ALTERATIONS OF GABA_A RECEPTOR FUNCTION AND ALLOSTERIC MODULATION DURING DEVELOPMENT OF STATUS EPILEPTICUS

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ABSTRACT

Partial limbic seizures in rodents induced by pilocarpine progress from stages I-II (mouth and facial movements; head nodding) to stage III (forelimb clonus) and then progress rapidly to stages IV-V (generalized limbic seizures; rearing and rearing with falling) followed by status epilepticus (SE). While limbic seizures in rodents are terminated by benzodiazepines, a group of GABA$_A$ receptor positive modulators, significant pharmacoresistance to benzodiazepine develops within minutes during SE. The alterations of GABA$_A$ receptor function and allosteric modulation during development of SE are poorly understood. We induced seizures in juvenile rats by administration of lithium followed by pilocarpine, and whole cell recordings of miniature inhibitory post-synaptic currents (mIPSCs) were obtained from hippocampal dentate granule cells in brain slices. Compared with a sham-treated group, mIPSC amplitude was reduced and decay was accelerated at onset of the first occurrence of stage III (S3) seizures [S3(0)], resulting in a reduction in the total charge transfer at S3(0). Recovery of mIPSC amplitude and prolongation of mIPSC decay occurred 30 minutes after onset of S3 seizures [S3(30)]. The mIPSC frequency was not altered for S3(0) and S3(30) neurons compared with sham neurons. The net enhancement of total charge transfer by diazepam was smaller for S3(30) than for sham and S3(0) neurons; however, the net reduction of total charge transfer by zinc was greater for S3(30) than for sham and S3(0) neurons. These findings suggest that substantial plastic changes of GABA$_A$ receptor function and allosteric modulation occur rapidly in neurons from juvenile animals during development of SE.
Administration of pilocarpine in rodents induces progressive seizures that evolve from partial limbic seizures to generalized seizures and culminate in status epilepticus (SE) (Kapur and Macdonald 1997; Jones et al. 2002; Kubova et al. 2004). In the rodent pilocarpine model, EEG studies demonstrated that seizures begin in the hippocampus (Turski et al. 1989) and that stage III seizures (S3, forelimb clonus) (Racine 1972) represent the transition from partial to generalized seizures (Jones et al. 2002). Substantial changes in neuronal excitability, receptor pharmacology and receptor distribution have been demonstrated to occur early in SE, suggesting that significant molecular changes occur soon after the onset of generalized seizures. For example, SE at early stages can be effectively treated with benzodiazepines, but pharmacoresistance to benzodiazepines develops rapidly (Walton and Treiman 1988, 1991; Kapur and Macdonald 1997; Mazarati et al. 1998; Jones et al. 2002). Studies using the pilocarpine model of SE in juvenile animals demonstrated that pharmacoresistance to some anticonvulsants including benzodiazepines occurred within minutes after onset of S3 seizures (Jones et al. 2002). However, the mechanistic basis for the alteration of drug sensitivity during the development of SE remains unclear.

GABA<sub>A</sub> receptors mediate the majority of inhibition in the mammalian brain, and two types of GABAergic inhibition have been described: phasic and tonic inhibition (Mody and Pearce 2004; Farrant and Nusser 2005). GABA<sub>A</sub> receptors are pentameric chloride ion channels formed by GABA<sub>A</sub> receptor subunit subtypes selected from more than sixteen subunit subtypes (Olsen and Macdonald 2002). It has been demonstrated that αβγ and αβδ receptors are the predominant isoforms present in the brain (McKernan and Whiting 1996), primarily mediating phasic and tonic inhibition, respectively (Saxena and Macdonald 1994; Stell et al. 2003; Farrant
and Nusser 2005). GABA\(_A\) receptors are allosterically modulated in a subunit-dependent manner by a variety of structurally different positive and negative modulators, including benzodiazepines, barbiturates, neurosteroids, penicillin and zinc (Olsen and Macdonald 2002). For example, benzodiazepines are positive modulators for \(\gamma_2\) subunit-containing receptors, and multiple domains of the \(\gamma_2\) subunit are involved in their allosteric modulation (Boileau and Czajkowski 1999; Jones-Davis et al. 2005). In contrast, zinc is a negative modulator for \(\gamma_2\) subunit-containing receptors, whose action may involve overlapping \(\gamma_2\) subunit domains with those for benzodiazepines (Nagaya and Macdonald 2001). Based on these studies in recombinant receptors, it is expected that benzodiazepines and zinc may exert different effects on GABAergic phasic inhibition. Acute alterations of GABA\(_A\) receptor function and/or pharmacology have been observed shortly after SE onset (Kapur and Macdonald 1997; Goodkin et al. 2005; Naylor et al. 2005) and after development of epilepsy weeks or months after SE (Cohen et al. 2003; Leroy et al. 2004; Peng et al. 2004; Zhang et al. 2004). However, the alterations of GABA\(_A\) receptor function and allosteric modulation during development of SE are still poorly understood. By recording miniature inhibitory post-synaptic currents (mIPSCs) from hippocampal dentate granule cells in brain slices, we examined the alteration of mIPSC properties and modulation of mIPSC by diazepam and zinc at onset of the first occurrence of S3 seizures [S3(0)] and 30 minutes after onset of S3 seizures [S3(30)]. We demonstrated that mIPSC total charge transfer (mIPSC area) was reduced for S3(0) but enhanced for S3(30) neurons compared with sham neurons. In addition, we found that diazepam sensitivity was decreased and zinc sensitivity was increased at S3(30). These findings indicate that GABA\(_A\) receptor function and allosteric modulation undergo substantial plastic changes in neurons from juvenile animals during development of SE.
MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) at 25-28 days old were used in the study. The rats were housed one per cage in a temperature-controlled animal facility on a 14-hr/10-hr light/dark cycle, with free access to food and tap water. Studies were performed in accordance with the guide of National Institutes of Health for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Laboratory Animal Care and Use Committee of Vanderbilt University School of Medicine.

Induction of status epilepticus

Seizures were induced by i.p. injection of 3 mEq/kg LiCl (Sigma-Aldrich, St. Louis, MO) followed 20-24 hr later by 50 mg/kg pilocarpine hydrochloride (Sigma-Aldrich) (Jones et al. 2002). The animals exhibited five distinct stages of limbic seizures after pilocarpine administration, including mouth and facial movements (S1), head nodding (S2), forelimb clonus (S3), rearing (S4), and rearing and falling (S5) (Racine 1972). An EEG study suggested that S1 and S2 seizures were partial seizures, and S4 and S5 seizures were generalized seizures. S3 seizures occurred at the transition stage for partial seizures progressing to generalized seizures (Jones et al. 2002). Once an animal began having S3 seizures, the seizures rapidly progressed to S4 or S4 followed by S5 seizures. As previously described (Jones et al. 2002), pilocarpine-induced seizures progressed from S1 to S4 or S5 seizures and were followed by a return to S2 seizures, which then progressed to S4 or S5 seizures again in a cycle recurring multiple times.

Since a previous behavioral study showed that the development of pharmacoresistance to some anticonvulsants including benzodiazepines occurred within minutes after onset of S3 seizures in juvenile rats (Jones et al. 2002), four groups of juvenile rats were used in the
electrophysiological recordings to determine the mechanistic basis for benzodiazepine resistance and the alteration of GABA_A receptor function during the development of SE. The first group was treated with neither LiCl nor pilocarpine. The second group was treated only with LiCl (3 mEq/kg), and brain slices were prepared 20-24 hr later. The third group [S3(0)] received LiCl followed by pilocarpine, and brain slices were prepared at the onset of the first occurrence of S3 seizures. Because SE is traditionally defined as clinical or electrographic seizures lasting for at least 30 minutes (Kapur and Macdonald 1997), the fourth group [S3(30)] received LiCl followed by pilocarpine, and brain slices were made 30 minutes after onset of the first occurrence of S3 seizures.

**Electrophysiological recordings**

Brain slices were prepared as previously described (Mathews and Diamond 2003; Zsiros and Maccaferri 2005). Since most anesthetics affect GABA_A receptor function, decapitation of juvenile rats was performed without anesthesia using methodology described in the approved institutional protocols. Brains were removed quickly and hemisected and then using a Vibratome (The Vibratome Company, St. Louis, MO) were sectioned into 400-µm coronal slices in ice-cold oxygenated solution, which was composed of (in mM) 1.25 NaH_2PO_4, 28 NaHCO_3, 2.5 KCl, 0.5 CaCl_2, 7 MgCl_2, 7 glucose, 234 sucrose, 1 ascorbic acid and 3 pyruvic acid. The slices then were incubated in the storage chamber at room temperature (19-23 °C) for at least one hour prior to transfer to the recording chamber. In both the storage and recording chambers, the slices were kept in ACSF with the following composition (in mM): 119 NaCl, 2.5 KCl, 1.3 MgCl_2, 2.5 CaCl_2, 1 NaH_2PO_4, 26.2 NaHCO_3 and 11 glucose. The ACSF was continually bubbled with 95% O_2 and 5% CO_2. During recordings, the slice was perfused with ACSF containing 40 µM DL-2-amino-5-phosphono-pentanoic acid (AP5) and 10 µM 6-cyano-7-
nitroquinoxaline-2,3-dione disodium salt (CNQX) to block glutamate receptors. Tetrodotoxin (TTX, 1 µM) was also added to ACSF when miniature inhibitory post-synaptic currents (mIPSCs) were recorded. The pipettes for whole cell recording were pulled from thin-wall borosilicate glass (i.d. = 1.12 mm, o.d. = 1.5 mm) (World Precision Instruments Inc., Sarasota, FL) on a PP-830 Puller (Narishige, Japan). Recording pipette resistances were 4-6 MΩ when filled with an internal solution consisting of (in mM): 130 CsCl, 10 HEPES, 10 EGTA, 2 MgATP, 0.2 NaGTP and 1 QX-314 (lidocaine N-ethyl bromide) (pH 7.4, 290 mOsm). All chemicals were purchased from Sigma-Aldrich.

Dentate gyrus granule cells were identified visually using an upright microscope (Olympus America Inc., Woodbury, NY) equipped with infrared differential interference contrast video imaging. Cells were voltage clamped at – 60 mV, and whole cell recordings were obtained at room temperature (19-23 °C) using an Axopatch-1D amplifier (Molecular Devices, Foster City, CA) and Digidata 1322A data acquisition system (Molecular Devices). Currents were low-pass filtered at 2 kHz, sampled at 10 kHz, and digitally stored on disk for off-line analysis. Series resistance (~10-18 MΩ) was not compensated. However, it was checked frequently throughout the experiment, and recordings were terminated when series resistance increased by >20%.

Diazepam (in DMSO) and zinc chloride (both from Sigma-Aldrich) were prepared as stock solutions that were diluted with ACSF on the day of the experiment to the desired concentrations to make working solutions. The final concentration of DMSO in working solutions was <0.2%. The drugs were perfused using a peristaltic pump (Gilson, Middleton, WI), and a 5-minute wash-in was allowed prior to mIPSC recordings.
Data analysis

Detection of individual mIPSCs was performed offline using the Mini Analysis program (Synaptosoft Inc., Fort Lee, NJ). The detection threshold was set at 15 pA. The mIPSCs were automatically detected by the program initially and then manually analyzed based on the criteria that only those events with a total rise time <1.5 ms were included in the analysis. Events with slower rise times may involve other complicated factors such as dendritic filtering or activation of extrasynaptic receptors that may confound interpretation of mIPSC kinetics (McIntyre et al. 2002; Kobayashi and Buckmaster 2003; Mody and Pearce 2004). The events within mIPSC area <50 fC (these events were too small to be considered mIPSCs) and overlapping mIPSCs were excluded from analysis. For each neuron, mIPSC kinetics and total charge transfer (mIPSC area) were analyzed on averaged events that were aligned by rise times. At least 100 events were averaged for each neuron, and preliminary analysis indicated that further increase in the number of events did not significantly alter these parameters. The decay of the average mIPSCs was fitted using the formula of $\sum a_n e^{-t/\tau_n}$, where $a$ denotes the relative amplitude of the exponential component, $\tau$ represents the time constant and $n$ is the number of exponential components. Although the decay of some average mIPSCs could only be fitted by one exponential component, the majority of average mIPSCs were fitted by two exponential components. A weighted $\tau$ in the form of $(a_1*\tau_1 + a_2*\tau_2)/(a_1 + a_2)$ was used to compare the rate of decay among different groups of neurons, where $a_1$ and $a_2$ are the relative amplitudes of the exponential components at time zero.

All mIPSC amplitudes, weighted decay time constants and total charge transfer were reported as mean $\pm$ SEM. All mIPSC properties were compared using one-way ANOVA followed by Newman-Keuls multiple comparison test among different neuronal groups. The
paired Student’s t test was used to compare mIPSC properties prior to and after drug application. Differences were considered to be statistically significant if p was less than 0.05.
**RESULTS**

The mIPSCs were recorded from hippocampal dentate granule cells in the presence of 40 µM AP5, 10 µM CNQX and 1 µM TTX. Since the mIPSC properties were not significantly different between the first (treated with neither LiCl nor pilocarpine) and second (treated only with LiCl) group of rats, the data from these two groups of rats were pooled as a sham control group. The mIPSCs were recorded from slices obtained from 15 sham, 14 S3(0) and 15 S3(30) rats. Recordings were made from only one neuron in each slice, and for each neuron, only one drug (either diazepam or zinc) was applied to reduce the possible contamination by the other drug. Sometimes recordings were made from more than one slice from the same rat, but a minimum of 4 rats were used for each drug treatment. In these cases, the drug application system was adequately washed between recordings from different slices to minimize the drug contamination.

**Seizure-induced alterations of mIPSC kinetics**

The onsets of the first S3 seizures [S3(0)] occurred approximately 20 minutes after pilocarpine injection, and the S3(0) animals were sacrificed at that time, whereas the S3(30) animals were sacrificed 30 minutes after this point. We examined the mIPSC properties at both time points (Figure 1A, B). The mIPSC frequencies were not different among the sham (0.30 ± 0.05 Hz, n = 18), S3(0) (0.27 ± 0.04 Hz, n = 17) and S3(30) (0.31 ± 0.04 Hz, n = 20) neurons. However, compared with sham neurons, the amplitudes of mIPSCs were smaller for S3(0) neurons but were not altered for S3(30) neurons (Figure 1A, C). The mean mIPSC amplitude for S3(0) neurons (38.9 ± 1.8 pA) was smaller than that for either sham (46.9 ± 2.0 pA; p<0.01) or S3(30) neurons (49.1 ± 2.0 pA; p<0.01) (Figure 1E). The mean amplitude of mIPSCs for S3(30) neurons was not different from that for sham neurons (Figure 1E).
The decay of mIPSCs was differentially altered for S3(0) and S3(30) neurons compared with sham neurons (Figure 1B, D). The mean weighted decay time constant ($\tau_w$) for S3(0) neurons (29.8 ± 0.9 ms) was smaller than that for sham neurons (39.2 ± 1.2 ms; p<0.05) (Figure 1F). However, the mean weighted decay time constant for S3(30) neurons (48.3 ± 3.8 ms) was greater than that for sham neurons (p<0.05). The mean weighted decay time constant for S3(30) neurons was also greater than that for S3(0) neurons (p<0.001) (Figure 1F).

GABAergic phasic inhibition is determined by mIPSC frequency, amplitude and time course (i.e. decay) of synaptic events. Since mIPSC frequency was not different among the sham, S3(0) and S3(30) neurons, the total charge transfer (mIPSC area) was calculated to compare the magnitude of phasic inhibition among three groups of neurons. The mIPSC area was smaller for S3(0) neurons (1188.4 ± 64.5 fC) than for sham (1683.5 ± 48.0 fC; p<0.001) and S3(30) neurons (1922.6 ± 114.2 fC; p<0.001). The total charge transfer for S3(30) neurons was greater than that for sham neurons (p<0.05) (Figure 1G).

**Modulation of mIPSC kinetics by diazepam for sham, S3(0) and S3(30) neurons**

The effects of seizures on diazepam modulation of mIPSCs recorded from sham, S3(0) and S3(30) animals were determined (Figure 2). Diazepam (1 µM) did not alter the mIPSC frequency for sham, S3(0) and S3(30) neurons. However, it enhanced mIPSC amplitudes for these three groups of neurons (Figure 2A, C). Diazepam increased the mean mIPSC amplitude of sham neurons from 45.3 ± 3.3 pA to 66.3 ± 4.3 pA (n = 7, p<0.01). The mean mIPSC amplitude for S3(0) neurons was augmented from 40.9 ± 3.0 pA to 61.9 ± 3.1 pA by diazepam (n = 5, p<0.01). For S3(30) neurons, diazepam enhanced the mIPSC amplitude as compared with controls (50.1 ± 2.3 pA vs 58.7 ± 2.1 pA) (n = 8, p<0.01). However, the net increase (%) in
mIPSC amplitude by diazepam was not significantly different among the sham, S3(0) and S3(30) neurons.

Diazepam also prolonged the mIPSC decay for sham, S3(0) and S3(30) neurons (Figure 2B, D). The mean mIPSC weighted decay time constant for sham neurons was increased from 40.0 ± 1.9 ms to 58.8 ± 3.6 ms by diazepam (p<0.001). Diazepam increased the mean weighted decay time constant from 31.9 ± 2.3 ms to 54.7 ± 1.5 ms for S3(0) neurons (p<0.001) and from 45.3 ± 4.0 ms to 58.0 ± 2.1 ms for S3(30) neurons (p<0.01). However, the net increase in mIPSC weighted decay time constant by diazepam was smaller for S3(30) neurons (33.5 ± 9.2%) than for S3(0) neurons (74.2 ± 10.1%) (p<0.05), although no significant difference was observed compared to sham with either S3(0) or S3(30) neurons.

The total charge transfer (mIPSC area) was increased by diazepam for sham, S3(0) and S3(30) neurons (Figure 2E). Diazepam increased the mean mIPSC area from 1661.7 ± 83.3 fC to 3338.0 ± 329.1 fC for sham neurons (p<0.01). The mean mIPSC area was increased by diazepam from 1318.5 ± 121.7 fC to 2960.3 ± 136.7 fC for S3(0) neurons (p<0.01) and from 2086.5 ± 221.5 fC to 3003.8 ± 146.2 fC for S3(30) neurons (p<0.001). The net increase in mIPSC area by diazepam was smaller for S3(30) neurons (51.4 ± 11.0%) than for sham (100.6 ± 15.3%) (p<0.05) and S3(0) neurons (131.7 ± 21.6%) (p<0.01). The net increase in mIPSC area for sham neurons was not significantly different from that for S3(0) neurons (Figure 2E).

*Modulation of mIPSC kinetics by zinc for sham, S3(0) and S3(30) neurons*

The effects of seizures on zinc inhibition of mIPSCs recorded from sham, S3(0) and S3(30) animals were determined (Figure 3). Zinc (300 µM) did not affect mIPSC frequency but decreased the mIPSC amplitudes for sham, S3(0) and S3(30) neurons (Figure 3A, C). The mean
mIPSC amplitude was reduced by zinc from 51.2 ± 3.2 pA to 41.8 ± 2.3 pA for sham neurons (n = 7, p<0.05). Zinc also inhibited mIPSC amplitude from 39.8 ± 2.7 pA to 36.3 ± 1.7 pA for S3(0) neurons (n = 9, p<0.05) and from 49.2 ± 3.3 pA to 33.9 ± 2.4 pA for S3(30) neurons (n = 9, p<0.001). The net decrease in mIPSC amplitude by zinc was greater for S3(30) neurons (30.4 ± 3.2%) as compared with S3(0) neurons (7.4 ± 3.3%) (p<0.001). The net decrease in mIPSC amplitude for sham neurons (17.7 ± 3.7%) was greater than S3(0) (p<0.05) but smaller than S3(30) neurons (p<0.05).

Zinc accelerated the mIPSC decay for sham and S3(30) neurons but did not alter that for S3(0) neurons (Figure 3B, D). Zinc reduced the mean weighted decay time constant from 37.5 ± 1.9 ms to 32.0 ± 2.5 ms for sham neurons (p<0.05). For S3(30) neurons, the mean weighted decay time constant was decreased from 46.7 ± 4.9 ms to 31.1 ± 1.6 ms by zinc (p<0.01). Zinc did not significantly change the mean weighted decay time constant for S3(0) neurons. The net decrease in weighted decay time constant by zinc was greater for S3(30) neurons (29.3 ± 5.0%) than for either sham (14.4 ± 5.5%) (p<0.05) or S3(0) neurons (1.5 ± 2.0%) (p<0.001). The net decrease in mean weighted decay time constant was not significantly different between sham and S3(0) neurons.

The total charge transfer (mIPSC area) was reduced by zinc for sham, S3(0) and S3(30) neurons (Figure 3E). Zinc decreased mean mIPSC area from 1705.7 ± 89.9 fC to 1287.3 ± 81.0 fC for sham neurons (p<0.01). The mean mIPSC area was also reduced by zinc from 1182.2 ± 89.8 fC to 1035.7 ± 56.0 fC for S3(0) neurons (p<0.05) and from 1818.9 ± 131.9 fC to 1043.6 ± 54.8 fC for S3(30) neurons (p<0.001). The net decrease in mIPSC area by zinc was greater for S(30) neurons (41.1 ± 3.5%) than for S3(0) neurons (10.9 ± 3.3%) (p<0.001). The net decrease
in mIPSC area for sham neurons (24.0 ± 4.2%) was different from that for S3(0) (p<0.05) and S3(30) neurons (p<0.01) (Figure 3E).
DISCUSSION

In the present study, we report that GABA_A receptor function was altered acutely during the evolution of status epilepticus. Both the amplitude and the weighted decay time constant of mIPSCs were altered as seizures progressed from partial to generalized seizures, resulting in less total charge transfer per synaptic event. Pharmacoresistance to benzodiazepines, as determined by the effect of diazepam on mean total charge transfer, developed by 30 minutes after onset of S3 seizures. The net increase in mIPSC weighted decay time constant by diazepam was smaller for S3(30) than for S3(0) neurons, and the net increase in mIPSC area was smaller for S3(30) than for sham and S3(0) neurons. The net decreases in both amplitude and weighted decay time constant by zinc were greater for S3(30) neurons than for sham and S3(0) neurons. The net decrease in mIPSC area was smaller for S3(0) and greater for S3(30) neurons compared with sham neurons. These data demonstrated that significant plastic changes of GABA_A receptor function and allosteric modulation occurred rapidly in neurons from juvenile animals during development of SE.

Plastic changes of GABA_A receptor function during development of SE

A previous EEG study suggested that pilocarpine-induced seizures develop from partial to generalized seizures culminating in SE (Jones et al. 2002). Both mIPSC amplitude and weighted decay time constant were significantly reduced at S3(0), the transition point from partial to generalized seizures, leading to a reduction of total charge transfer compared with sham neurons. Associated with this compromised GABA_A receptor function at S3(0), a previous behavioral study demonstrated that progression to S4 or S4 followed by S5 seizures was rapid once S3 was reached (Jones et al. 2002). It would be interesting to know if the reduction of GABAergic phasic inhibition contributes to seizure spread and generalization. The mIPSC amplitude recovered and decay was prolonged for S3(30) neurons, resulting in an increased total charge transfer per synaptic event.
charge transfer compared with sham neurons. Pilocarpine-induced seizures progressed from S1 to S4 or S5 seizures and were followed by a return to S2 seizures, which then progressed to S4 or S5 seizures again in a cycle recurring multiple times (Jones et al. 2002). It would be interesting to know if the enhanced GABAergic function contributed to the periodic reduction of seizure severity. The weighted decay time constant of mIPSCs in the present study was larger than previously reported values at room temperature (Perrais and Ropert 1999; McIntyre et al. 2002). However, our recordings were performed at lower temperature range (19–23 °C) than the above studies (22–26 °C). In addition, IPSC decay can be affected by other factors such as the phosphorylation state of the GABA_A receptors (Jones and Westbrook 1997). All these factors may have contributed to the slower mIPSC decay in this study.

The alterations of GABAergic phasic inhibition during prolonged acute seizures may be related to short-term changes of GABA_A receptors such as post-translational modifications, receptor trafficking and receptor endocytosis. Rapid internalization of GABA_A receptors has been observed at 1 hour after SE in pilocarpine model (Naylor et al. 2005) and as early as 10 minutes in an in vitro model of SE (Goodkin et al. 2005). Thus, the functional alterations of GABA_A receptors in the current study were likely due to receptor internalization. The mIPSC amplitude was not altered 30 minutes after S3 seizures as compared with sham neurons. A parsimonious explanation is that although surface GABA_A receptor number was reduced by prolonged seizures, the receptor efficacy might have been enhanced by some factors. Although there are likely to be additional mechanisms affecting GABAergic inhibition during prolonged seizures, changes in chloride gradient for example, the changes in mIPSC kinetics we observed were more consistent with alterations of GABA_A receptors. Weeks or months after SE, spontaneous recurrent seizures (epilepsy) could develop in these rats, and GABA_A receptor
functional alterations have been reported. It was shown that GABA<sub>A</sub> receptor-mediated inhibition was reduced in the epileptic rats (Isokawa 1996; Kobayashi and Buckmaster 2003). Several other studies, interestingly, demonstrated that mIPSC amplitude was enhanced in these animals (Nusser et al. 1998; Cohen et al. 2003; Leroy et al. 2004; Gavrilovici et al. 2006; Sun et al. 2007). In contrast to the possible mechanisms for the acute changes we observed, alterations of GABAergic phasic inhibition during epilepsy are likely related to long-term changes in GABA<sub>A</sub> receptor expression and/or function such as regulation of receptor gene expression and degradation or synthesis of multiple receptor subunit proteins.

**Seizure-induced alterations of GABA<sub>A</sub> receptor allosteric modulation**

The net increase in total charge transfer by diazepam was significantly smaller for S3(30) than for sham and S3(0) neurons, suggesting that the sensitivity of GABA<sub>A</sub> receptors to benzodiazepines was decreased 30 minutes after onset of S3 seizures. This finding is consistent with our previous report using isolated, dissociated dentate granule cells that diazepam sensitivity was reduced after prolonged seizures (Kapur and Macdonald 1997), supporting the idea that a reduction in allosteric modulation of GABA<sub>A</sub> receptors by diazepam may contribute to the rapid diazepam pharmacoresistance observed in behavioral studies (Walton and Treiman 1988; Jones et al. 2002). Although the underlying cellular and molecular changes of GABA<sub>A</sub> receptors in the current study may be different from those occur during epilepsy, the pharmacoresistance to diazepam was also observed in epileptic rats weeks or months after SE (Cohen et al. 2003; Leroy et al. 2004).

Diazepam consistently augmented mIPSC amplitude in the present study, which is in contrast with several previous reports that benzodiazepines prolonged the mIPSC decay but did not enhance amplitudes in normal animals (Poncer et al. 1996; Cohen et al. 2003; Leroy et al. 2004).
Diazepam modulation of mIPSC amplitude may be age-dependent; potentiation of GABA-evoked currents (Zhang et al. 2004) and mIPSC amplitudes (Nusser et al. 1997; Defazio and Hablitz 1998; Perrais and Ropert 1999; Cohen et al. 2000; Hajos et al. 2000) has been observed in juvenile neurons including hippocampal dentate granule cells. In addition, the potentiating effect of diazepam may also be temperature-dependent (Perrais and Ropert 1999).

The net change in total charge transfer by zinc was significantly decreased for S3(0) as compared with sham neurons, suggesting that zinc sensitivity was reduced for S3(0) neurons. The reduction of zinc sensitivity during development of SE was reported previously in hippocampal and cortical neurons (Kapur and Macdonald 1997; Banerjee et al. 1999). The net change evoked by zinc on total charge transfer was significantly greater for S3(30) neurons compared with that for sham and S3(0) neurons, demonstrating that the sensitivity of GABA_A receptors to zinc was significantly increased 30 minutes after onset of S3 seizures. Interestingly, it has been demonstrated that GABA_A receptor sensitivity to zinc was significantly increased in epileptic rats (Cohen et al. 2003) and in amygdala kindled rats (Buhl et al. 1996). A recent study demonstrated that pilocarpine or pilocarpine-induced seizures did not result in changes of distribution of endogenous zinc in hippocampus up to 2 hrs after pilocarpine injection (Noyan et al. 2007). Therefore, the zinc sensitivity alterations we observed for S3(0) and S3(30) neurons were unlikely to be caused by endogenous zinc distribution changes but more likely to be due to seizure-evoked alteration of GABA_A receptor allosteric modulation.

Zinc had no effects on mIPSC amplitude and decay of dentate granule cells from normal adult animals (Buhl et al. 1996; Hollrigel and Soltész 1997; Cohen et al. 2003). However, it inhibited the mIPSC amplitude and accelerated the decay in juvenile neurons including dentate granule cells (Hollrigel and Soltész 1997; Defazio and Hablitz 1998). Consistent with this, we
found that zinc reduced mIPSC total charge transfer in normal juvenile rats. This age-dependent effect of zinc on phasic currents is reminiscent of that of diazepam. It is not known if these two phenomena share similar cellular and/or molecular mechanisms.

The molecular mechanisms underlying the altered allosteric modulation of GABAergic phasic currents by diazepam and zinc during acute seizures are poorly understood. One possibility is alteration of surface GABA_A receptor subunit composition considering that several receptor isoforms exhibit differential sensitivity to benzodiazepines and zinc (Olsen and Macdonald 2002). In the chronic epilepsy model, multiple subunit mRNA and/or protein changes have been observed days or weeks after SE (Brooks-Kayal et al. 1998; Peng et al. 2004). It is interesting to note that the alterations of phasic current sensitivity to diazepam and zinc during acute seizures in the present study were similarly observed in epileptic rats (Buhl et al. 1996; Cohen et al. 2003; Leroy et al. 2004). As mentioned above, the alterations of allosteric modulation during acute seizures may result from short-term changes in GABA_A receptors whereas those that occur once epilepsy is established may result from long-term changes in GABA_A receptors. It seems that the outcomes of GABA_A receptor changes with these two scenarios sometimes may be similar. Further experiments are needed to explore the underlying mechanisms.

It is also possible that altered sensitivity to diazepam or zinc may be contributed by phosphorylation or dephosphorylation of GABA_A receptors. Allosteric modulation of GABA_A receptors by benzodiazepines was altered by PKC phosphorylation in vitro (Leidenheimer et al. 1993). It was also reported that GABA_A receptor subunit protein dephosphorylation occurred rapidly after seizures (Sanchez et al. 2005), and a reduction of CaM kinase II activity was
associated with amygdala kindling as well as SE (Wasterlain and Farber 1984; Singleton et al. 2005).

In summary, we demonstrated that significant plastic changes of both GABA_A receptor function and allosteric modulation rapidly occurred in neurons from juvenile animals during development of SE.

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**FIGURE LEGENDS**

*Figure 1. mIPSC kinetics were altered for S3(0) and S3(30) neurons.*

A, Examples of mIPSC traces recorded from a sham control rat (sham), a rat exhibiting onset of S3 seizures [S3(0)] and a rat with continued seizures 30 minutes after S3 seizures [S3(30)]. B, Typical examples of average mIPSCs from sham, S3(0) or S3(30) neurons, showing the alterations of mIPSC decay in both S3(0) and S3(30) recordings. The dashed grey line indicated normalization of mIPSC amplitude in S3(0) and S3(30) recordings to sham recording. The grey traces were from the same sham neuron. C, D, The cumulative probability plots of mIPSC amplitude and decay time constant (τ) for neurons in panel B are presented. E, Comparison of the mean mIPSC amplitude among sham, S3(0) and S3(30) neurons. The mean amplitude was significantly smaller for S3(0) neurons compared with that for sham and S3(30) neurons, but the mean amplitude for S3(30) neurons was not significantly different from that for sham neurons. F, Comparison of the mean mIPSC weighted decay time constant (τ_w) among sham, S3(0) and S3(30) neurons. The mean decay τ_w was significantly smaller for S3(0) but significantly greater for S3(30) neurons compared with that for sham neurons. The mean decay τ_w for S3(30) neurons was significantly greater than that for S3(0) neurons. G, Comparison of total charge transfer (mIPSC area) among sham, S3(0) and S3(30) neurons. Compared with sham neurons, mIPSC area was significantly reduced for S3(0) neurons but was significantly increased for S3(30) neurons. The mIPSC area for S3(0) neurons was also significantly smaller than that for S3(30) neurons. Error bars represent the SEM.

* Significantly different from sham neurons at p<0.05; ** p<0.01; *** p<0.001
++ Significantly different from S3(0) neurons at p<0.01; +++ p<0.001
**Figure 2. Modulation of mIPSC kinetics by diazepam for sham, S3(0) and S3(30) neurons.**

A, Typical examples of average mIPSCs and cumulative probability plots prior to and after application of diazepam (DZP, 1 µM) for sham, S3(0) and S3(30) neurons, demonstrating the changes of mIPSC amplitudes induced by this drug. The grey traces indicated the control currents, and the black traces denoted the currents after drug application. B, For the same neurons in panel A, the control mIPSC amplitudes were normalized to those after drug application to demonstrate the alterations of mIPSC decay induced by DZP. The decay alterations were also shown using cumulative probability plots. C, Comparison of the mean net increase in mIPSC amplitude by DZP among sham, S3(0) and S3(30) neurons. The % increase in mIPSC amplitude was not different among the three groups of neurons. D, Comparison of the mean net increase in mIPSC weighted decay time constant (τ_w) by DZP among sham, S3(0) and S3(30) neurons. The % increase in decay τ_w was significantly reduced for S3(30) neurons compared with S3(0) neurons. Compared with sham neurons, the % increase in decay τ_w was not significantly altered for S3(0) and S3(30) neurons. E, Comparison of the mean net increase in total charge transfer (mIPSC area) by DZP among sham, S3(0) and S3(30) neurons. The mean net increase in mIPSC area by diazepam was significantly reduced for S3(30) neurons as compared with both sham and S3(0) neurons. The mean net increase in mIPSC area for sham neurons was not significantly different from that for S3(0) neurons. Error bars represent the SEM.

* Significantly different from sham neurons at p<0.05

+ Significantly different from S3(0) neurons at p<0.05; ++ p<0.01
Figure 3. Modulation of mIPSC kinetics by zinc for sham, S3(0) and S3(30) neurons.

A, Typical examples of average mIPSCs and cumulative probability plots prior to and after application of zinc (Zn\textsuperscript{2+}, 300 µM) for sham, S3(0) and S3(30) neurons, demonstrating the changes of mIPSC amplitudes induced by this drug. The grey traces indicated the control currents, and the black traces denoted the currents after drug application. B, For the same neurons in panel A, the mIPSC amplitudes after drug application were normalized to those for control currents to demonstrate the alterations of mIPSC decay induced by zinc. The decay alterations were also shown using cumulative probability plots. C, Comparison of the mean net decrease in mIPSC amplitude by zinc among sham, S3(0) and S3(30) neurons. The % decrease in mIPSC amplitude was significantly greater for S3(30) neurons as compared with S3(0) neurons. The % decrease in mIPSC amplitude for sham neurons was significantly different from that for S3(0) and S3(30) neurons. D, Comparison of the mean net decrease in mIPSC weighted decay time constant (\(\tau_w\)) by zinc among sham, S3(0) and S3(30) neurons. The % decrease in decay \(\tau_w\) was significantly greater for S3(30) neurons compared with sham and S3(0) neurons. The % decrease in decay \(\tau_w\) was not significant between sham and S3(0) neurons. E, Comparison of the mean net decrease in total charge transfer (mIPSC area) by zinc among sham, S3(0) and S3(30) neurons. The mean net decrease in mIPSC area by zinc was significantly greater for S3(30) as compared with S3(0) neurons. The mean net decrease in mIPSC area was significantly smaller for S3(0) but was significantly greater for S3(30) neurons as compared with sham neurons. Error bars represent the SEM.

* Significantly different from sham neurons at p<0.05; ** p<0.01
+++ Significantly different from S3(0) neurons at p<0.001
Fig 1

127x209mm (600 x 600 DPI)
Fig 2

127x230mm (600 x 600 DPI)
Fig 3