibers after SE, in keeping with previous reports that seizure-like activity is followed by the emergence of a monosynaptic GABAergic signal in CA3 pyramidal neurons (Gutierrez and Heinemann, 2001). Thus, to explain the loss of heterosynaptic depression after SE, it is necessary to postulate either that (1) the source of GABA mediating heterosynaptic depression is not mossy fibers but interneurons, or (2) a change downstream of GABA release is responsible.

Heterosynaptic depression is not restored by blocking mGluRs or GABA uptake

If the GABA that mediates heterosynaptic depression is released from interneurons, a possible explanation for the effect of SE is impaired recruitment of interneurons, which has been described in several models of epilepsy (Sloviter, 1991; Lothman et al., 1996; Doherty and Dingledine, 2001). Reduced recruitment of inter-

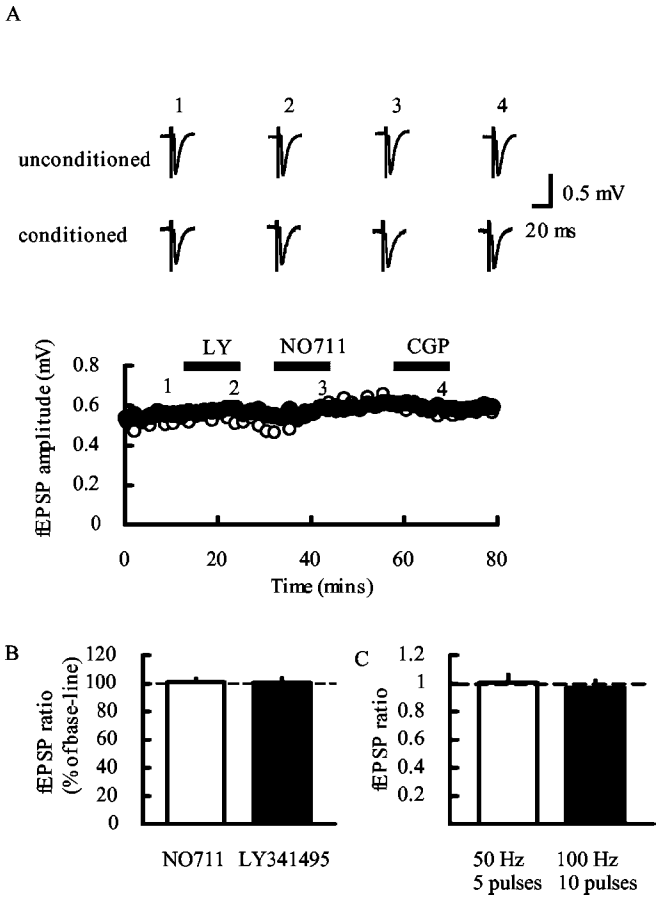

Figure 5. Heterosynaptic depression could not be rescued after SE by blocking group II metabotropic glutamate receptors by blocking GABA uptake or by increasing the number of conditioning stimuli. *A*, Example of one experiment. The group II metabotropic glutamate receptor antagonist LY341495 (LY; 500 nM) failed to restore heterosynaptic depression in a slice obtained after pilocarpine-induced SE. Application of the GABA transporter GAT1 blocker NO711 (20 μM) also failed to affect the fEPSP ratio. Sample traces, fEPSPs in control conditions and in the presence of LY34195, NO711, and CGP52432 (each trace is the average of 5 trials). *B*, Summary of effects of NO711 ($n = 4$) and LY341495 ($n = 5$) on the fEPSP ratio in animals after pilocarpine-induced SE. *C*, A 100 Hz train did not rescue heterosynaptic depression in slices after SE. The fEPSP ratio did not change significantly when the train was increased from 50 to 100 Hz ($p = 0.4$; paired *t* test).

neurons in the dentate hilus after SE has been proposed to be mediated by enhanced activity of group II mGluRs (Doherty and Dingledine, 2001). We therefore asked whether heterosynaptic depression could be rescued in slices from post-status animals by blocking group II mGluRs. We applied the group II mGluR antagonist LY341495 (500 nM) during the two-pathway experiment to slices from animals 24 hr after pilocarpine-induced SE. This had no significant effect on the magnitude of heterosynaptic depression ($p = 0.18$; paired *t* test; $n = 5$) (Fig. 5*A*,*B*). This concentration of LY341495 was sufficient to antagonize the effect of the group II mGluR agonist DCG-IV on mossy fiber transmission completely ($n = 3$; data not shown). We then asked whether changing the stimulation protocol to enhance the recruitment of interneurons could rescue depression. We applied the same stimulation protocol of five pulses at 50 Hz and then in the same slices increased the train to 10 pulses at 100 Hz (we tested this protocol in slices from four rats, two of which had pilocarpine-induced status epilepticus and two of which had perforant path stimulation-induced status epilepticus). Heterosynaptic depression was not rescued. The fEPSP ratio was 1 ± 0.1 during the 50 Hz protocol and 0.97 ± 0.05 during the 100 Hz protocol. The fEPSP ratio was not significantly different between 50 and 100 Hz trains (Fig. 5*C*). This provides no evidence for the hypothesis that impaired recruitment of interneurons accounts for the loss of heterosynaptic depression after SE.

Another possible explanation is that GABA uptake is enhanced. Altered GABA transport has been implicated in the pathogenesis of epilepsy (Patrylo et al., 2001). The antiepileptic drug tiagabine is a blocker of neuronal GABA transporter 1 (GAT1) (Andersen et al., 1993). A decrease in immunostaining for GAT1 has been reported in the sensorimotor cortex (Silva et al., 2002) and the hippocampus (Andre et al., 2001). We therefore attempted to rescue heterosynaptic depression by interfering with the major hippocampal GABA transporter GAT1 with the selective blocker NO711. We applied the same two-pathway protocol as described previously in slices from rats 24 hr after pilocarpine-induced SE. Bath application of 20 μM NO711 (which is sufficient to evoke a tonic $GABA_A$ receptor-mediated current in pyramidal neurons; Semyanov et al., 2003) failed to restore heterosynaptic depression ($n = 4$; $p = 0.88$; paired *t* test) (Fig. 5*A*,*B*). These results thus lend no support to the hypothesis that heterosynaptic depression was lost because of increased uptake.

Status epilepticus alters $GABA_B$ receptors on mossy fibers

A further explanation for loss of heterosynaptic depression is an alteration in the target $GABA_B$ receptors. Altered expression of $GABA_B$ receptors in CA3 pyramidal neurons has been reported in human temporal lobe epilepsy patients (Princivalle et al., 2002), and decreased presynaptic $GABA_B$ receptor activity has been proposed to underlie increased inhibitory activity in the dentate gyrus during epileptogenesis (Haas et al., 1996). We therefore asked whether there was loss of $GABA_B$ receptor function after SE. We stimulated a single mossy fiber pathway at 0.05 Hz in acute hippocampal slices taken either from control animals or from animals after pilocarpine-induced SE and measured the sensitivity of the fEPSP to increasing concentrations of the $GABA_B$ receptor agonist baclofen. Baclofen was less potent in reducing the fEPSP in the postpilocarpine SE slices compared with its effect in control slices (controls, IC_{50}, 0.47 ± 0.14 μM, $n = 6$; SE, IC_{50}, 1.17 ± 0.27 μM, $n = 7$; $p < 0.05$ for difference) (Fig. 6). There was no significant difference in the I_{max} by baclofen (controls, I_{max}, 0.95 ± 0.05, $n = 6$; SE, I_{max}, 0.87 ± 0.06, $n = 7$; $p = 0.31$ for difference). Loss of $GABA_B$ receptor function may contribute to the development of self-sustaining seizures in SE. This could lead to increased excitability in the hippocampus and could play a role in the later development of spontaneous seizures. We therefore asked whether this change in $GABA_B$ receptor function was persistent. We treated six animals with pilocarpine to induce 90 min of SE. These were killed 3 weeks later when they were exhibiting spontaneous seizures (all animals had several limbic seizures per day). We recorded fEPSPs in the stratum lucidum while stimulating in the dentate gyrus as above. Although the increase in IC_{50} for baclofen did not reach significance when compared with that in controls, the I_{max} was significantly reduced (0.74 ± 0.05; $p = 0.007$; unpaired *t* test) (Fig. 6*B*).

We then asked whether the reduced potency of baclofen at 24 hr was attributable to a change in the number or functional properties of $GABA_B$ receptors. We used [^{3}H]CGP62349 autoradiography to estimate the density of $GABA_B$ receptors in the stratum lucidum in control and post-pilocarpine-induced SE tissue (24 hr after SE). The radiolabeled antagonist was applied at concentrations of 0.5, 0.75, 1, 2, 4, and 8 nM. The mean K_D for post-SE

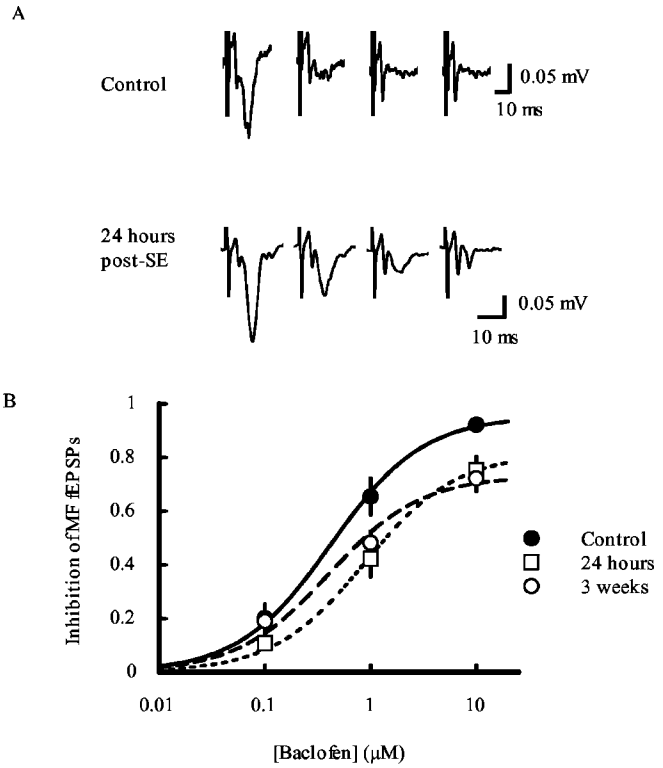

Figure 6. Mossy fiber fEPSPs become less sensitive to the $GABA_B$ receptor agonist baclofen after SE. *A*, fEPSPs (averages of 5 traces) illustrating reduced sensitivity to baclofen 24 hr after SE. Left to right, Traces are shown at baseline and in 0.1, 1, and 10 μM baclofen. *B*, Baclofen has a less potent effect on mossy fiber fEPSPs 24 hr after SE ($p < 0.05$; unpaired *t* test on IC_{50} values; 6 control slices and 7 post-SE slices), and the I_{max} was significantly reduced in slices 3 weeks after SE ($p = 0.007$; $n = 6$).

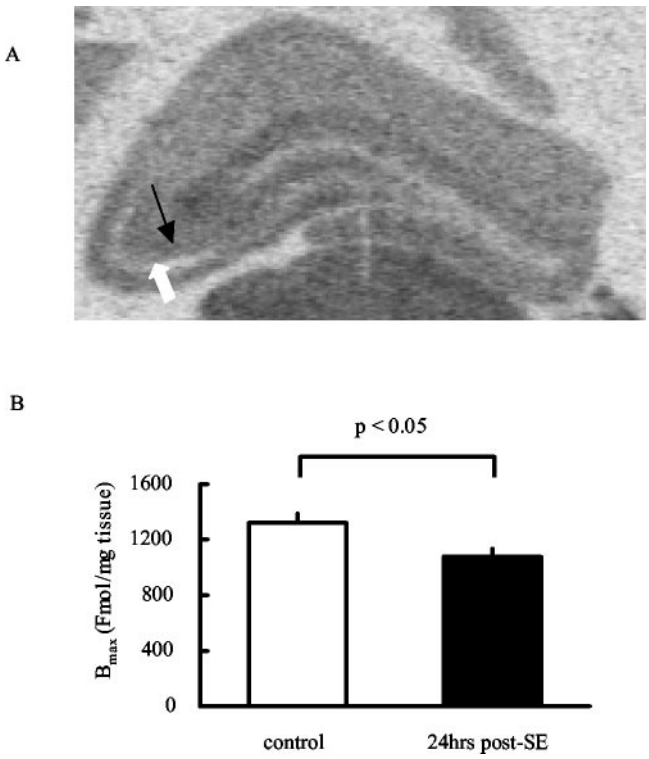

Figure 7. Reduction of binding of [^{3}H]CGP62349 to $GABA_B$ receptors in the stratum lucidum after pilocarpine-induced SE. *A*, Example of an autoradiograph from an animal after pilocarpine-induced SE. We measured binding in the stratum lucidum, (black arrow). The stratum pyramidale of CA3 is marked with a white arrow. *B*, The binding parameter B_{max}, which reflects maximal binding of the radioactive ligand to $GABA_B$ receptors, was significantly lower than that of the control after SE (control, $n = 5$; SE, $n = 4$; $p = 0.033$ for difference).

animals was not significantly different from that for control animals, implying that the affinity of the $GABA_B$ receptors was unchanged (controls, 1.28 ± 0.34 nM, $n = 5$; SE, 1.09 ± 0.10 nM, $n = 4$; $p = 0.67$). However, the mean B_{max} (a measure of $GABA_B$ receptor density) 24 hr after SE was significantly less than in controls (controls, 1322 ± 68 fmol/mg of tissue, $n = 5$; SE, 1080 ± 56 fmol/mg of tissue, $n = 4$; $p < 0.05$ for difference) (Fig. 7). We also observed a nonsignificant trend for the B_{max} to be reduced at 3 weeks (1144 ± 54 fmol/mg of tissue; $n = 4$; $p = 0.09$ for difference from control).

Is the reduction in $GABA_B$ receptor density specific to the presynaptic $GABA_B$ receptors on mossy fiber terminals, or is it a global change that also occurs in the dentate granule cell dendrites? To address this question, we measured binding in the molecular layer of the dentate gyrus. The mean B_{max} and K_D in 24 hr post-SE tissue were not significantly different from those of controls (B_{max}: controls, 925 ± 74 fmol/mg of tissue, $n = 5$; SE, 812 ± 71 fmol/mg of tissue, $n = 4$; $p = 0.29$; K_D: controls, 0.69 ± 0.20 nM; SE, 0.82 ± 0.23 nM; $p = 0.83$).

Thus, the loss of $GABA_B$ receptor-mediated heterosynaptic depression at 24 hr is accompanied by a selective reduction in $GABA_B$ receptor density in the stratum lucidum and an associated reduction in the sensitivity of mossy fiber signaling to $GABA_B$ receptor agonists. The 18% reduction in $GABA_B$ receptor binding in the stratum lucidum is nevertheless modest compared with the complete loss of $GABA_B$ receptor-mediated heterosynaptic depression. Among possible explanations for this discrepancy are nonlinear amplification of the intracellular signaling cascade linking receptors to transmitter release and the fact that, within the stratum lucidum, other cell types also express $GABA_B$ receptors, some of which may be upregulated (see Discussion).

Discussion

We have demonstrated that $GABA_B$ receptor-mediated heterosynaptic depression at the rat mossy fiber synapse is lost after SE, whether this is evoked by pilocarpine or perforant path stimulation. This loss is not attributable to a compensatory heterosynaptic facilitation or to depletion of GABA from mossy fiber terminals. We could not rescue it by applying group II mGluR antagonists or GABA transporter inhibitors. Instead, we find that the sensitivity of mossy fiber synapses to $GABA_B$ receptor agonists diminishes after SE, and this is mirrored by a decrease in $GABA_B$ receptor binding density. Thus, SE results in a loss of $GABA_B$ receptors that is sufficient to impair the detection of physiological levels of their endogenous ligand.

The role of heterosynaptic interactions among mossy fibers in gating excitatory afferent traffic to the hippocampus *in vivo* remains to be determined. Impairment of mutual activity-dependent depression among mossy fibers could contribute to lowering the threshold for seizure spread through mesial temporal structures. These changes could play a role in the development of temporal lobe epilepsy (TLE) and the ancillary damage to principal neurons in the hippocampus. The plasticity revealed in the present study must be seen within the broader context of other changes in inhibition occurring in the hippocampus during epileptogenesis, such as those occurring within the dentate gyrus and in the hippocampus proper. Altered inhibition is observed frequently in both experimental and human epilepsy. Both in-

creases (Haas et al., 1996) and decreases (Sloviter, 1987; Bekenstein and Lothman, 1993; Doherty and Dingledine, 2001) in hippocampal inhibition have been reported. Most studies have, however, concentrated on changes in $GABA_A$ receptor-mediated inhibition (Buhl et al., 1996; Kapur and Macdonald, 1997; Brooks-Kayal et al., 1998).

The loss of heterosynaptic depression observed in this study did not appear to be attributable to decreased GABA release or increased GABA uptake. There are two potential sources of GABA release after the conditioning train: mossy fibers themselves and interneurons recruited by mossy fibers, such as mossy fiber associated interneurons in the stratum lucidum (Vida and Frotscher, 2000) and other interneurons in the hilus. Mossy fibers have been shown to contain GABA in addition to glutamate and zinc, and recent evidence suggests that mossy fibers may release GABA in an action potential-dependent manner (Gutierrez and Heinemann, 2001; Walker et al., 2001). After perforant path stimulation, the GABA content of mossy fibers increases (Sloviter et al., 1996). We have confirmed that this is also the case after SE induced by pilocarpine administration using immunogold labeling for GABA. This increase in GABA content is associated with an increased expression of vesicular GABA transporter and an associated enhancement of the putative GABAergic mossy fiber signal (Gutierrez and Heinemann, 2001; Lamas et al., 2001; Gutierrez et al., 2003). Decreased GABA release from mossy fibers, therefore, cannot explain our observation of a loss of heterosynaptic depression because, if anything, the increased GABA released from mossy fibers should enhance heterosynaptic depression.

Interneurons recruited by mossy fibers are other possible sources of GABA. Loss of interneurons could undoubtedly contribute to a decrease in GABA release and so a decrease in heterosynaptic depression. Although populations of interneurons are lost after SE, interneurons in CA3 are relatively well preserved after lithium- and pilocarpine-induced seizures (Andre et al., 2001). Nevertheless, we cannot exclude interneuronal dysfunction as a contributor to the loss of heterosynaptic depression in our models. There are also other possible mechanisms, including decreased recruitment of interneurons via enhancement of presynaptic group II mGluR depression of mossy fiber transmission (Doherty and Dingledine, 2001). We were, however, unable to rescue heterosynaptic depression by applying a group II mGluR antagonist. This argues against the hypothesis that overactivity of group II mGluRs explains the loss of heterosynaptic depression after SE.

The concentration of GABA detected by mossy fiber terminals depends not only on the amount of GABA released but also on the efficiency of GABA uptake. The loss of heterosynaptic depression could thus be explained by enhanced GABA uptake after SE. Anatomical and functional studies have, however, suggested that GABA uptake is decreased after SE. The predominant GABA transporter GAT1 is downregulated in rodent epilepsy models (Andre et al., 2001), and functional impairment of GABA transport has also been reported in tissue obtained either from patients with mesial temporal sclerosis or from rats after kainic acid-induced SE (Patrylo et al., 2001). Our finding that inhibiting GABA uptake does not lead to enhanced heterosynaptic depression in epileptic animals further argues that increased GABA uptake does not play a part in the loss of heterosynaptic depression.

Finally, we explored the possibility that the target $GABA_B$ receptors are lost or fail to modulate transmitter release by measuring the effect of the $GABA_B$ receptor agonist baclofen on the amplitude of mossy fiber fEPSPs. We observed reduced sensitivity of the mossy fiber fEPSP to baclofen after SE. Autoradiography revealed that the $GABA_B$ receptor density is reduced in the stratum lucidum without decreased binding affinity. This was unlikely to be attributable to a change in the number of mossy fibers because granule cells tend to be relatively resistant to seizures, and mossy fiber sprouting starts several days after excitotoxic insults (Mello et al., 1993). $GABA_B$ receptors have been strongly implicated in the pathogenesis of absences (Liu et al., 1992), and rats lacking the $GABA_{B1}$ subunit have spontaneous absence, clonic, and tonic–clonic seizures (Schuler et al., 2001). Their role in temporal lobe epilepsy, however, is unclear. Changes in $GABA_B$ receptors have been observed in both humans with TLE and experimental models of epilepsy. Binding to $GABA_B$ receptors in the CA3 pyramidal neuronal layer is reduced in human patients with TLE (Princivalle et al., 2002), although $GABA_B$ receptors are upregulated when the data are corrected for cell loss. In the dentate gyrus, both presynaptic (Buhl et al., 1996; Haas et al., 1996) and postsynaptic $GABA_B$ receptor functions (Wasterlain et al., 1996) are downregulated after kainic acid-induced seizures and perforant path stimulation, respectively. Also, Wu and Leung (1997) showed a decrease in efficacy of $GABA_B$ receptors on CA1 interneurons. Our results are surprising in that the loss of $GABA_B$ receptors occurs so soon after SE. However, others have shown that receptors can alter rapidly after SE (Kapur and Macdonald, 1997).

Thus, our data are consistent with a loss of presynaptic $GABA_B$ receptors on mossy fiber terminals. This parallels the recent finding that $GABA_{B1}$ and $GABA_{B2}$ receptor mRNA levels are transiently reduced after kainic acid-induced SE (Furtinger et al., 2003). Loss of presynaptic $GABA_B$ receptors renders mossy fibers relatively resistant to GABA, whether this is released directly from the mossy fibers themselves or from interneurons recruited by mossy fibers, explaining the loss of heterosynaptic depression after SE. A possible weakness of this hypothesis is that there is a quantitative discrepancy between the complete loss of heterosynaptic depression and the ~20% decrease in [^{3}H]CGP62349 binding density. This can be partly explained by the $GABA_B$ receptor binding detected in the stratum lucidum being not exclusively attributable to receptors on presynaptic mossy fiber terminals. Indeed, there are also interneurons present in this area that express $GABA_B$ receptors, and increased expression of $GABA_B$ receptors in these interneurons with seizures (Kokaia and Kokaia, 2001) could mask the loss at mossy fiber terminals. Also, intracellular amplification of metabotropic receptor-mediated actions may lead to a nonlinear relationship between receptor density and sensitivity to GABA spillover.

Could changes in the extracellular space contribute to the loss of heterosynaptic depression? Status epilepticus induces CNS edema, which may last several days (Roch et al., 2002). Cell swelling during excessive neuronal activity can result in a 30% decrease in the volume of the extracellular space (Lux et al., 1986) and could change concentrations of extracellular ions and transmitters. Although an experimental investigation of this possibility was beyond the scope of this study, a decrease in the extracellular space might be expected to increase the extracellular GABA concentration after exocytosis and, therefore, to increase $GABA_B$ receptor-mediated heterosynaptic depression. However, if this is accompanied by increased tortuosity of the extracellular space, the diffusional path for GABA molecules from their release sites to the receptors could be increased, with the opposite effect on heterosynaptic depression.

Loss of heterosynaptic depression may lead to propagation of

excessive excitatory afferent traffic into the hippocampus proper. This could also play a role in epileptogenesis if it allows further seizure spread through the entorhinal cortex–dentate gyrus–hippocampus–entorhinal cortex circuit. It could also contribute to the excitotoxic pattern of principal cell loss in the hippocampus proper, which is a hallmark of chronic temporal lobe epilepsy. In addition, because heterosynaptic depression is recruited by synchronous mossy fiber discharges, it could contribute to seizure termination; a breakdown in this phenomenon could thus lead to the persistence of seizure activity during status epilepticus. Others have noted a downregulation of $GABA_B$ receptors on presynaptic terminals of inhibitory cells in the dentate gyrus in animals 2 weeks after kainic acid-induced seizures, leading to reduced disinhibition and an increase in GABA release (Haas et al., 1996). Although we were unable to detect a significant loss in autoradiographic labeling in the molecular layer of the dentate gyrus, such an effect could reflect a compensatory antiepileptic effect in the chronic stages of epilepsy. Complex changes in $GABA_B$ receptor expression have also been reported in the entorhinal cortex during epileptogenesis, including presumed loss of postsynaptic $GABA_B$ receptors (Kokaia and Kokaia, 2001) and an increase in presynaptic $GABA_B$ receptor-mediated autoinhibition in layer III of the entorhinal cortex (Gloveli et al., 2003). These changes are likely to have a proepileptic effect, enhancing the entorhinal cortex to hippocampus signaling. Thus, changes in $GABA_B$ receptor expression at multiple sites in the hippocampal formation during epileptogenesis may have complex effects on excitability.

In summary, we have shown that $GABA_B$ receptor-mediated heterosynaptic depression at the mossy fiber synapse is reduced acutely after SE, despite an increase in the GABA concentration in mossy fibers. This is associated with a decrease in the sensitivity of the mossy fiber response to the $GABA_A$ agonist baclofen, a change that persists for at least 3 weeks. This can be explained by a loss of presynaptic $GABA_B$ receptors on mossy fiber terminals, revealed by autoradiographic detection of bound $GABA_B$ receptor radioligand in the stratum lucidum. Reduced $GABA_B$ receptor-mediated heterosynaptic depression after SE may play a role in the maintenance of self-sustaining seizure activity and the later development of spontaneous seizures.

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