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Subunit-Specific Trafficking of $GABA_A$ Receptors during Status Epilepticus

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It is proposed that a reduced surface expression of $GABA_A$ receptors (GABARs) contributes to the pathogenesis of status epilepticus (SE), a condition characterized by prolonged seizures. This hypothesis was based on the finding that prolonged epileptiform bursting (repetitive bursts of prolonged depolarizations with superimposed action potentials) in cultures of dissociated hippocampal pyramidal neurons (dissociated cultures) results in the increased intracellular accumulation of GABARs. However, it is not known whether this rapid modification in the surface-expressed GABAR pool results from selective, subunit-dependent or nonselective, subunit-independent internalization of GABARs. In hippocampal slices obtained from animals undergoing prolonged SE (SE-treated slices), we found that the surface expression of the GABAR β2/3 and γ2 subunits was reduced, whereas that of the δ subunit was not. Complementary electrophysiological recordings from dentate granule cells in SE-treated slices demonstrated a reduction in GABAR-mediated synaptic inhibition, but not tonic inhibition. A reduction in the surface expression of the γ2 subunit, but not the δ subunit was also observed in dissociated cultures and organotypic hippocampal slice cultures when incubated in an elevated KCl external medium or an elevated KCl external medium supplemented with NMDA, respectively. Additional studies demonstrated that the reduction in the surface expression of the γ2 subunit was independent of direct ligand binding of the GABAR. These findings demonstrate that the regulation of surface-expressed GABAR pool during SE is subunit-specific and occurs independent of ligand binding. The differential modulation of the surface expression of GABARs during SE has potential implications for the treatment of this neurological emergency.

Key words: **$GABA_A$ receptor; synapse; plasticity; endocytosis; status epilepticus; neuronal excitability**

Introduction

$GABA_A$ receptors (GABARs) are heteropentameric ligand-gated chloride channels that mediate both synaptic and tonic inhibition (Semyanov et al., 2004; Kullmann et al., 2005). Although there are multiple potential combinations of the 16 different GABAR subunits and splice variants, the majority of GABARs are composed of α and β subunits in combination with either a γ or δ subunit (Mohler, 2006). The subunit composition of a GABAR affects its functional and pharmacological properties (Macdonald and Olsen, 1994) as well as its cellular localization (Brunig et al., 2002; Wei et al., 2003; Mangan et al., 2005) and potentially its trafficking (Kittler et al., 2005). GABARs mediating synaptic inhibition typically contain a γ2 subunit (Alldred et al., 2005), which is required for diazepam sensitivity. In contrast, tonic inhibition is mediated by extrasynaptic benzodiazepine-insensitive GABARs composed of a δ subunit in combination with an α4 subunit as well as zolpidem-insensitive GABARs composed of a γ2 subunit in combination with an α5 subunit (for review, see Scimemi et al., 2005; Mtchedlishvili and Kapur, 2006; Glykys and Mody, 2007).

Status epilepticus (SE) is a neurological emergency characterized by a prolonged, self-sustained seizure that can result in death or neurological sequelae. During SE, there is a reduction in the GABAR-mediated inhibition of hippocampal principal neurons and a reduced response of these neurons to benzodiazepines (Kapur et al., 1989; Kapur and Coulter, 1995; Kapur and Macdonald, 1997). Based on these electrophysiological studies, it has been posited that a rapid modification of the postsynaptic GABAR population contributes to the pathogenesis of SE.

The results of two studies (Goodkin et al., 2005; Naylor et al., 2005) suggest that a reduction in the surface expression of GABARs caused by an increase in the rate of GABAR internalization during SE may partially account for the alteration in the complement of postsynaptic GABARs. Goodkin et al. (2005) found that recurrent sustained epileptiform bursting in a network of cultured hippocampal pyramidal neurons resulted in a reduction in GABAR-mediated synaptic inhibition and an increase in the intracellular accumulation of β2/3 subunit-containing GABARs. In an *in vivo* study (Naylor et al., 2005), SE resulted in altered GABA-mediated inhibition and a diminished colocalization of the β2/3 and γ2 GABAR subunits with synaptic markers. Although the second study found an increase in tonic inhibition, neither study directly assessed changes in the trafficking of the extrasynaptic δ subunit-containing GABARs, the prime mediators of tonic inhibition (Glykys and Mody, 2007; Mody et al.,

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2007). These studies also offered two different explanations for the increased trafficking of GABARs during SE. One suggested that the increase in GABAR internalization was the result of a ligand-independent mechanism that was triggered by an increase in neuronal excitability as the result of stimulation of excitatory amino acid receptors (ligand independent) whereas the other posited an increase in ligand-dependent GABAR internalization as the result of an increase in the extracellular GABA concentration.

To address these unresolved issues, biochemical, electrophysiological, and immunohistochemical methods were used in this study to test for subunit-specific trafficking of GABARs during SE.

Materials and Methods

All animals were treated per the guidelines set by the University of Virginia Health Sciences Center for Animal Research Committee. All efforts were made to minimize animal stress and discomfort.

In vivo *SE*

SE was induced in male Sprague Dawley rats [postnatal day 30 (P30) or older] by either chemical induction using a combination of lithium chloride and pilocarpine (Honchar et al., 1983) or continuous hippocampal stimulation (Lothman et al., 1989).

Lithium-pilocarpine SE. Rats were pretreated with intraperitoneal lithium chloride (LiCl; 3 mEq/kg) followed 20–24 h later by intraperitoneal pilocarpine (50 mg/kg). After pilocarpine injection, rats were observed continuously for occurrence of behavioral seizures. One hour after the first observed stage 5 seizure (Racine, 1972) the rats were anesthetized with halothane and then decapitated. The brain was dissected free and 300 μm horizontal slices were cut with a Vibratome (Camden Instruments, Loughborough, UK) while the brain was immersed in an ice-cold (2–4°C) dissection buffer containing (in mM) 65.5 NaCl, 2 KCl, 5 $MgSO_4$, 1.1 KH_2PO_4, 1 $CaCl_2$, 10 dextrose, and 113 sucrose (300 mOsm). The slices were placed in an artificial CSF (aCSF) at 29–30°C and allowed to equilibrate for a minimum of 30 min before being used for measurement of surface-expressed GABARs via a biotinylation pull-down assay or for electrophysiological recordings. The aCSF contained (in mM) 127 NaCl, 2 KCl, 1.5 $MgSO_4$, 25.7 $NaHCO_3$, 10 dextrose, and 1.5 $CaCl_2$ (osmolarity 300 mOsm). The dissection buffer and aCSF were equilibrated with 95% O_2, 5% CO_2.

Continuous hippocampal stimulation SE. Bipolar insulated stainless steel electrodes were implanted stereotaxically in the left posterior ventral hippocampus (coordinates: anteroposterior −5.3 mm, mediolateral −4.9 mm, dural touch point −5.0 mm, incisor bar at −3) under ketamine/xyalzine anesthesia and secured using dental acrylic. After a 7 d recovery, SE was induced in each rat by continuous hippocampal stimulation with 1 ms biphasic square wave electrical pulses (50 Hz, 400 mA) in 10 cycles/s trains applied every 11 s for 90 min during which time seizures occurred. Poststimulation seizure activity was monitored by electroencephalography. Those rats which continued to have continuous seizures (electrographic stage III) for 60 min after the end of stimulation were anesthetized with halothane, decapitated, and hippocampal slices were obtained as described above.

Hippocampal neuronal cultures

Cultures of dissociated hippocampal pyramidal neurons (dissociated cultures) were obtained and maintained using methods previously described (Goslin et al., 1998; Mangan and Kapur, 2004; Swanwick et al., 2004). Organotypic hippocampal slice cultures (organotypic cultures) were obtained using a method modified from that of Gogolla et al. (2006) using hippocampi isolated from P7–P10 rat pups. The hippocampi were placed into a Gey's balanced buffered salt solution containing (in mM) 137 NaCl, 5 KCl, 0.25 $MgS0_4$, 1.5 $CaCl_2$, 1.05 $MgCl_2$, 0.84 Na_2HPO_4, 0.22 K_2HPO_4, 2.7 $NaHCO_3$ 2.7, and 5.6 dextrose supplemented with 6.5 mg/ml glucose. The hippocampi were sliced on a McIlwain tissue chopper (section thickness, 350 μm) and then cultured on Millicell culture inserts (0.4 μm membrane thickness, 30 mm diameter) for 7–8 d in a humidified incubator at 37°C with 5% CO_2. The culture medium contained 50% MEM, 25% heat-inactivated horse serum, 25% HBSS, 0.5% glutamax II, 10 mM HEPES, and 6.5 mg/ml glucose. The culture medium was changed every 2 d.

Biotinylation

Surface expression of GABAR subunits in acutely obtained hippocampal slices or organotypic cultures was studied using techniques previously described (Nosyreva and Huber, 2005). The slices were obtained as per the methods outlined above. Surface proteins were biotinylated by incubating the slices in ice-cold aCSF or PBS containing 1 mg/ml sulfosuccinimidyl-6-(biotin-amido)hexanoate (Pierce Biotechnology, Rockford, IL) for 30 min at 4°C with gentle shaking. Unbound biotin was removed by washing the slices in Tris-buffered saline containing (in mM) 25 Tris, pH 7.4, 137 NaCl, 5 KCl, 2.3 $CaCl_2$ and 0.5 $MgCl_2$. The hippocampus was dissected free from each slice and five hippocampi were pooled together. The hippocampi were lysed and sonicated using a Branson sonifier (five pulses, output control 5, duty cycle 30%) in an ice-cold standard radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1 mM sodium orthovanadate and a protease inhibitor mixture (Cocktail set I; Calbiochem, La Jolla, CA). The nonsoluble fraction was removed after centrifugation of the lysate at 14,000 × *g* for 15 min at 4°C. Protein concentration was measured with a DC Protein Assay (Bio-Rad, Hercules, CA) and 30 μg of total protein was removed for later calculation of the surface/total protein ratio (see below). Biotin-tagged proteins were separated by incubating 500 μg of protein with 100 μl of Ultralink immobilized NeutrAvidin beads (Pierce Biotechnology) for 2 h at 4°C followed by extensive washing of the beads with a RIPA-lysis buffer and elution of the biotin-tagged protein in a nonreducing sample buffer for 5 min at 95°C.

The biotin-tagged protein and the previously separated 30 μg of total protein were subjected to electrophoresis on 10% SDS polyacrylamide gels and then transferred to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences, Piscataway, NJ). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with 1% Tween 20 (TBST) for 2 h followed by overnight incubation at 4°C with an anti-β2/3 GABAR subunit antibody (1:1000; Millipore, Billerica, MA), an anti-δ GABAR subunit antibody (1:1000; a gift from Dr. Werner Sieghart, Medical University, Vienna, Austria), or an anti-γ2 GABAR subunit antibody (1:500; Millipore) diluted in TBST containing 1% bovine serum albumin. These antibodies were characterized previously (Swanwick et al., 2006). After incubation, the membranes were washed in TBST and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Bio-Rad) diluted in TBST for 1 h at room temperature and developed using a Western lightning chemiluminescence reagent kit (PerkinElmer, Boston, MA). The signal from the immunoreactive band was detected using a Kodak (Carestream Health Molecular Imaging, New Haven, CT) gel Logic 2200 imaging system. Signal intensity was determined by densitometric scanning and the ratio of surface to total proteins was calculated. After scanning, all blots were stripped and re-probed with an anti-β-actin antibody (1:5000; Sigma, St. Louis, MO) diluted in TBST to assure the absence of contamination of the biotin-tagged surface protein fraction with cytoplasmic proteins and equal loading of the total protein fraction. In all experiments, a β-actin signal was absent from the surface protein fraction and the total protein fractions were equally loaded (data not shown).

Whole-cell electrophysiological recording

Whole-cell patch-clamp recordings of GABAR currents from dentate granule cells (DGCs) in hippocampal slices were performed using standard techniques described previously (Hamill et al., 1981; Mtchedlishvili and Kapur, 2006). Slices were maintained in continuously oxygenated aCSF, at 32°C, in a holding chamber for a minimum of 30 min and then at room temperature in a recording chamber mounted on the stage of an Nikon (Tokyo, Japan) E600FN microscope equipped with a 40× water-immersion objective, infrared-differential interference contrast optics, and video. DGCs were identified in the dentate granule layer as small- and medium-sized neurons with typical oval-shaped soma and single process.

Patch electrodes were pulled from thick walled 1.5 mm (outer diameter) × 0.86 mm (inner diameter) borosilicate glass (Sutter Instruments, Novato, CA) on a horizontal P-97 Flaming-Brown microelectrode puller (model P-97; Sutter Instruments) using a three-stage pull to a final resistance of 2–5 MΩ. Electrode tips were filled with a filtered internal recording solution consisting of (in mM) 153.3 CsCl, 1 $MgCl_2$, 10 HEPES, and 5 EGTA, pH adjusted to 7.2 with CsOH (osmolarity, 285–290 mOsM). The electrode shank contained ATP Mg^{2+} salt in a concentration of 4 mM. Voltage-clamp recordings were performed at a holding potential of −60 mV with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Whole-cell capacitance and series resistance were compensated by 70% at 10 μs lag. Membrane currents were low-pass filtered at 5 kHz with an eight-pole Bessel filter before digitization (10 kHz), display, and storage.

For recordings of miniature IPSCs (mIPSCs), glutamate receptor-mediated synaptic currents were blocked by adding 50 μM DL-2-amino-5-phosphonopentanoic acid (DL-AP5) and 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) to the aCSF. Action potentials were blocked by adding 1 μM tetrodotoxin (TTX) to the aCSF. For recordings of GABAR-mediated tonic inhibition, TTX was omitted, and the membrane-impermeable Na^+ channel blocker QX-314 (lidocaine *N*-ethyl bromide; 10 μM) was added to the internal solution to prevent the clamped cell from firing action potentials.

Analysis of synaptic and tonic currents

Off-line analysis of mIPSC characteristics (amplitude, 10–90% rise time, decay time constants, and frequency) and tonic current was performed using MiniAnalysis software (Synaptosoft, Fort Lee, NJ) as described previously (Goodkin et al., 2005; Mangan et al., 2005; Mtchedlishvili and Kapur, 2006). The threshold for mIPSC detection was set at five times the root mean square of the baseline noise. The accuracy of detection was visually confirmed.

The tonic current was measured by quantification of two features of the holding current, the current required to voltage clamp the DGC to a holding voltage of −60 mV. The first, I_{avg} or mean holding current, was defined as an arithmetic mean of peak-to-peak amplitudes of individual points during a 50 ms epoch (500 amplitude measurements; 10 kHz digitization rate) in the absence of phasic currents. The second, I_{rms} or noise, was defined as square root of the average of the squares of the deviation from I_{avg} during the same 50 ms epoch. For each DGC, I_{avg} and I_{rms} was calculated for sixty epochs separated by 500 ms before and 5 min after application of either one of two competitive GABAR antagonists [bicuculline or 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid hydrobromide (SR 95531)] or the open channel blocker penicillin. Before and after values for an individual neuron were compared using the Kolmogorov–Smirnov (KS) test (www.physics.csbsju.edu/stats/KS-test.n.plot_form.html). In addition, for each neuron, a mean I_{avg} and mean I_{rms} before and 5 min after drug application were calculated by averaging the individual before and after I_{avg} and I_{rms} values. By subtracting the mean I_{avg} and mean I_{rms} after drug application from the mean I_{avg} and mean I_{rms} before drug application, a ΔI_{avg} and ΔI_{rms} was calculated for each neuron. For the population of control and SE-treated DGCs, the mean ΔI_{avg} and mean ΔI_{rms} were calculated and compared using a Student's *t* test.

Immunofluorescence

Cultures of dissociated, living hippocampal neurons 14–18 d *in vitro* (DIV) were incubated at room temperature in an external medium for 0, 5, 15, 30, or 60 min. The standard, control external medium contained (in mM) 146 NaCl, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.3–7.4 (osmolarity 300–320). For experiments performed in a high-potassium external environment (10 $[KCl]_o$), the 2.5 mM KCl was replaced with 10 mM KCl. For experiments performed in a high-GABA external environment, GABA 100 μM was added to the standard, control medium. After the initial incubation, the neurons were fixed with 4% paraformaldehyde/4% sucrose in 1× PBS, blocked with 4% normal goat serum, and incubated at 4°C overnight in the presence of either an anti-γ2 GABAR subunit antibody (2 μg/ml; Millipore) or an anti-δ GABAR subunit antibody (5 μg/ml; Gift from Dr. Werner Sieghart) in the absence of permeabilization so that only surface receptors containing the subunit of interest were tagged. The following morning, the neurons were washed with 1× PBS and then incubated with a fluorochrome-conjugated secondary antibody (Alexa Fluor 488 goat anti-rabbit; Invitrogen, Eugene, OR) on a shaker at room temperature in darkness. The coverslips were mounted on slides with Gel/Mount (Biomeda, Foster City, CA). The edge of each coverslip was sealed with clear nail polish, and slides were stored at −20°C.

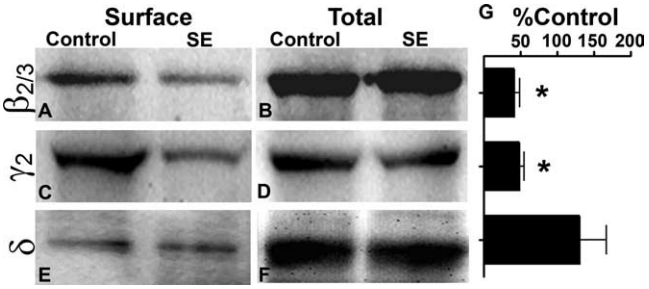

Figure 1. Status epilepticus results in a reduction in the surface expression of the GABAR β2/3 and γ2 subunits, but not the δ subunit. ***A–F***, Sample Western blots of the surface protein fraction (***A***, ***C***, ***E***) and the total protein fraction (***B***, ***D***, ***F***) of the GABAR β2/3 (***A***, ***B***), γ2 (***C***, ***D***), and δ (***E***, ***F***) subunits in hippocampal slices acutely obtained from animals in status epilepticus of 1 h in duration (SE-treated slices) and in age-matched controls. Status epilepticus was induced by the intraperitoneal injection of LiCl 3 mEq/kg followed 20–24 h later by the intraperitoneal injection of pilocarpine 50 mg/kg. ***G***, Quantitative data of the ratio of surface to total β2/3, γ2, or δ subunits in SE-treated slices expressed as a percentage of the same ratio in control slices. Error bars represent SEM. *$p < 0.01$.

Fluorescent and brightfield images of cells were captured on a Roper Scientific/Photometrics (Tucson, AZ) CoolSNAPcf CCD camera mounted on a Nikon Eclipse TE200 fluorescent microscope equipped with a mercury lamp using a 60× 1.4 numerical aperture lens as described previously (Goodkin et al., 2005). Off-line analysis of surface immunoreactivity and surface area was performed using the MetaMorph imaging software system (Molecular Devices, Downington, PA). For each captured neuron, the immunoreactive area of surface antibody-tagged receptors and cell surface area were determined by thresholding the fluorescent and brightfield images as described previously (Swanwick et al., 2004; Goodkin et al., 2005). Values are reported as the mean ± SEM. Statistical comparisons were performed by either a two-tailed Student's *t* test or repeated-measures ANOVA. Adobe (San Jose, CA) Photoshop CS2 was used to increase overall brightness for final production.

Results

Differential changes in the surface expression of GABAR subunits (subunit-specific trafficking)

We compared the surface expression of the GABAR β2/3, γ2, and δ subunits in hippocampal slices acutely removed from animals in SE (SE-treated) and age-matched controls using a biotinylation pull-down assay. Compared with the controls, the surface expression of the β2/3 and γ2 subunits was reduced in the SE-treated slices (Fig. 1*A–D*).

Representative Western blots of the β2/3 subunit in the surface (biotin-tagged) protein fraction and total protein fraction of SE-treated and control hippocampal slices are displayed in Figure 1, *A* and *B*. Although the surface expression of the β2/3 subunit was reduced in the SE-treated slices, as indicated by the weaker signal in Figure 1*A*, the total expression of the β2/3 subunit was similar in the SE-treated and control slices (Fig. 1*B*) consistent with the previous finding of an increase in the intracellular accumulation of receptors containing this subunit during prolonged epileptiform bursting (Goodkin et al., 2005).

The surface expression of the GABAR subunits was quantified by scanning densitometry and expressed as a ratio of the immunoreactivity of the surface fraction to the total fraction. For the

Western blots displayed in Figure 1, *A* and *B*, the surface/total ratio for the $\beta 2/3$ subunit for the SE-treated and control slices was 0.29 and 0.64, respectively. In 4 replicates, the surface expression of the $\beta 2/3$ subunit in the SE-treated slices was 41 ± 7% of controls (Fig. 1*G*) ($p < 0.01$, *t* test).

For the Western blots displayed in Figure 1, *C* and *D*, the surface/total ratio for the $\gamma 2$ subunit for the SE-treated slices was 0.48 and for the control slices was 0.85. In four replicates, the surface expression of the $\gamma 2$ subunit in the SE-treated slices was 48 ± 5% of controls (Fig. 1*G*) ($p < 0.01$, *t* test).

In contrast, when compared with controls, the surface expression of the δ subunit was not reduced in the SE-treated slices. For the Western blots displayed in Figure 1, *E* and *F*, the surface/total ratio for the δ subunit for the SE-treated slices was 0.46 and for the control slices was 0.40. Across the four replicates, the surface expression of δ subunit in the SE-treated slices was 120 ± 36% of controls (Fig. 1*G*) ($p > 0.05$, *t* test).

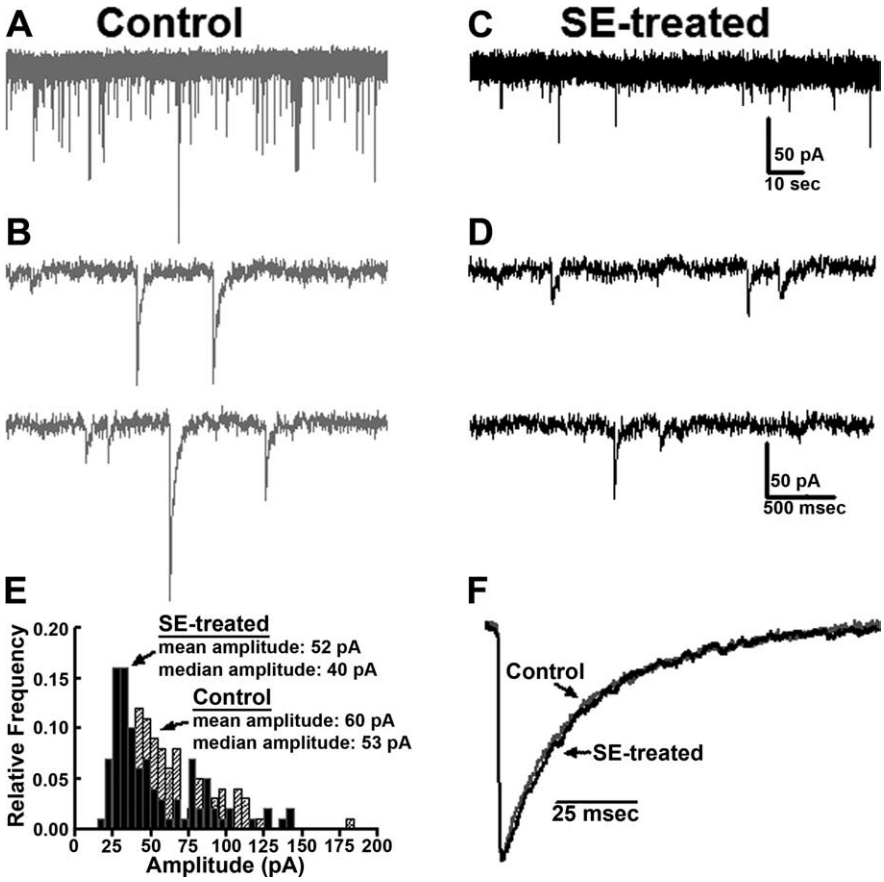

Figure 2. Diminished GABAergic synaptic transmission in DGCs in SE-treated slices. ***A–D***, Current traces recorded from a control DGC (***A***, ***B***; expanded temporal resolution) and a SE-treated DGC (***C***, ***D***; expanded temporal resolution) voltage clamped to a holding potential of −60 mV. ***E***, Amplitude distribution histogram for mIPSCs recorded from the control (crosshatched bars) and SE-treated (black bars) DGCs displayed in ***A–D***. ***F***, Normalized averaged mIPSC traces for the control and SE-treated neurons displayed in ***A–D***.

Altered GABA-mediated inhibition after prolonged *in vivo* status epilepticus

We observed previously that the amplitude of GABA-mediated mIPSCs was reduced after a period of prolonged epileptiform bursting in a network of cultured hippocampal neurons and that this reduction was partially the result of an increase in the intracellular accumulation of GABARs (Goodkin et al., 2005). As one determinant of mIPSC amplitude is the number of GABARs clustered at the synapse (De Koninck and Mody, 1994; Poncer et al., 1996), a similar reduction in the amplitude of mIPSCs in hippocampal slices acutely obtained from animals in SE was expected given the finding of a reduction in the surface expression of the GABAR $\beta 2/3$ and $\gamma 2$ subunits.

To test this hypothesis, we measured GABAR-mediated synaptic inhibition on DGCs in hippocampal slices acutely removed from animals in SE (SE-treated DGCs) and age-matched controls (control DGCs). Whole-cell membrane ruptured patch-clamp recordings of mIPSCs were recorded from SE-treated DGCs ($n = 8$ from 3 animals) and control DGCs ($n = 8$ from 3 animals) in an isotonic chloride environment in the presence of TTX, D-APV, and DNQX with the neuron voltage clamped to −60 mV. Bath application of bicuculline (50 μM) abolished these events verifying that they were GABA-mediated mIPSCs (data not shown).

In Figure 2, traces recorded from a control DGC (Fig. 2*A*,*B*) and a SE-treated DGC (Fig. 2*C*,*D*) are displayed demonstrating that the amplitude of mIPSCs recorded from SE-treated DGCs was reduced. The peak of the mIPSC amplitude distribution histogram recorded from the SE-treated DGC was shifted leftwards, toward smaller values, compared with the histogram from the control DGC (Fig. 2*E*). The mean peak amplitude recorded from the SE-treated DGC was 52 pA and that recorded from the control neuron was 60 pA. However, as the mIPSC amplitude distribution is skewed, the median mIPSC amplitude was calculated. The median mIPSC amplitude for the SE-treated DGC was 40 pA whereas the median mIPSC amplitude for the control DGC was 53 pA. For the population of SE-treated DGC, the mean of the median peak mIPSC amplitudes was 42 ± 2 pA, an ~25% reduction compared with that of the control DGC population (55 ± 2 pA; $p < 0.01$, *t* test).

A decrease in the frequency of mIPSCs was also observed. For the recordings displayed in Figure 2, the frequency of the mIPSCs for the SE-treated DGC was 0.2 Hz and for the control DGC was 0.8 Hz. For the population of SE-treated and control DGC, there was an ~50% decrease in the frequency of mIPSCs (0.3 ± 0.1 Hz vs 0.6 ± 0.1 Hz; $p < 0.01$, *t* test). A reduction in mIPSC amplitude could potentially contribute to this decrease in mIPSC frequency as there may be more mIPSCs that fall below the level of detection. In addition, changes in mIPSC frequency can also result from modification in presynaptic factors such as changes in presynaptic GABA release probability or from the loss of synapses. A loss of proximal synapses is supported by a small increase in the mIPSC 10–90% rise time. The population mean for 10–90% rise time for the SE-treated DGCs was 1.7 ± 0.1 ms and for the control DGCs was 1.3 ± 0.1 ms ($p < 0.01$, *t* test). These factors were not explored further.

The mIPSC decay phase was essentially unchanged. Given that mIPSC amplitude in the SE-treated DGCs was decreased compared with controls, analysis of mIPSC decay was limited to those mIPSCs with similar 10–90% rise times to assure that a similar

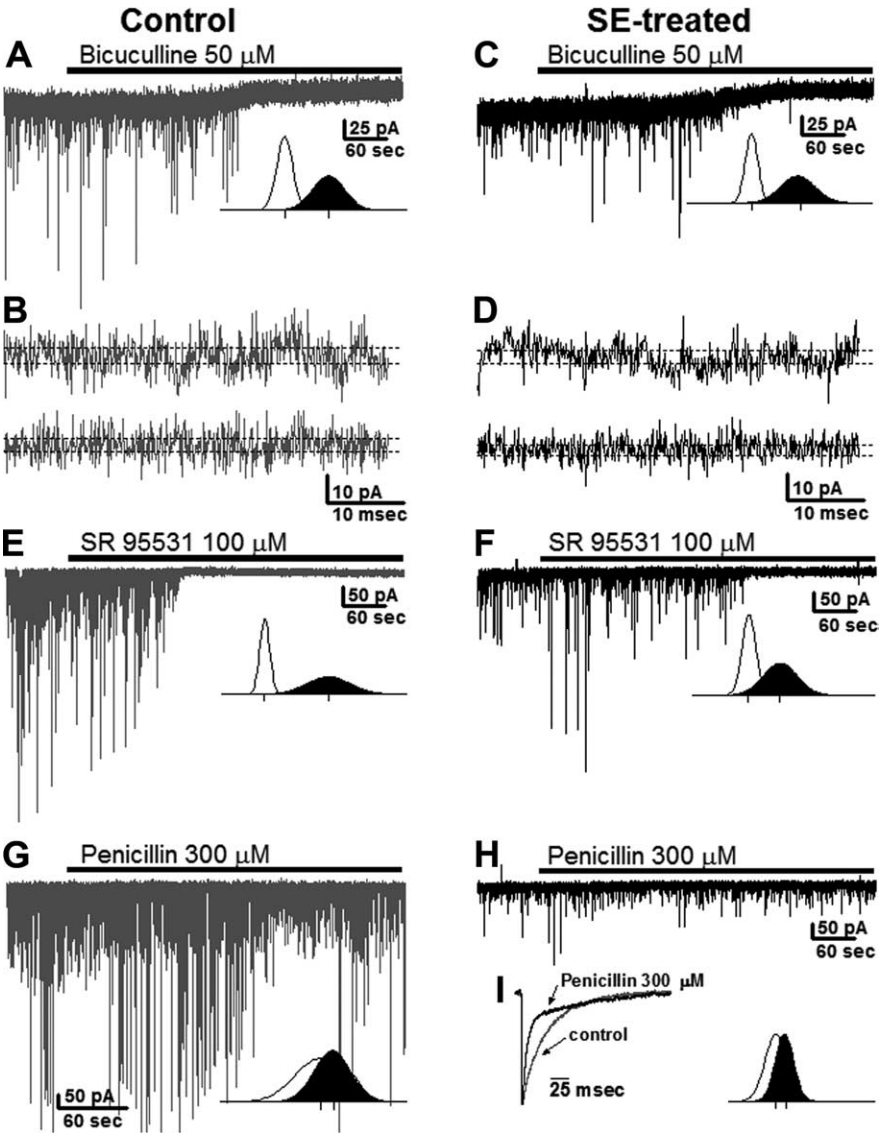

Figure 3. ΔI_{avg} and ΔI_{rms} in response to the competitive GABAR antagonists bicuculline and SR 95531 and the open channel blocker penicillin were unchanged in DGCs in SE-treated slices in the presence of the ambient GABA concentration. ***A***, ***C***, ***E–H***, Current traces recorded from control DGCs (***A***, ***E***, ***G***) and SE-treated DGCs (***C***, ***F***, ***H***) before and after the application of bicuculline 50 μM (solid line; ***A***, ***C***), SR 95531 100 μM (solid line; ***E***, ***F***), and penicillin 300 μM (solid line; ***G***, ***H***). ***B***, ***D***, Current traces of baseline noise (I_{rms}) for 100-ms epochs before (top traces) and in the presence (bottom trace) of bicuculline 50 μM for a control DGC (***B***) and SE-treated DGC (***D***). The I_{rms} for 60 100 ms epochs, void of transient currents, before and after drug application, were calculated and the values were fit to separate before (filled) and after (open) Gaussian distributions (insets ***A***, ***C***, ***E–H***). ***I***, Averaged spontaneous IPSCs for the control neuron before and after penicillin application displayed in ***G***. In the presence of penicillin, there was a shortening of the decay phase and no change in amplitude.

subset of fast mIPSCs was compared between the SE-treated and control DGCs. In Figure 2*F*, the averaged mIPSC, scaled to peak amplitude, recorded from the SE-treated DGC and control DGC are displayed. For these neurons, the mean fast ($\tau 1$) and mean slow ($\tau 2$) decays were similar ($\tau 1$, 13 ms vs 14 ms; $\tau 2$, 84 ms vs 89 ms). The population means of the mIPSC decay constants recorded from the SE-treated DGCs were $\tau 1 = 12 \pm 1$ ms and $\tau 2 = 69 \pm 6$ ms, and those from the control DGCs were $\tau 1 = 10 \pm 1$ ms and $\tau 2 = 74 \pm 4$ ms ($p > 0.05$, *t* test).

To control for the possibility that the changes observed in GABA-mediated synaptic inhibition were the result of either a direct or indirect effect of either lithium or pilocarpine and not in response to the prolonged seizure, a second model of *in vivo* SE that does not require chemical induction was used. We acutely obtained hippocampal slices from SE-treated animals after 60 min of self-sustaining SE induced via continuous hippocampal stimulation. At this time point of self-sustaining SE, benzodiazepine pharmacoresistance is well established (Mazarati et al., 1998). The mIPSCs were recorded from 11 SE-treated DGCs (five animals) and 13 control DGCs (six animals). As observed in the lithium-pilocarpine model, the strength of GABA-mediated synaptic inhibition on SE-treated DGCs was reduced. The amplitude was reduced (42 ± 3 pA vs 53 ± 3 pA; $p < 0.01$, *t* test), the frequency declined (0.4 ± 0.1 Hz vs 1.0 ± 0.1 Hz; $p < 0.01$, *t* test), there was a prolongation of 10–90% rise-time (2.5 ± 0.3 ms vs 1.8 ± 0.1 ms; $p < 0.01$, *t* test), and decay was unchanged ($\tau 1$, 11 ± 1 ms vs 8 ± 1 ms, $p > 0.05$, *t* test; $\tau 2$, 61 ± 5 ms vs 53 ± 4 ms, $p > 0.05$, *t* test).

We also characterized tonic inhibition of the DGC after SE via whole-cell ruptured membrane patch-clamp recordings from SE-treated and control DGC. As δ subunit-containing GABARs are one of the prime mediators of tonic inhibition (for review, see Glykys and Mody, 2007; Mody et al., 2007), we hypothesized that in the absence of a reduction in the surface expression of the δ subunit in the SE-treated slices that GABA-mediated tonic inhibition is not reduced during the prolonged seizures of SE.

Tonic currents were initially measured in the presence of the ambient GABA concentration in the control and SE-treated hippocampal slices. After obtaining a baseline recording, we bath applied the competitive GABAR antagonist bicuculline. A typical recording for a control DGC before and after application of bicuculline is displayed in Figure 3*A*,*B*. With the application of bicuculline, the rapid phasic currents are lost just before the onset of a slow outward current with a corresponding reduction in baseline noise. For this neuron, there was a 20 pA reduction in I_{avg} (KS test, $p < 0.01$) and 1 pA decrease in I_{rms} (KS test, $p < 0.01$). For each of the four control DGCs, the I_{avg} and I_{rms} after bath application of bicuculline was less than that during the baseline period (KS test, $p < 0.01$); and the mean population reduction in I_{avg} (ΔI_{avg}) was 19.4 ± 1.0 pA and the mean population reduction in I_{rms} (ΔI_{rms}) was 1.2 ± 0.1 pA.

Similar changes after bath application of bicuculline were observed for the SE-treated neurons. For the SE-treated neuron displayed in Figure 3, *C* and *D*, there was a 24 pA reduction in I_{avg} (KS test, $p < 0.01$) and a 1 pA decline in the mean I_{rms} (KS test, $p < 0.01$). For each of the six SE-treated DGCs, the I_{avg} and I_{rms} after bath application of bicuculline was less than that during the baseline period (KS test, $p < 0.01$), and the mean ΔI_{avg} (16.82 ± 4.1 pA) and mean ΔI_{rms} (1.2 ± 0.2 pA) were not statistically

different ($p > 0.05$, *t* test) than those recorded in the control DGCs.

To confirm these findings of similar changes in tonic currents after the bath application of bicuculline, we measured the effect of a second GABAR antagonist, SR 95531, on tonic inhibition of the SE-treated and control DGCs. At high concentrations, SR 95531 has been shown to block those GABARs which mediate tonic inhibition (Stell and Mody, 2002; Yeung et al., 2003; Mtchedlishvili and Kapur, 2006). This GABAR antagonist had a similar effect on the tonic current of the DGC in the SE-treated ($n = 4$, 2 animals) and control ($n = 5$, 3 animals) slices (Fig. 3*E*,*F*). In both, SR 95531 inhibited synaptic currents, had a small effect on I_{avg}, and decreased I_{rms} compared with the baseline period. The mean ΔI_{avg} for the SE-treated and control DGCs was 4.0 ± 3.2 pA and 7.3 ± 3.3 pA, respectively ($p > 0.05$; *t* test). The mean ΔI_{rms} for the SE-treated and control DGCs was 0.72 ± 0.42 pA and 0.96 ± 0.18 pA, respectively ($p > 0.05$, *t* test).

The competitive GABAR antagonists bicuculline and SR 95531 prevent closed unbound GABARs from opening. Once the receptor is bound and open, these antagonists are ineffective (Bianchi and Macdonald, 2001). As persistently open bound and unbound GABARs may contribute to tonic inhibition (Mtchedlishvili and Kapur, 2006; McCartney et al., 2007), we choose to also measure tonic inhibition in SE-treated and control DGCs in response to the open channel blocker penicillin (Fig. 3*G*,*H*).

After bath application of this noncompetitive GABAR antagonist, no change was observed in the frequency, rise time, or amplitude of synaptic currents recorded from seven SE-treated DGCs and four control DGCs (data not shown). As expected (Mtchedlishvili and Kapur, 2006), there was an ~50% decrease in the decay phase (Fig. 3*I*) of the synaptic currents recorded from these 11 neurons.

As with the competitive GABAR antagonists, measures of the tonic current in response to penicillin were similar in the SE-treated and control DGCs. After the application of penicillin, small but significant decreases in I_{avg} were observed in six of the seven SE-treated DGCs and three of the four control DGCs (KS test, $p < 0.05$) and there was a significant decrease of I_{rms} in all neurons (KS test, $p < 0.01$). The mean ΔI_{avg} for the SE-treated and control DGCs was 2.0 ± 1.7 pA and 2.8 ± 2.0 pA, respectively ($p > 0.05$; *t* test). The mean ΔI_{rms} for the SE-treated and control DGCs was 0.42 ± 0.12 pA and 0.40 ± 0.08 pA, respectively ($p > 0.05$, *t* test).

Although the tonic currents measured in response to bicucculine, SR 95531, and penicillin varied, the ΔI_{avg} and ΔI_{rms} measured for each agent were similar in the SE-treated and control slices. This result supports the finding obtained using the biotinylation pull-down assay that the surface expression of the δ subunit was not reduced in the SE-treated slices. However, as the ambient GABA concentration in the SE-treated and control slices may vary because of alterations in GABA synthesis, release, or uptake during SE or because of differences in washout (Glykys and Mody, 2007), we were concerned that a reduction in the surface expression of δ subunit-containing receptors and a corresponding increase of the GABA concentration in the SE-treated slices was a potential alternative explanation for these results. Therefore, the effect of bicuculline on tonic inhibition of the DGC was also measured in the presence of the GABA uptake blocker 1-[2[[diphenylmethylene)imino]oxy]ethyl]-2,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711) (10 μM) and GABA (1 μM).

After bath application of bicuculline, the rapid phasic currents were lost and there was a slow outward current and reduction in baseline noise as observed in the ambient GABA condition. For each of the seven control DGCs (3 animals), the I_{avg} and I_{rms} after the bath application was less than that during the baseline period (KS test, $p < 0.01$). The ΔI_{avg} was 77.2 ± 7.7 pA and the ΔI_{rms} was 4.4 ± 0.6 pA. As with the control DGCs, for each of the six SE-treated DGCs (four animals), the I_{avg} and I_{rms} after bath application of bicuculline was less than that during the baseline period (KS test, $p < 0.01$). However, the ΔI_{avg} in SE-treated DGCs (109.5 ± 9.9 pA) was greater than the ΔI_{avg} recorded in the control DGCs ($p < 0.05$, *t* test), whereas the mean change in ΔI_{rms} (5.1 ± 0.5 pA) was not statistically different from the ΔI_{rms} recorded in the control DGCs ($p > 0.05$, *t* test). This finding is not consistent with the potential alternative explanation of a reduction in the surface expression of the δ subunit-containing GABARs in the presence of an increased GABA concentration.

Changes in the surface expression of GABARs are independent of ligand-binding

To confirm and extend the findings of differential trafficking of GABARs during SE, we compared the surface expression of the γ2 and δ subunits in dissociated cultures (DIV 14 or older) incubated in either the standard, control external medium (control) or the standard, control medium supplemented with 10 mM KCl ($10[KCl]_o$-treated) for 5, 15, 30, and 60 min at room temperature. The elevation of KCl in the extracellular media results in prolonged neuronal depolarization and an increase in the spontaneous frequency of action potentials.

Images of representative processes from control neurons and $10[KCl]_o$-treated neurons incubated for 5, 15, 30, and 60 min before fixation and antibody-tagging of the γ2 subunit in the absence of permeabilization are displayed in Figure 4*A–H*. The surface expression of the tagged γ2 subunits was quantified as the ratio of cell-surface immunoreactivity normalized to the cell-surface area. For the control neurons, the surface immunoreactivity ratio was similar at each time point (Fig. 4*I*) ($n = 15$ from 3 replicates for each time point). In contrast, incubating the neurons for longer periods of time in $10[KCl]_o$ resulted in a decrease in the surface expression of the γ2 subunit (Fig. 4*I*) ($n = 15$ from 3 replicates for each time point).

When the cultured hippocampal neurons were incubated in $10[KCl]_o$ supplemented with 350 nM sucrose ($10[KCl]_o$ plus sucrose) for 30 min, the surface immunoreactivity ratio was higher than for $10[KCl]_o$-treated neurons (Fig. 5). Because hyperosmolar sucrose is known to inhibit clathrin-mediated endocytosis (Hansen et al., 1993; Kittler et al., 2000), this finding suggests the reduction in the total surface receptor pool of γ2 subunit-containing GABARs that occurred in the setting of $10[KCl]_o$ is, in part, dependent on the endocytosis of receptors from the surface.

To confirm the *in vivo* finding of a similar surface expression of the δ subunit in SE-treated and control hippocampal slices, we compared the surface expression of the δ subunit in $10[KCl]_o$-treated and control neurons in dissociated cultures. Images of representative processes from control neurons and $10[KCl]_o$-treated neurons incubated for 5, 15, 30, and 60 min before fixation and antibody-tagging of the δ subunit in the absence of permeabilization are displayed in Figure 6*A–H*. For the control and $10[KCl]_o$-treated neurons, surface immunoreactivity was similar at each time point (Fig. 6*I*) ($n = 15$ from 3 replicates for each time point), consistent with the previous findings.

Because dissociated hippocampal neuronal cultures are an imperfect model of the well organized intrinsic circuitry of the hippocampus, additional studies were performed in the organotypic cultures. A biotinylation pull-down assay was used to com-

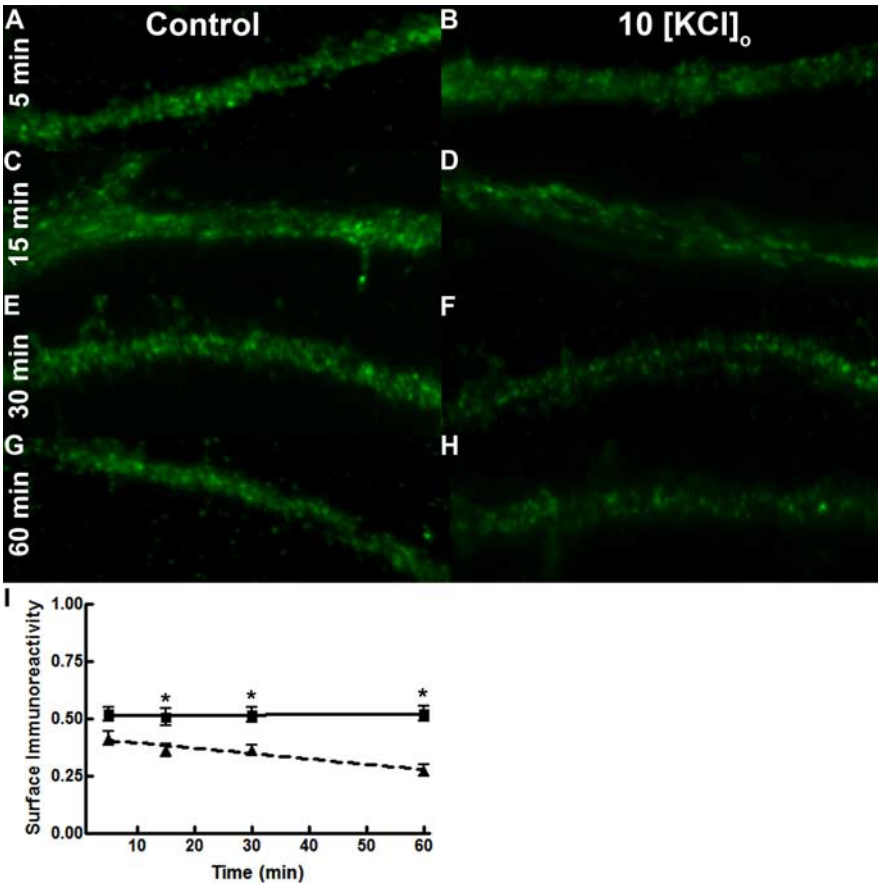

Figure 4. The surface expression of the GABAR γ2 subunit in dissociated cultures was reduced after incubation in a high potassium external medium (10[KCl]$_o$). ***A–H***, Images of representative processes of cultured pyramidal neurons that were incubated in a standard, control external medium (see Materials and Methods) (***A***, ***C***, ***E***, ***G***) or in 10[KCl]$_o$ (***B***, ***D***, ***F***, ***H***) for 5 min (***A***, ***B***), 15 min (***C***, ***D***), 30 min (***E***, ***F***) or 60 min (***G***, ***H***) before fixation and antibody tagging of the γ2 subunit (green) using a primary antibody directed against the N terminus of that subunit. ***I***, Quantification of the γ2 subunit surface expression for neurons incubated in standard, control external medium or in 10[KCl]$_o$ for 0, 15, 30, and 60 min before fixation and antibody tagging. The surface immunoreactivity ratio was defined as the ratio of the surface immunoreactivity to the surface area of the neuronal process. The ratio for each time point was obtained by pooling data from 15 neuronal processes (3 replicates). For each time point, the mean and SEM are displayed. Solid line, Time course of γ2 subunit surface expression in standard, control external medium. Dotted line, Time course of GABAR γ2 subunit surface expression in 10[KCl]$_o$. *$p < 0.01$.

pare the surface expression of the γ2 and δ subunit-containing receptors in organotypic cultures (DIV 8) incubated in a standard aCSF supplemented with 10 mM KCl and 10 μM NMDA (10[KCl]$_o$+10[NMDA]$_o$-treated) for 1 h with the surface expression of these subunits in slice cultures incubated in a standard aCSF (control) for 1 h (Fig. 7). Representative Western blots for the γ2 subunit in the surface (biotin-tagged) and total protein fractions of 10[KCl]$_o$+10[NMDA]$_o$-treated and control slices are displayed in Figure 7, *A* and *B*. For these Western blots, the surface/total ratio for the γ2 subunit for the 10[KCl]$_o$+10[NMDA]$_o$-treated and control slices was 0.32 and 0.56, respectively. In four replicates, the surface expression of the γ2 subunit in the 10[KCl]$_o$+10[NMDA]$_o$-treated slices was 35.8 ± 6% of controls ($p < 0.01$, *t* test).

In comparison, the surface expression of the δ subunit in 10[KCl]$_o$+10[NMDA]$_o$-treated slices was similar to that in control slices. Representative Western blots for the δ subunit in the surface and total protein fractions of 10[KCl]$_o$+10[NMDA]$_o$-treated and control organotypic slice cultures are displayed in 7C and 7D. For these Western blots, the surface/total ratio for the δ subunit in the 10[KCl]$_o$+10[NMDA]$_o$-treated and control slices was 0.32 and 0.22, respectively. In 5 replicates, the surface expression of the δ subunit in the 10[KCl]$_o$+10[NMDA]$_o$-treated slices was 110 ± 11% of controls (Fig. 7*E*) ($p > 0.05$, *t* test).

In the dissociated cultures, incubation in 10[KCl]$_o$ was sufficient to induce a decrease in the surface expression of the γ2 subunit (Fig. 4). In contrast, in preliminary studies (data not shown), we found that the surface expression of the γ2 subunit in organotypic hippocampal slice cultures incubated in a standard aCSF supplemented with 10 mM KCl for 1 h was similar to the surface expression in control slices and that supplementation with NMDA was necessary to induce a reduction in the γ2 surface expression in this system. To determine whether NMDA was sufficient to induce a reduction in the surface expression of γ2 subunit-containing GABARs, a biotinylation pull-down assay was used to compare the surface expression of the γ2 subunit-containing GABARs in organotypic cultures incubated in a standard aCSF supplemented with 10 μM NMDA (10[NMDA]$_o$-treated) for 1 h with the surface expression of the γ2 subunits in slices incubated in a standard aCSF (control) for 1 h. Representative Western blots for the γ2 subunit in the surface and total protein fractions of 10[NMDA]$_o$-treated and control slice cultures are displayed in Figure 7, *E* and *F*. For these Western blots, the surface/total ratio for the γ2 subunit in the 10[NMDA]$_o$-treated and control slices was 0.26 and 0.70, respectively. In six replicates, the surface expression of the γ2 subunit in the 10[NMDA]$_o$-treated slices was 58 ± 21% of controls. Although reduced, this decrease did not reach statistical significance ($p = 0.08$, *t* test).

A reduction in the surface expression of a subset of GABARs during SE could occur in response to either a ligand-independent mechanism (Stelzer and Shi, 1994; Chen and Wong, 1995; Lu et al., 2000) or a ligand-dependent mechanism (Tehrani and Barnes, 1991; Calkin and Barnes, 1994). The finding that NMDA was, at a minimum, necessary to induce a reduction in the surface expression of the γ2 subunit in the organotypic culture is consistent with a ligand-independent mechanism. However, because GABA may be released in response to cell depolarization or activation of excitatory amino acid receptors (Harris and Miller, 1989), a ligand-dependent mechanism cannot be excluded based on these results.

To test the relative contribution of a ligand-dependent mechanism on the regulation of the surface expression of the γ2 subunit, we first compared the surface expression of the GABAR γ2-subunit in dissociated cultures that were incubated in either the standard, control external medium (control) or the standard, control medium supplemented with GABA 100 μM (GABA-treated neurons) for 30 min at room temperature before fixation. Images of representative processes from a control neuron (Fig. 8*A*) and a GABA-treated neuron (Fig. 8*B*) are displayed. Under

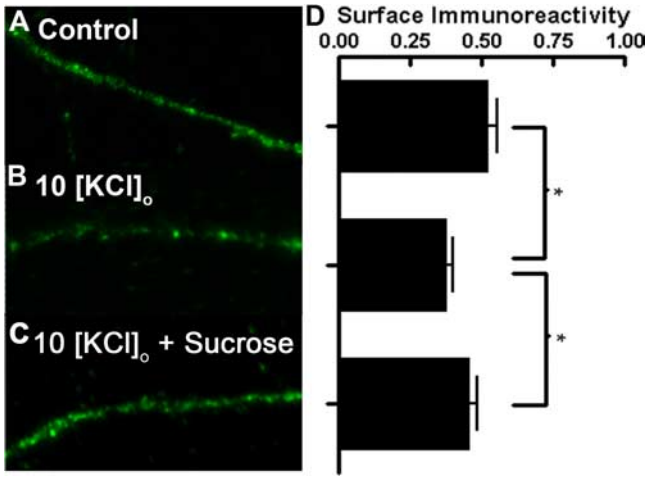

Figure 5. The reduction in the surface expression of the GABAR γ2 subunit in dissociated cultures that occurred in a high potassium external medium was inhibited in the presence of a hyperosmolar external medium. ***A–C***, Representative neuronal processes of cultured pyramidal neurons incubated for 30 min in the standard, control external medium (***A***), 10[KCl]$_o$ (***B***), or 10[KCl]$_o$ supplemented with 350 mM sucrose (***C***) before fixation and antibody tagging of the γ2 subunit (green). ***D***, Quantification of surface immunoreactivity under the three conditions. Surface immunoreactivity was calculated for 15 neurons (3 replicates). Error bars indicate SEM. $^*p < 0.05$.

both conditions, the γ2 surface immunoreactivity was widespread with frequent immunoreactive clusters. The surface immunoreactivity ratio for the control neurons (0.46 ± 0.02; $n = 15$ from 3 replicates) and GABA-treated neurons (0.47 ± 0.02; $n = 15$ from 3 replicates) was similar ($p > 0.05$, t test). Although internalization of GABARs may occur in response to ligand-binding, this result suggested that changes in the total surface expression of GABARs containing the γ2 subunit was not dependent on the concentration of GABA in the extracellular environment during the 30 min time period studied.

To confirm and extend this finding, a biotinylation pull-down assay was used to compare the surface expression of the GABAR γ2 subunit in organotypic cultures that were incubated in either a standard, control aCSF (control slices) or the standard, control aCSF supplemented with GABA 100 μM (GABA-treated slices) for 1 h. As in the dissociated cultures, surface expression of the γ2 subunit was similar in the GABA-treated and control slices. Representative Western blots for the γ2 subunit in the surface and total protein fractions of GABA-treated and control slices are displayed in Figure 8, *C* and *D*. For these Western blots, the surface/total ratio for the γ2 subunit in the GABA-treated and control slices was 0.20 and 0.28, respectively. In five replicates, the surface expression of the γ2 subunit in the GABA-treated slices was 146 ± 31% of controls ($p > 0.05$, t test).

Because of concern that the external GABA concentration may have declined over the 1 h incubation period because of active GABA uptake and metabolism, the surface expression of the GABAR γ2 subunit in organotypic cultures incubated in either a standard aCSF supplemented with GABA 100 μM and NO-711 10 μM (GABA plus NO-711) or in standard aCSF supplemented with muscimol 1 μM was compared with controls using a biotinylation pull-down assay. Under both conditions, the surface expression was not different from that in controls. With GABA plus NO-711, surface expression of the γ2 subunit was 139 ± 70% of controls ($n = 5$ replicates; $p > 0.05$, t test) and with muscimol was 161 ± 37% of controls ($n = 5$ replicates; $p > 0.05$, t test).

Discussion

The major findings of this study are that (1) subunit-specific trafficking of GABARs during *in vivo* SE results in a reduced surface expression of the β2/3 and γ2 subunits, but not the δ subunit, with a corresponding reduction in GABAR-mediated synaptic, but not tonic inhibition and (2) ligand binding to the GABAR was insufficient to induce a reduction in the surface expression of the γ2 subunit in dissociated and organotypic cultures.

Implications of subunit-specific trafficking for the treatment of SE

A key determinant of inhibitory synaptic strength is the number of postsynaptic GABARs (De Koninck and Mody, 1994; Poncer et al., 1996; Wan et al., 1997; Wang et al., 2003). It has been demonstrated that recurrent seizures and SE are correlated with a use-dependent decline in GABAR-mediated inhibition (Kapur and Lothman, 1989; Kapur et al., 1989; Michelson et al., 1989; Sayin and Rutecki, 1997). The findings of this study provide additional evidence supporting the hypothesis (Goodkin et al., 2005; Naylor et al., 2005) that a reduction in the surface expression of synaptic GABARs containing the β2/3 and γ2 subunits is one of the mechanisms by which this reduction in GABAR-mediated inhibition occurs.

Tonic inhibition is mediated predominantly by extrasynaptic δ subunit-containing GABARs (Glykys and Mody, 2007; Mody et al., 2007). A unique finding of this study was that, unlike the surface expression of the β2/3 and γ2 subunits, the surface expression of the δ subunit in the SE-treated slices was not reduced after *in vivo* SE. Likewise, a reduction in tonic inhibition was not observed when tonic currents were recorded in SE-treated slices under conditions of ambient GABA. Instead, similar to the finding of Naylor et al. (2005), there was a greater change in the holding current (ΔI_{avg}) of DGCs in SE-treated slices than in control slices in response to bicuculline when GABA uptake was blocked. Our finding that the surface expression of the δ subunit in the SE-treated slices was similar to controls suggests that this increase in tonic inhibition with GABA uptake blocked was not the result of an increase in surface expression of δ subunit-containing GABARs. However, although the change in surface expression in the SE-treated slices measured by the biotinylation assay was not statistically significant, there was a trend toward an increase in the SE-treated slices (120 ± 36% of controls). Whether this trend is enough to account for this change in tonic inhibition is not known. Alternatively, as demonstrated by Scimemi et al. (2005), higher GABA concentrations may activate other receptor subtypes or other mechanisms that contribute to tonic inhibition independent of δ subunit-containing GABARs. Given previous findings that the extracellular GABA concentration is potentially increased in the hippocampus during SE (but see Lehmann et al., 1985; Walton et al., 1990), these findings suggest that GABAR-mediated tonic inhibition, unlike GABAR-mediated synaptic inhibition, may be increased during SE.

The differential modulation of the surface expression of synaptic GABARs containing a γ2 and extrasynaptic GABARs containing a δ subunit during SE has ramifications for the treatment of SE. Current treatment protocols (Riviello and Holmes, 2004) recommend a benzodiazepine as the initial agent for treatment of SE. However, these agents often fail to treat prolonged episodes of SE. If other first line agents fail (phenytoin and phenobarbital), anesthetic agents are re-

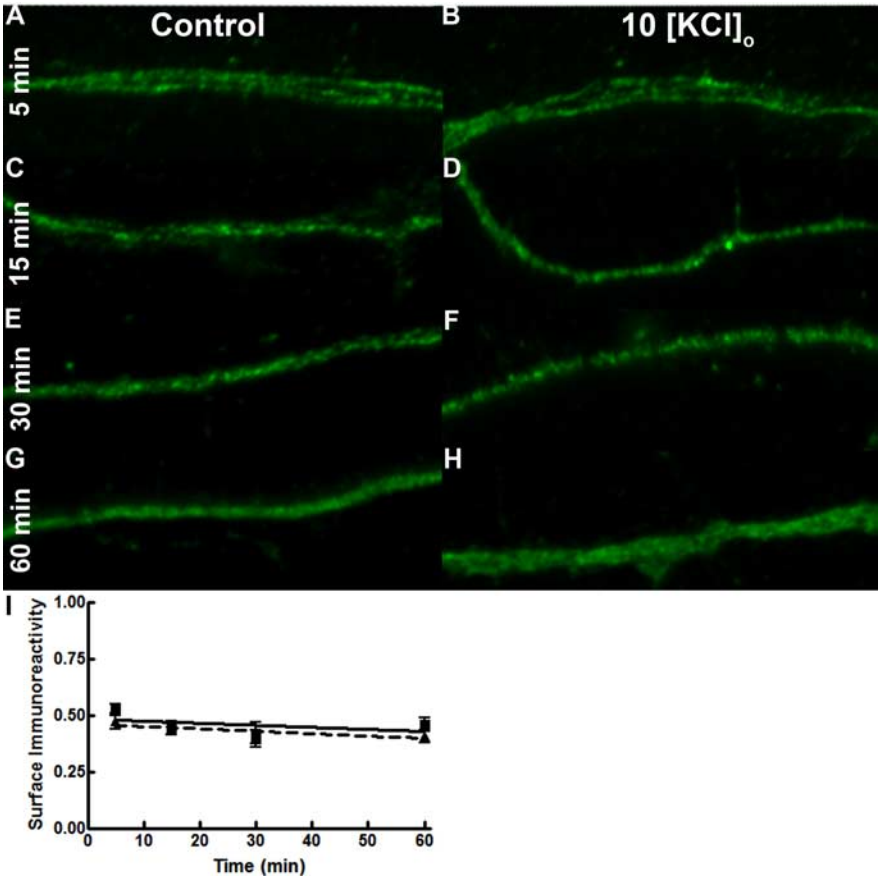

Figure 6. A high potassium external medium (10[KCl]$_o$) had no effect on the surface expression of the GABAR δ subunit in dissociated cultures. ***A–H***, Images of representative processes of cultured pyramidal neurons that were incubated in a standard, control external medium (***A***, ***C***, ***E***, ***G***) or with neuronal excitability increased in 10[KCl]$_o$ (***B***, ***D***, ***F***, ***H***) for 5 min (***A***, ***B***), 15 min (***C***, ***D***), 30 min (***E***, ***F***) or 60 min (***G***, ***H***) before fixation and antibody tagging of the δ subunit (green) using a primary antibody directed against the N terminus of that subunit. ***I***, Quantification of δ subunit surface expression for neurons incubated in standard, control external medium or in 10[KCl]$_o$ for 0, 15, 30, and 60 min before fixation and antibody tagging. The ratio for each time point was obtained by pooling data from 15 neuronal processes (3 replicates). For each time point, the mean and SEM are displayed. Solid line, Time course of GABAR δ subunit surface expression in standard, control external medium. Dotted line, Time course of GABAR δ subunit surface expression in 10[KCl]$_o$.

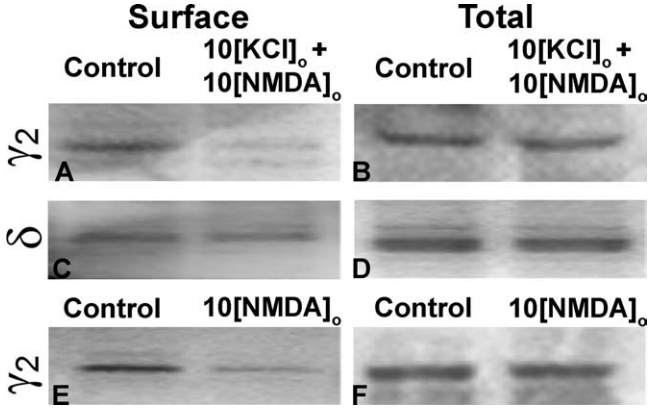

Figure 7. Surface expression of the γ2 subunit but not δ subunit was reduced after incubation of organotypic cultures in a high potassium external medium supplemented with NMDA (10[KCl]$_o$ + 10[NMDA]$_o$). ***A–D***, Sample Western blots of the surface (***A***, ***C***) and total (***B***, ***D***) protein fraction of the GABAR γ2 (***A***, ***B***) and δ (***C***, ***D***) subunits in organotypic cultures incubated in either a standard external aCSF or 10[KCl]$_o$ + 10[NMDA]$_o$ for 1 h. ***E***, ***F***, Sample Western blots of the surface (***E***) and total (***F***) protein fractions of the GABAR γ2 subunit in organotypic cultures incubated in either a standard external aCSF (control) or the standard aCSF supplemented with NMDA (10[NMDA]$_o$).

quired. The response of GABARs to allosteric modulators is dependent on subunit composition. The minimum requirement to form a benzodiazepine-sensitive GABAR is the presence of an α and γ subunit (Pritchett et al., 1989). Receptors containing a δ subunit are benzodiazepine insensitive (Quirk et al., 1995; Brown et al., 2002). In contrast, GABARs that contain either a γ or δ subunit are sensitive to general anesthetics such as propofol and pentobarbital (Lees and Edwards, 1998; Brown et al., 2002; Wohlfarth et al., 2002; Feng and Macdonald, 2004; Feng et al., 2004). Therefore, the finding of reduced surface expression of benzodiazepine-sensitive γ2 subunit-containing GABARs and preserved expression of the benzodiazepine-insensitive δ subunit containing GABARs is a potential mechanism to partially explain the development of benzodiazepine pharmacoresistance during SE. In addition, our finding is in agreement with a current suggestion that SE treatment protocols may need to be revised to allow for the earlier use of general anesthetics once the benzodiazepines have failed (Bleck, 2005, 2006).

Subunit-specific trafficking of GABARs

Modulation of the surface expression of GABARs could occur at single or multiple steps in the GABAR trafficking pathway (Chen and Olsen, 2007; Michels and Moss, 2007). Previously, Kittler et al. (2005) demonstrated that there is direct binding of the μ2 subunit of AP2 to the β and γ GABAR subunits and that posttranslational modification of two serine residues in the β3 subunit can affect synaptic strength likely through regulation of receptor internalization. Such findings demonstrate a role for post-translational modification of specific GABAR subunits in the regulation of synaptic strength and GABAR trafficking. To date, there has been little focus on the trafficking of the extrasynaptic δ subunit-containing GABARs. Although the δ subunit may also interact with the AP2 complex (Kittler et al., 2005), our findings suggest that the regulation of the surface expression of the extrasynaptic δ subunit-containing GABARs occurs via mechanisms independent of those controlling the surface expression of other GABAR subtypes.

During SE, the signaling cascade modulating GABAR surface expression could be initiated by a ligand-independent process such as an increase in neuronal excitability as the result of stimulation of excitatory amino acid receptors (Stelzer et al., 1987; Kapur and Lothman, 1990; Stelzer, 1990; Min et al., 1999; Lu et al., 2000) or initiated by excessive extracellular GABA (ligand dependent). Our findings suggest that control of the surface expression of γ2 subunit-containing GABARs occurs via a ligand-independent process.

The surface immunoreactivity of the γ2 subunit in the dissociated cultures was reduced when neuronal excitability was increased via a high external concentration of KCl. It was also re-

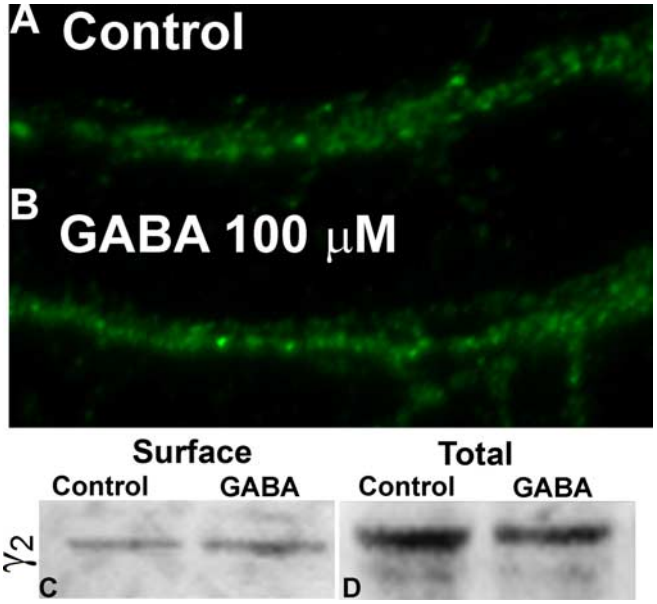

Figure 8. Surface expression of the GABAR γ2 subunit was unchanged after incubation in standard control external medium supplemented with GABA 100 μM. ***A***, ***B***, Images of representative neuronal processes of cultured pyramidal neurons incubated for 30 min in the standard, control external medium (***A***) or the standard, control external medium supplemented with GABA 100 μM (***B***) before fixation and antibody tagging of the GABAR γ2 subunit (green). ***C***, ***D***, Sample Western blots of surface (***C***) and total (***D***) protein fractions of the GABAR γ2 subunit in organotypic cultures incubated for 1 h in either a standard external aCSF (control) or the standard aCSF supplemented with GABA.

duced in the organotypic cultures when a high external concentration of KCl was supplemented with NMDA. We suspect that supplemental NMDA was required in the organotypic cultures as 10 mM KCl may be insufficient to induce a significant change in glutamate release in these cultures (Takahashi and Hashimoto, 1996; Muzzolini et al., 1997, Oldenziel et al., 2007) and because of the preserved connectivity in this *in vitro* model compared with the dissociated cultures. These findings are consistent with a ligand-independent process. Although NMDA receptor activation was necessary to induce a reduction in the surface expression of the γ2 subunit in the organotypic cultures, NMDA receptor activation was not sufficient to induce a statistically significant decrease in the surface expression of the γ2 subunits. This suggests that other changes in neuronal excitability induced by the high external concentration of potassium were required for the reduction in the surface expression of this subunit.

In contrast, when dissociated cultures and organotypic cultures were incubated in excessive extracellular GABA, the surface expression of the γ2 subunit was unchanged. Studies in which the organotypic cultures were exposed to either excessive extracellular GABA concentration with GABA uptake blocked or to the high affinity GABAR agonist muscimol also demonstrated that modulation of the surface expression of the γ2 subunit was not dependent on direct ligand binding of the receptor. Although previous studies have demonstrated a ligand-dependent mechanism for the regulation of the surface expression of GABARs, these studies (Tehrani and Barnes, 1991; Calkin and Barnes, 1994) used longer incubation periods and higher concentrations of GABA than that used in the present study.

Other potential modifications during SE

Our findings demonstrate that a rapid modification in the complement of GABARs during SE results from a selective decrease in the surface expression of GABARs containing a β2/3 and γ2 subunit; however, other potential mechanisms may also contribute to the reduction in GABA-mediated inhibition during SE. For example, we found a reduction in mIPSC frequency and an increase in 10–90% rise time. These findings support presynaptic changes in GABA-mediated inhibition resulting from either a modification of presynaptic mechanisms that regulate neurotransmitter release during SE similar to that observed in models of chronic temporal lobe epilepsy (Hirsch et al., 1999) or from the loss of proximal inhibitory inputs to the DGC. Studies evaluating the relative contribution of presynaptic changes to the reduced GABA-mediated inhibition are required.

In summary, these *in vitro* and *in vivo* findings are consistent with the differential modulation of the surface expression of GABARs during the prolonged seizures of SE that is independent of ligand binding to the receptor. The net result of this differential modulation is that the surface expression of the benzodiazepine-sensitive γ2-containing GABARs was reduced, but the expression of the benzodiazepine-insensitive δ-containing GABARs was unchanged. These findings confirm and extend our findings (Goodkin et al., 2005) and those of others (Naylor et al., 2005) regarding the trafficking of GABARs during SE, and uniquely demonstrate the subunit-specificity of this trafficking. These findings have direct implications for the treatment of SE. Future experiments are required to better define the cellular mechanism that results in the subunit-specific reduction in the surface expression of γ2-containing GABARs during SE.

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