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Receptors with low affinity for neurosteroids and GABA contribute to tonic inhibition of granule cells in epileptic animals

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Abstract

Neurosteroid sensitivity of GABA_A receptor mediated inhibition of the hippocampal dentate granule cells (DGCs) is reduced in animal models of temporal lobe epilepsy. However, the properties and subunit composition of GABA_A receptors mediating tonic inhibition in DGCS of epileptic animals have not been described. In the DGCs of epileptic animals, allopregnanolone and L-65708 sensitivity of holding current was diminished and δ subunit was retained in the endoplasmic reticulum and its surface expression was decreased in the hippocampus. Ro15–4513 and lanthanum had distinct effects on holding current recorded from DGCs of control and epileptic animals. The pharmacological properties of GABA_A receptors maintaining tonic inhibition in DGCs of epileptic animals were similar to those containing the $\alpha 4\beta\gamma 2$ subunits. Furthermore, surface expression of the $\alpha 4$ subunit increased and a larger fraction of the subunit was co-immunoprecipitated with the $\gamma 2$ subunit in hippocampi of epileptic animals. Together these studies revealed that functional $\alpha 4\beta\gamma\delta$ and $\alpha 5\beta\gamma 2$ receptors were reduced in the hippocampi of epileptic animals, and that novel $\alpha 4\beta\gamma 2$ receptors contributed to the maintenance of tonic inhibition. The presence of $\alpha 4\beta\gamma 2$ receptors resulted in low GABA affinity and neurosteroid sensitivity of tonic currents in the DGCs of epileptic animals that could potentially increase seizure vulnerability. These receptors may represent a novel therapeutic target for anticonvulsant drugs without sedative actions.

Introduction

Temporal lobe epilepsy (TLE) is a common form of epilepsy and nearly a third of patients may be refractory to anticonvulsants. An array of pathological changes in neuronal circuits occur in the hippocampal dentate gyrus of experimental animals with TLE including, sprouting of mossy fibers and loss and dysfunction of GABAergic interneurons (Dudek and Sutula, 2007; Sutula and Dudek, 2007; Obenaus et al., 1993; Kobayashi and Buckmaster, 2003; Sun et al., 2007a; Zhang and Buckmaster, 2009). These changes render the hippocampus susceptible to generating and propagating seizures. However, there are endogenous molecules such as neurosteroids that can suppress seizures. Neurosteroids are molecules derived from circulating steroids and synthesized de novo from cholesterol. Neurosteroids modulate GABA_A receptors

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and have an anticonvulsant action (Majewska et al., 1986; Kokate et al., 1994; Reddy et al., 2004; Lawrence et al., 2010).

There is growing evidence that neurosteroid modulation of GABA_A receptors on hippocampal dentate granule cells (DGCs) is diminished in TLE (Mtchedlishvili et al., 2001; Peng et al., 2004; Zhang et al., 2007; Sun et al., 2007b). GABA_A receptors are pentamers composed of subunits derived from α , β , γ , δ , ϵ , and π gene families, and the majority of receptors are composed of 2 α , 2 β , and a γ or δ subunit (Sieghart and Sperk, 2002). Receptors containing the δ subunit are particularly sensitive to neurosteroids (Mihalek et al., 1999; Wohlfarth et al., 2002; Belelli et al., 2006). In DGCs, GABA_A receptors containing the $\alpha 4$ and δ subunit are targeted to the extrasynaptic membrane, where they contribute to neurosteroid-sensitive leak conductance often referred to as tonic inhibition (Wei et al., 2003; Stell and Mody, 2002; Sun et al., 2004; Mtchedlishvili and Kapur, 2006; Herd et al., 2007). In TLE, there is diminished expression of δ subunit mRNA and polypeptide in DGCs with associated diminished neurosteroid sensitivity of tonic currents (Zhang et al., 2007; Schwarzer et al., 1997a; Nishimura et al., 2005; Peng et al., 2004).

Previous studies investigating the neurosteroid modulation of tonic inhibition have not addressed several issues. Despite loss of neurosteroid sensitivity and reduced expression of the δ subunit, tonic inhibition of DGCs appears to be preserved in animals with TLE however; the identity and properties of these GABA_A receptors have not been described. Studies in δ subunit knockout animals demonstrate a residual tonic current suggesting that GABA_A receptors containing subunits other than the δ subunit can maintain tonic inhibition (Stell et al., 2003; Wei et al., 2004). Recent studies indicate that GABA_A receptors containing the $\alpha 5$ subunit maintain residual tonic currents in DGCs (Glykys et al., 2008), suggesting the possibility that upregulation of $\alpha 5$ subunit-containing receptors can maintain tonic inhibition in DGCs of epileptic animals (Zhan and Nadler, 2009). Alternately, a previous study proposed that tonic inhibition of epileptic DGCs could be maintained by GABA_A receptors containing the $\alpha 4$ and $\gamma 2$ subunits (Zhang et al., 2007). These possibilities have not been tested directly, and assembly of the $\gamma 2$ subunit with the $\alpha 4$ subunit remains untested.

The current study demonstrates that receptors containing the $\alpha 4$ and $\gamma 2$ subunit contribute to the maintenance of tonic inhibition of DGCs in epileptic animals. These receptors have a lower affinity for GABA and neurosteroids, which is likely to increase vulnerability to seizures.

Materials and Methods

Induction of TLE

All experimental procedures were performed on adult male Sprague-Dawley rats in accordance with the protocol approved by the University of Virginia Animal Use and Care Committee. TLE was induced in rats using the continuous hippocampal stimulation (CHS) protocol as described previously (Lothman et al., 1989). The rats were anesthetized with ketamine (50 mg/kg) and xylazine (40 mg/kg) and implanted with a pair of bipolar stimulating electrodes in the left posterior ventral hippocampus (AP 3.6, ML 4.0, DV 5.0 from dura; incisor bar at +5.0). After 1 week of recovery, the left hippocampus was stimulated with 10s trains of 50 Hz, 1 ms, 400 mA biphasic square wave current pulses delivered every 13 s for 90 min to induce status epilepticus (SE) (Lothman et al., 1990). Approximately 4–6 weeks after stimulation, the rats developed spontaneous limbic seizures with a motor component. For this study, seizures were documented by either continuous EEG recording or by direct observation of the spontaneous seizure (Bertram et al., 1997). The epileptic animals were sacrificed at least 24 hours after the last seizure. All animals were housed individually under conditions of a 12h/12h light/dark cycle and had free access to food and water. Age-matched adult male Sprague-Dawley rats (150–200 g) were used as controls.

Electrophysiology

Animals were anesthetized with isoflurane and decapitated. The brain was dissected free and immersed in cold (2–4°C) ACSF composed of (in mM) 65.5 NaCl, 2 KCl, 5 MgSO₄, 1.1 KH₂PO₄, 25 NaHCO₃, 10 dextrose, 113 sucrose, and 1 CaCl₂ (osmolarity 300 mOsm) saturated with 95% O₂-5% CO₂. Brains were mounted on a vibratome stage (Camden Instruments, UK) and 300 µm thick horizontal sections containing the right ventral hippocampus were cut. Slices were maintained in continuously oxygenated ACSF containing (in mM) 127 NaCl, 2 KCl, 1.5 MgSO₄, 25.7 NaHCO₃, 10 Dextrose, 1.5 CaCl₂ (pH 7.4; 300 mOsm) at 32°C in a holding chamber for 30–45 min and then at room temperature in a recording chamber mounted on the stage of an Olympus BX51 microscope equipped with a 40× water-immersion objective, IR-DIC optics, and video. Patch electrodes (final resistances 4–6 MΩ) were pulled from borosilicate glass (Sutter Instruments, Novato, CA) on a horizontal Flaming-Brown microelectrode puller (model P-97, Sutter Instruments), using a 2-stage pull protocol. Electrode tips were filled with a filtered internal recording solution consisting of (in mM): CsCl 153.3, MgCl₂ 1.0, N-[2-Hydroxyethyl] piperazine-N'-[2-ethansulfonic acid] (HEPES) 10.0, and glycol-bis (α-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 5.0, pH 7.2 (with CsOH), osmolarity was 285–295 mOsm. The electrode shank contained 3 mM ATP Mg²⁺ salt. GABA_A receptor mediated currents were recorded from DGCs voltage-clamped to –65 mV with an Axopatch 200B amplifier (Molecular Devices, CA) in the presence of 50 µM DL-2-Amino-5-phosphonopentanoic acid (DL-AP5) and 20 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris-Cookson Ellisville, MO). Whole cell capacitance and series resistance were compensated by 80% at a 7 µs lag. Recordings were performed when series resistance after compensation was less than 20 MΩ. Access resistance was frequently monitored with a 10 ms –5 mV test pulse. If the series resistance increased by 25% at any time during the experiment, the recording was terminated. Currents were filtered at 5 kHz, digitized at 10 kHz using a Digidata 1440A digitizer, and acquired using Axoscope 10.2 software (Molecular Devices, CA) on an IBM PC-compatible computer hard drive.

Determination of tonic inhibition

To assess the potential alterations in tonic inhibition, we evaluated either the shift in baseline holding current (I_{hold}) (Mtchedlishvili et al., 2006; Rajasekaran et al., 2009), or changes in its root mean square amplitude of noise (RMS) (Mtchedlishvili et al., 2006) following application of antagonists or agonists. The GABA_A receptor mediated component of the holding was confirmed by applying bicuculline and demonstrating a reduction in holding current. To determine the shifts in I_{hold} (I_{Δ}), the digitized current traces were analyzed with Clampfit 10.2 (Molecular Devices, CA). The mean I_{hold} for each recording was determined by measuring the mean current (I_{avg}) sampled every 50 ms at 500 ms intervals. Fifty such data points were collected immediately before drug application and at least 10 min after drug application to allow steady state response after drug equilibration. To minimize the contribution of synaptically present receptors to our measures of I_{hold} , epochs containing synaptic events that could be visually identified or were larger than the RMS noise were eliminated from the analysis. Epochs having an unstable baseline were also eliminated from the analysis. The drug effects on individual neurons were assessed by comparing the distribution of the mean holding current before and after drug application by means of a Kolmogorov-Smirnov (KS) test (available online at http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html). The changes in I_{hold} between the experimental groups were not normalized to cell capacitance since we did not find any difference in whole cell capacitance between control and epileptic DGCs (data not shown). The difference in the mean I_{hold} after drug application relative to baseline was calculated and I_{Δ} in the two experimental groups were then compared using an unpaired t test.

RMS noise (I_{rms}) was defined by the equation

$$I_{rms} = \left[\frac{(I_{avg} - I_1)_2 + (I_{avg} - I_2)_2 + \dots + (I_{avg} - I_n)_2}{n} \right]^{1/2}$$

where I_{avg} is the average current amplitude, I_n is the amplitude of an individual point and n is the number of measurements in an epoch. Each epoch was 50 ms in duration and contained 2500 amplitude measurements. The time interval between 2 epochs was 500 ms. Sixty epochs were analyzed for each experimental condition (60 control and 60 after the drug application in each cell). In order to assess the effect of a drug on I_{rms} in individual neurons, the distribution of I_{rms} in epochs before application of the drug (during the baseline period) was compared to that following drug application by means of a KS test. In order to compare data obtained from a group of neurons, I_{rms} values in individual epochs before and after drug application were averaged. Mean I_{rms} values were compared using unpaired t-test.

Since differences in the washout of slices or pathophysiological changes leading to altered GABA synthesis, release, or clearance could potentially alter ambient GABA concentrations and confound experimental determination of changes in tonic currents (Glykys and Mody, 2007), most experiments were performed in the presence of 10 μ M of the GABA uptake inhibitor, NO-711 (Sigma, St. Louis, MO), and 3 μ M GABA (Sigma, St. Louis, MO). However, in experiments designed to measure RMS noise, the uptake blocker or GABA was not included in the ACSF.

A total of 40 epileptic animals and 30 age-matched controls were used for electrophysiological experiments. The age matched control group included animals that were implanted with electrodes but not stimulated and animals that were stimulated but did not develop acute status epilepticus since the physiological properties tested were similar to those of naïve control animals.

Biotinylation and western blotting

Whole hippocampus was used for all biochemical studies. Since stimulation and recording electrodes were implanted in the left ventral hippocampus, the tissue used for analysis consisted of the right hippocampus. Surface expression of GABA_A receptor subunits was studied by biotinylation (Goodkin et al., 2008). The brain slices (300 μ m thickness) were prepared as described above and incubated in oxygenated ACSF at 28°C for 1 hr. Surface proteins were biotinylated by incubating the slices in ice-cold ACSF containing 1 mg/ml sulfo-NHS-LC-biotin (Pierce Biotechnology, Rockford, IL) for 30 min at 4°C with gentle shaking. Unbound biotin was removed by washing the slices twice in TBS (25 mM Tris pH 7.4, 137 mM NaCl, 5 mM KCl, 2.3 mM CaCl₂, and 0.5 mM MgCl₂). Tissue was lysed in ice-cold RIPA lysis buffer containing 100 μ M sodium orthovanadate and protease inhibitor cocktail set I (Calbiochem, San Diego, CA), and sonicated using a Branson Sonifier (5 pulses, output control 5, duty cycle 30%). The lysates were cleared by centrifugation at 14,000 $\times$ g for 15 min. Biotinylated proteins were purified by incubating lysates corresponding to 500 μ g protein with 100 μ l neutravidin-agarose beads (Pierce Biotechnology, Rockford, IL) overnight at 4°C, followed by extensive washing of the beads with RIPA-lysis buffer. The pulled-down proteins were eluted in non-reducing sample buffer, denatured at 95°C for 5 min, and separated on 10% SDS-PAGE gels. Expression of the δ and $\alpha 4$ subunits was detected by standard western blotting procedure using rabbit polyclonal anti- δ (1:1000 dilution) and anti- $\alpha 4$ (1:500 dilution) subunit antibodies (kind gift from Dr. Sieghart, Medical University, Vienna, Austria), directed against the N-terminal extracellular epitope of respective protein and which have been previously characterized for their specificity (Sperk et al, 1997). Expression of the δ and $\alpha 4$ subunits was also studied in 30 μ g total protein for normalization. Blots were reprobed with the cytoplasmic protein β -actin

(1:5000 dilution, Sigma, St. Louis, MO) to confirm specificity of the biotinylation reaction. Signal obtained in the western blots was quantified by scanning densitometry and the ratio of surface/total expression of the δ or $\alpha 4$ subunits was calculated. Total expression of the protein was normalized with β -actin expression. A total of 6 epileptic and age-matched control animals were used for the biochemical studies.

Isolation of microsomes

Whole hippocampus was used to isolate microsomal membranes and tissue from one animal represents single replicate. Hippocampal tissue was dissected out in ice-cold oxygenated ACSF and homogenized in homogenization buffer (0.32M sucrose, 10 mM HEPES-NaOH pH 7.2) containing protease inhibitor cocktail set I (Calbiochem, San Diego, CA) in a glass douncer with 10 strokes. All the steps were carried out at 4°C. Homogenates were first cleared twice at $1000 \times g$ for 10 min and then at $9000 \times g$ for 15 min. The resulting supernatant (S2) was centrifuged at $200,000 \times g$ for 1 h to pellet out total microsomal fraction. The microsomal fractions were suspended in a non-reducing sample buffer, denatured at 95°C for 5 min, and separated on 9% SDS-PAGE gels. Presence of the δ subunit was detected by Western blotting as described above. Expression of the endoplasmic reticulum (ER) marker proteins calnexin and Glucose responsive protein (Grp78/BIP) was also studied by Western blotting. Four pairs of epileptic and control animals were used.

Co-immunoprecipitation

Whole hippocampus was used in immunoprecipitation studies and each animal represents a single replicate. Six pairs of epileptic and control animals were used to study co-immunoprecipitation between the $\alpha 4$ or $\alpha 1$ and $\gamma 2$ subunits. Co-immunoprecipitation of the $\alpha 4$ and $\gamma 2$ subunit was performed in the whole hippocampus using the ProFound mammalian co-immunoprecipitation kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Anti-GABA_A receptor $\gamma 2$ subunit antibody (50 μ g, Alpha Diagnostics, San Antonio, TX, directed against the N-terminal extracellular domain) was used to pull-down the $\gamma 2$ subunit and associated proteins. The expression of the $\alpha 4$, $\alpha 1$, and $\gamma 2$ subunits was studied using standard SDS-PAGE and Western blotting procedures. The intensity of the $\alpha 4$ and $\gamma 2$ signals in pull-down and total protein was determined by densitometric scanning and the pull-down signal was normalized with total expression of the respective subunit. A ratio of normalized $\alpha 4$ to $\gamma 2$ was obtained and compared between control and epileptic animals. Association of the $\alpha 4$ subunit with the $\gamma 2$ subunit was expressed as percent fraction of that in naïve animals. As a negative control co-immunoprecipitation between the δ and $\gamma 2$ subunits which are mutually exclusive *in vivo* (Arajuo et al, 1998) was also studied.

Results

Neurosteroid modulation of GABA mediated tonic currents is diminished in TLE

We investigated the role of allopregnanolone modulation of tonic currents in epileptic DGCs. Physiological GABA_A receptor modulating concentrations (30 and 60 nM) of allopregnanolone were bath-applied in separate experiments to control and epileptic DGCs. Modulation of I_{hold} was studied with an elevated extracellular GABA concentration obtained by blocking GABA uptake and including 3 μ M GABA in the ACSF. Allopregnanolone (30 nM) increased I_{hold} in control but not epileptic DGCs (Fig 1A). In all control DGCs, the mean I_{hold} during 50 epochs after drug application was larger than that at baseline ($p < 0.05$, KS test, $n = 8$ cells / 4 animals). In contrast, drug application did not change ($p > 0.05$, KS test) the mean I_{hold} in 5 out of 7 epileptic DGCs ($n = 5$ animals). Allopregnanolone caused a smaller change in I_{Δ} in epileptic DGCs (0.10 ± 0.9 pA) than in control DGCs (6.61 ± 2.0 pA; $p = 0.01$, unpaired t test).

A higher concentration of allopregnanolone (60 nM) robustly enhanced I_{hold} in control DGCs but had minimal effect in epileptic DGCs (Fig 1B). In all control DGCs ($n=6$ cells / 3 animals) 60 nM allopregnanolone increased I_{hold} ($p < 0.05$, KS test), whereas in epileptic DGCs ($n = 6$ cells / 4 animals), it caused minimal (4 / 6 cells, $p < 0.05$, KS test) or no change in I_{hold} (2 / 6 cells, $p > 0.05$, KS test). The mean population I_{Δ} in epileptic DGCs (3.18 ± 1.6 pA) was significantly less than that in control DGCs (25.60 ± 8.3 pA; $p = 0.02$, unpaired t-test).

Role of the δ subunit

GABA_A receptors containing the δ subunit are particularly sensitive to neurosteroids. Based on our initial findings with allopregnanolone, we tested whether the expression of this subunit was diminished in the hippocampi of epileptic animals by Western blot technique. There expression of the δ subunit was reduced in the membranes prepared from the hippocampi of epileptic animals (Fig. 2A). The expression of β -actin was similar between control and epileptic animals confirming equal protein loading. The δ subunit band was normalized to the β -actin band and the δ subunit protein level in the hippocampi of epileptic animals was $80 \pm 11\%$ of that in the hippocampi of controls ($n = 7$, $p < 0.001$, t-test). Loss of principal neurons in epileptic animals may have contributed to decreased expression of the δ subunit. In addition, the diminution of total δ subunit expression was modest in comparison to loss of allopregnanolone sensitivity observed in the physiological studies. Furthermore, the δ subunit immunoreactivity in tissue lysates is likely to detect intracellular polypeptides besides that on surface, which are physiologically active.

We therefore tested whether δ subunit surface expression was reduced in epileptic animals. In biotin-labeled hippocampal membrane proteins obtained from TLE animals, the δ protein signal was weaker than that in the membrane proteins from control animals (Fig. 2 A). To quantify surface expression of the δ subunit, expression of the δ subunit was determined by scanning densitometry. Intensity of the surface signal was 19486 ± 2934 units in control rats and 6191 ± 532 units in epileptic rats ($n=5$) and expressed as a fraction of total δ expression. In controls, the ratio of surface to total expression was 0.34 ± 0.05 ($n=5$), whereas in epileptic animals it was 0.13 ± 0.01 ($n=5$). Thus the surface expression of the δ subunit in epileptic animals was 60% less than that in controls ($p < 0.01$, t-test).

The extent of reduction in the surface fraction of the δ subunit was higher than the decline in the total δ subunit expression, suggesting altered trafficking of the receptor. Defects in trafficking of receptor subunits to the cell surface could lead to their accumulation in the microsomal fraction, which contains the ER, Golgi apparatus, and endosomes. Expression of the δ subunit in the microsomal fraction isolated from hippocampi of control and epileptic animals was compared; and it was more in epileptic animals than that in controls (Fig 2B). Isolation of the microsomal fraction was confirmed by the expression of the ER marker proteins, calnexin and Grp78/BIP, which were similar in the hippocampi of epileptic and control animals indicating equal loading of proteins. The δ subunit in microsomal fraction isolated from control animals was 129214 ± 82768 units ($n=4$) less than that in epileptic animals (244227 ± 175212 units; $n=4$). The δ subunit expression in microsomal fraction was normalized to total δ protein. This was $65 \pm 8\%$ higher in epileptic animals (0.89 ± 0.07 , $n=4$) than in controls (0.54 ± 0.05 , $n=4$, $p < 0.05$, t-test). The microsomal δ subunit expression was also normalized to the ER marker protein calnexin, which revealed a ratio 0.7 ± 0.2 in control and 1.2 ± 0.3 in epileptic animals, demonstrating a $75 \pm 27\%$ higher expression in the epileptic animals ($n=4$, $p=0.05$). Taken together, these studies demonstrated altered trafficking of the δ subunit in epilepsy.

GABA_A receptors containing the $\alpha 5$ subunit were not responsible for modulating tonic currents in epileptic DGCs.

We then compared bicuculline sensitivity of I_{hold} in control and epileptic DGCs. Bicuculline was bath-applied after 5 minutes of baseline recording, which caused a slow outward current, accompanied by loss of spontaneous inhibitory post synaptic currents. Bicuculline reduced the mean I_{hold} in each control ($n = 9$ cells / 5 animals, $p < 0.05$, KS test) and epileptic DGC tested ($n = 6$ cells / 5 animals, $p < 0.05$, KS test, Fig 3a). The mean I_{Δ} in epileptic DGCs (50.86 ± 7.80 pA) was similar to that in control DGCs (46.59 ± 5.1 pA, $p = 0.33$, unpaired t test, Fig 3b). Another competitive antagonist, SR 95531 (gabazine), similarly reduced the mean I_{hold} in both control and epileptic DGCs (data not shown).

To account for potential changes in the basal level of extracellular GABA and uptake mechanisms between control and epileptic DGCs, we compared bicuculline induced shift in I_{hold} in the absence of exogenous GABA and NO-711. In these conditions also, bicuculline induced a similar shift ($p > 0.05$) in the Gaussian mean of all-point histogram in both control (214.3 ± 11.3 , $n = 5$ cells / 3 animals) and epileptic DGCs (217.8 ± 30.2 pA, $n = 5$ cells / 3 animals, Fig 3c). Bicuculline also caused similar reductions in I_{rms} in epileptic DGCs and control DGCs ($p > 0.05$). In epileptic DGCs, it caused an I_{rms} shift from 4.21 ± 0.21 pA to 2.67 ± 0.07 pA ($n = 5$, $p < 0.0001$, KS test) and in control DGCs, it shifted I_{rms} from 7.449 ± 0.08 pA to 6.083 ± 0.03 pA ($n = 5$, $D = 0.69$, $p < 0.0001$, KS test). These findings are consistent with those reported in DGCs in epileptic mice and in CA1 pyramidal neurons of rats with TLE (Zhang et al., 2007; Scimemi et al., 2005).

Although tonic inhibition in hippocampal DGCs is mainly mediated by the $\alpha 4\delta$ subunit-containing GABA_A receptors, it can also be maintained by the $\alpha 5\gamma 2$ subunit-containing GABA_A receptors (Caraiscos et al., 2004; Glykys and Mody, 2008). Modulation of tonic currents in epileptic DGCs despite reduced surface expression of the δ subunit suggested that $\alpha 5\gamma 2$ subunit-containing GABA_A receptors may contribute to maintenance of tonic currents in these neurons.

We therefore compared the modulation of I_{hold} using the $\alpha 5$ subunit-specific benzodiazepine site inverse agonist, L-655,708 (Caraiscos et al., 2004; Scimemi et al., 2005). L-655,708 inhibited the I_{hold} in each control DGC ($n = 6$ cells / 5 animals; $p < 0.05$, KS test) but it did not do so in 4 out of 6 epileptic DGCs ($n = 5$ animals, $p > 0.05$, KS test, Fig 4). In data pooled from all cells, L-655,708 caused a larger I_{Δ} in control (11.2 ± 3.6 pA) compared to epileptic DGCs (0.38 ± 3.7 pA; $p = 0.04$, unpaired t test). Among the 2 epileptic DGCs in which L-655,708 altered the mean I_{hold} , the response in 1 epileptic DGC (mean $I_{\Delta} = 14.4$ pA) was similar to that observed in control DGC; however the response of the other cell was lower (mean $I_{\Delta} = 5.6$ pA) compared to that of control values. These results suggested diminished contribution of $\alpha 5$ subunit-containing GABA_A receptors to the maintenance of tonic currents in the epileptic DGCs.

Diminished modulation of tonic currents by drugs acting on putative $\alpha 4\delta$ - and the $\alpha 5\gamma 2$ -containing GABA_A receptors in epileptic DGCs suggested that in these neurons, tonic currents were maintained by GABA_A receptors containing novel subunit composition(s). We have previously demonstrated using electron microscopic studies that the $\alpha 4$ subunit expression increased in epileptic DGCs and with a specific increase in localization at synapses (Sun et al., 2007b). Using similar techniques, it has also been demonstrated in a mouse model of TLE that there is an increased localization of the $\gamma 2$ subunit of the GABA_A receptor in peri- and extra-synaptic sites (Zhang et al., 2007). Thus, GABA_A receptors containing the $\alpha 4\gamma 2$ subunits may potentially contribute to maintaining tonic currents in epileptic DGCs (Zhang et al., 2007).

Properties of GABA_A receptors contributing to tonic currents in epileptic DGCs

To test whether GABA_A receptors in epileptic DGCs have properties similar to those containing $\alpha 4\gamma 2$ subunits, we tested the effect of lanthanum (LaCl_3) and Ro15-4513 on I_{hold}

in control and epileptic DGCs. These drugs have distinct effects on GABA currents in recombinant receptors containing the $\alpha 4\delta$ or $\alpha 4\gamma 2$ subunits (Brown et al., 2002).

Lanthanum (LaCl_3) potently inhibits GABA_A receptor currents mediated by recombinant receptors containing the $\alpha 4\delta$ subunit, but has minimal effects on $\alpha 4\gamma 2$ subunit-containing receptors (Brown et al., 2002). LaCl_3 significantly decreased the mean I_{hold} in all control DGCs ($n = 10$ cells / 5 animals, $p < 0.05$, KS test Fig 5A)). In contrast, in epileptic DGCs, LaCl_3 modestly decreased (3 / 7 cells, $p < 0.05$, KS test) or had no effect (4 / 7 cells, $p > 0.05$, KS test) on the mean I_{hold} ($n = 5$ animals). The mean population I_{Δ} in control DGCs (12.60 ± 2.9 pA) was significantly different from that obtained in epileptic DGCs (0.95 ± 6.3 pA, $p = 0.047$, unpaired t-test). These data suggest a reduction of GABA_A receptors with pharmacological properties of the $\alpha 4\delta$ subunit-containing assembly, and the presence of GABA_A receptors with pharmacological properties similar to those that contain $\alpha 4\gamma 2$ subunits.

To further confirm the presence of putative $\alpha 4\gamma 2$ subunit-containing GABA_A receptors in epileptic DGCs, we tested the effect of the imidazobenzodiazepine, Ro15-4513 (ethyl-8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a] [1,4]benzodiazepine-3-carboxylate). Ro15-4513 binds to GABA_A receptors containing $\alpha 4\gamma 2$ subunit combinations, but not to the $\alpha 4\delta$ -containing receptors (Scholze et al., 1996). Application of Ro15-4513 (300 nM) did not significantly change mean I_{hold} in control DGCs ($n = 6$ cells / 3 animals, Fig 5B), but in 9 out of 10 epileptic DGCs ($n = 8$ animals, $p < 0.05$, KS test) it reduced the mean I_{hold} . The I_{Δ} in control DGCs much less than in epileptic DGCs (0.95 ± 6.3 pA vs 20.26 ± 5.9 pA, $p < 0.05$, unpaired t-test). Together, these data indicate that GABA_A receptors with pharmacological properties similar to that of recombinant receptors containing the $\alpha 4\gamma 2$ subunits contribute to maintenance of tonic currents in epileptic DGCs.

GABA affinity of receptors mediating tonic currents

Studies of recombinant GABA_A receptors containing $\alpha 4\gamma 2$ subunits suggest that these receptors have lower affinity for GABA than the $\alpha 4\delta$ subunit-containing receptors. We therefore probed the availability of high affinity GABA_A receptors on DGCs by directly applying a low concentration (1 μM) GABA and measuring I_{hold} and I_{rms} before and after drug application.

Application of 1 μM GABA revealed loss of high affinity GABA_A receptors on epileptic DGCs. In epileptic DGCs, the mean I_{hold} was not altered by 1 μM GABA (Fig 6A, B); it was 68.3 ± 0.14 pA at baseline, and 67.3 ± 0.13 pA after application of 1 μM GABA ($n = 6$ cells, $p > 0.05$, KS test). In contrast, in control DGCs, GABA increased the mean I_{hold} from 48.3 ± 0.14 pA to 53.9 ± 0.18 pA ($n = 6$ cells, $p < 0.05$, KS test). These findings suggested that 1 μM GABA increased tonic GABAergic current in control but not in epileptic DGCs.

To further confirm the absence of high affinity receptors in epileptic DGCs, we measured the changes in the slope conductance of GABA-activated channels. DGCs were voltage clamped to -65 , -35 , and -15 mV, and the mean RMS noise was determined before and after bath application of 1 μM GABA. We found differences in the absolute levels of I_{rms} as well as changes in the slope conductance between recordings obtained from control and epileptic DGCs (Fig 6C). Slope conductance was calculated from a linear regression of mean I_{rms} against the holding potential. In epileptic DGCs ($n = 5$), 1 μM GABA did not change the slope conductance or the elevations of line intercepts (Fig 6C). By contrast, in control DGCs ($n = 7$), application of 1 μM GABA changed the slope conductance, as well as the elevations of the line intercepts (Fig 6C).

The changes in the absolute baseline I_{rms} between control and epileptic DGCs suggested potential alterations in uptake mechanisms that may result in elevated extracellular GABA

levels in epileptic DGCs. Elevated extracellular GABA in turn could blunt the effects of low concentration of GABA in epileptic DGCs. In order to address this concern, we performed experiments with penicillin, the noncompetitive (Macdonald and Barker, 1977), open channel (Twyman and Macdonald, 1992) GABA_A receptor blocker. We have previously shown that penicillin can detect tonically open GABA_A receptors (Mtchedlishvili et al., 2006). If higher baseline I_{rms} in epileptic DGCs were due to more open GABA_A receptors than in control DGCs, they would be detected by penicillin. In control DGCs ($n = 6$ cells / 3 animals, Fig. 7A), 300 μ M penicillin inhibited I_{rms} in each cell tested (KS test). The mean I_{rms} was 4.845 ± 0.7 pA before and 3.899 ± 0.5 pA after application of 300 μ M penicillin ($p < 0.05$, Fig 7C). In contrast, penicillin application did not alter the mean I_{rms} in any epileptic DGCs ($n = 5$ cells / 3 animals; $p > 0.05$, KS test; Fig 7B). The mean I_{rms} was 3.680 ± 0.02 pA before and 3.805 ± 0.02 pA after application of penicillin (Fig. 7D) suggesting loss of high affinity GABA_A receptors on epileptic DGCs. Together, these data confirmed the loss of high affinity GABA_A receptors on epileptic DGCs.

Biochemical evidence for the $\alpha 4\gamma 2$ subunit-containing receptors

The pharmacological studies indicated that GABA_A receptors containing the $\alpha 4$ subunit contributed to maintenance of tonic currents in epileptic DGCs. The $\alpha 4$ subunit commonly associates with the δ subunit and diminished expression of the δ subunit in epileptic animals raised the possibility that the surface expression of the $\alpha 4$ subunit would also be diminished. We therefore studied the surface expression of the $\alpha 4$ subunit in the hippocampi of epileptic animals. Interestingly, in the epileptic animals, surface expression of the $\alpha 4$ subunit was higher than that of controls (Fig 8A). The intensity of surface signal for the $\alpha 4$ subunit in control animals was 29182 ± 11741 and that in epileptic animals was 39068 ± 15745 ($n=6$). The ratio of surface to total expression of the $\alpha 4$ subunit was 0.72 ± 0.13 ($n=6$) in control animals, and in epileptic animals, it was 1.13 ± 0.36 ($n=6$, an increase of $42 \pm 12\%$ $p < 0.05$, t-test). Thus, surface expression of the $\alpha 4$ subunit was increased, whereas that of the δ subunit was diminished in epileptic animals. This finding suggested that in the hippocampi of epileptic animals, the $\alpha 4$ subunit may potentially assemble with the $\gamma 2$ subunit.

To confirm presence of GABA_A receptors containing the $\alpha 4\gamma 2$ subunits in epileptic animals, we performed co-immunoprecipitation assays. The amount of $\alpha 4$ subunit associated with the $\gamma 2$ subunit was compared in hippocampi obtained from naïve and epileptic animals. In control animals, a weak signal for the $\alpha 4$ subunit was observed in the $\gamma 2$ pull-down samples suggesting minor association of the $\alpha 4$ and $\gamma 2$ subunits, a finding consistent with previous observations (Bencsits et al., 1999; Sur et al., 1999). In contrast, the signal for $\alpha 4$ subunit in proteins pulled-down along with the $\gamma 2$ subunit from epileptic animals was stronger (Fig 8B). The amount of $\alpha 1$ subunit coimmunoprecipitating with the $\gamma 2$ was similar in control and epileptic animals. Normal rabbit IgG failed to pull down proteins from tissue lysates, suggesting specific pull-down of the $\gamma 2$ subunit and associated proteins with the anti- $\gamma 2$ antibody used. Furthermore, association between the δ and the $\gamma 2$ subunit (that are mutually exclusive *in vivo*) was also tested in pull-down samples to confirm that interaction between $\alpha 4$ and $\gamma 2$ subunits represented an *in situ* interaction and not a processing artifact. In the 4 pairs of epileptic and control animals studied, expression of the δ subunit was not observed in proteins precipitated using anti $\gamma 2$ subunit antibody. A representative Western blot shows absence of the δ subunit signal in immunoprecipitated samples, whereas expression of the δ subunit was observed in total proteins, lower in epileptic animals than in naïve animals as described above (Fig 8B). The amount of $\alpha 4$ and $\gamma 2$ subunit expression was quantified using scanning densitometry in the pull-down and total protein and expressed as a ratio of the $\alpha 4$ subunit associated with the $\gamma 2$ subunit. The ratio of the $\alpha 4$ - $\gamma 2$ co-precipitation was 0.55 ± 0.02 in the control animals and 1.12 ± 0.24 in epileptic animals. Compared to naïve animals, the $\alpha 4$ and $\gamma 2$ co-precipitation in

epileptic animals was $199 \pm 33\%$ ($n=6$, $p<0.05$, t-test) more, demonstrating a 2 fold increased association between the $\alpha 4$ and $\gamma 2$ subunits.

Discussion

The major findings of this study were: 1) neurosteroid sensitivity of GABA_A receptors mediating tonic currents was diminished in epileptic DGCs; 2) surface expression of the δ subunit of GABA_A receptors was diminished and there was greater intracellular retention of the subunit; 3) tonic current was maintained in DGCs of epileptic animals but not mediated by high affinity GABA_A receptors; 4) pharmacological studies demonstrated that tonic currents recorded from epileptic DGCs was not mediated by the $\alpha 5\gamma 2$ subunit-containing receptors; 5) pharmacological properties of GABA_A receptors contributing to tonic currents in epileptic DGCs were similar to those of $\alpha 4\gamma 2$ subunit-containing GABA_A receptors; and 6) surface expression of the $\alpha 4$ subunit increased and a larger amount of the subunit co-immunoprecipitated with the $\gamma 2$ subunit in hippocampi of epileptic animals. Taken together, these findings suggest that $\alpha 4\gamma 2$ subunit-containing receptors contribute to maintenance of tonic currents in the DGCs of epileptic animals. However, because of their lower affinity for GABA and neurosteroids, the presence of these receptors may contribute to seizure vulnerability.

A combination of pharmacological and biochemical studies suggested that $\alpha 4\gamma 2$ subunit containing GABA_A receptors contribute to tonic inhibition in epileptic DGCs. Allopregnanolone and lanthanum sensitivity of tonic currents was diminished in epileptic DGCs. Recombinant GABA_A receptors composed of the $\alpha 4\delta$ subunits are blocked by lanthanum and enhanced by neurosteroids, whereas those composed of $\alpha 4\gamma 2$ subunits are less sensitive to these drugs (Brown et al., 2002). Ro15–4513, which binds and modulates the $\alpha \beta \gamma 2$ and $\alpha \beta \gamma 3$ subunit-containing receptors, but not to receptors composed of the $\alpha \beta$, $\alpha \beta \delta$, or $\alpha \beta \gamma 1$ subunits (Scholze et al., 1996; Ebert et al., 1996), differentially modulated tonic currents recorded from epileptic and control DGCs.

Biochemical studies revealed that the surface expression of the $\alpha 4$ subunit was increased while that of its partnering δ subunit was diminished. Although, our studies were performed on whole hippocampus, the results of our study are likely to be representative of changes in the granule cell region because (1) the majority of the $\alpha 4$ subunit expression in the hippocampus is found in the granule layer (Sperk et al, 1997), and (2) in the epileptic animals the $\alpha 4$ expression in the granule cells increased whereas that in the CA1 region decreased (Tsunashima et al, 1997; Schwarzer et al, 1997b; Laurie et al, 2003). Previous electron microscopic studies from our laboratory (Sun et al, 2007) and others (Zhang et al., 2007) were suggestive of a co-assembly of the $\alpha 4$ and $\gamma 2$ subunit. The results of the present study confirm the presence of functional $\alpha 4\gamma 2$ subunit containing GABA_A receptors in hippocampi of epileptic animals. A previous study reported co-assembly of the $\alpha 4$ and $\gamma 2$ subunits as early as 24 hr after lithium-pilocarpine-induced status epilepticus (Lund et al., 2008). However, the study did not address whether the receptors were functional or if they persisted in chronically epileptic animals. Co-assembly of the $\alpha 4$ and $\gamma 2$ subunits and increased binding of Ro15–4513 has also been observed on the removal of the δ subunit in knock out animals (Korpi et al, 2002) as well as in other conditions associated with increased seizure susceptibility such as ethanol intoxication and progesterone withdrawal (Kokka et al, 1993; Liang et al, 2006; Smith et al, 1998).

Receptors containing the $\alpha 4\gamma 2$ subunit have not been reported to mediate tonic inhibition of neurons in the past. In hippocampal DGCs, the majority of tonic inhibition is maintained by receptors containing the $\alpha 4\delta$ and $\alpha 5\gamma 2$ subunits. In cultured neurons, receptors composed of other subunit combinations, such as the $\alpha \beta$ dimers and ϵ subunit containing GABA_A receptors have been reported to contribute to tonic inhibition (Mortensen and Smart, 2006). We have

previously shown increased localization of the $\alpha 4$ subunit at synaptic and perisynaptic locations in epileptic DGCs and evidence for functional $\alpha 4\gamma 2$ subunit containing synaptic GABA_A receptors (Sun et al, 2007b). These receptors may have likely contributed to the overall measures of I_{hold} determined in the present study.

A recent report (Zhan and Nadler, 2009) suggested that tonic inhibition of epileptic DGCs was mediated by enhanced expression of $\alpha 5\gamma 2$ subunit-containing receptors, which is in contrast to our finding of a diminished component of $\alpha 5\gamma 2$ subunit-containing receptors. The discrepancy between our results and those of Zhan and Nadler could result from different epilepsy models used or due to differences in the methods used for measuring tonic inhibition.

The question of whether tonic inhibition of DGCs is preserved or compromised in epileptic animals largely depends on the GABA concentration in the extrasynaptic space. In contrast to competitive antagonists, Penicillin G is a non-competitive antagonist of the GABA_A receptor (Macdonald and Barker, 1977) which inhibits GABA_A receptors by blocking the chloride channel in all open states (Twyman and Macdonald, 1992). Since tonic inhibition is mediated by persistently open channels, an open channel blocker is likely to preferentially block GABA_A receptors that mediate tonic inhibition. A previous study demonstrated that penicillin preferentially blocked persistently open GABA_A receptors whereas transiently activated synaptic currents were partially inhibited by penicillin (Mchedlishvili and Kapur, 2006). The current study demonstrated a loss of penicillin-sensitive persistently open conductance in epileptic DGCs. It therefore appears that in epileptic DGCs, tonic inhibition is preserved under conditions where low affinity GABA_A receptors could be activated by elevating extracellular GABA concentration by applying 3 μM GABA with concomitant blockade of GABA transporters. Further, tonic inhibition in epileptic DGCs may not be mediated by high affinity, slowly desensitizing GABA_A receptors that can be activated by a low (1 μM) concentration of GABA in control DGCs. In these experiments we found that the baseline I_{rms} in control DGCs were lower than in epileptic DGCs. These differences may be due to differences in the absolute predrug baseline noise levels during recordings or due to altered physiology such that enhanced GABA rendered the receptors insensitive to 1 μM GABA.

Although spillover from synapses may be a source of GABA in the extracellular space, the concentration of extracellular GABA is largely regulated by the equilibrium potential of GABA transporters such as GAT-1 (Hamann et al., 2002; Wu et al., 2007; Wu et al., 2006). The intra- and extracellular concentration of GABA, Na^+ , and Cl^- , and the membrane potential determine the equilibrium potential of GAT-1. Under physiological conditions, it is estimated that GAT-1 maintains the extracellular concentration of GABA in the 0.1 – 0.6 μM range, which is supported by direct measurements of extracellular GABA (Glykys and Mody, 2007). Increasing intracellular GABA, depolarization of neurons, and intracellular accumulation of Na^+ are predicted to elevate extracellular GABA. It is currently unclear whether conditions in epileptic DGCs are sufficient to elevate extracellular GABA above 1 μM , which is necessary to activate low affinity receptors mediating tonic inhibition in epileptic DGCs. A previous study in the pilocarpine model of epilepsy found that the GAT-1 transporter-mediated GABA uptake or clearance remained unaffected in both DGCs and CA1 neurons (Frahm et al., 2003). Further, a recent study revealed that in patients with intractable TLE localized to the hippocampus, the levels of extracellular GABA seldom exceeded 1 μM (Pan et al., 2008). Thus it is possible that the extracellular concentration of GABA in epileptic dentate gyrus may be insufficient to activate $\alpha 4\gamma 2$ subunit-containing GABA_A receptors. Further studies with realistic estimates of extracellular GABA concentration are necessary to confirm whether tonic inhibition of DGCs is preserved or compromised in epileptic animals.

Neurosteroid modulation of tonic inhibition in epileptic DGCs was diminished. Neurosteroids are metabolic products derived from the circulating steroid hormones, progesterone,

testosterone, and cortisol (Compagnone and Mellon, 2000). Neurosteroids exert an anticonvulsant action, largely by potentiation of GABA_A receptor currents (Kokate et al., 1996; Lambert et al., 1996). The current study demonstrates that diminished neurosteroid modulation of tonic inhibition in epileptic DGCs is due in part to the loss of extrasynaptic δ subunit-containing receptors. We have also previously shown that while the response to the lower concentrations of allopregnanolone is diminished in epileptic DGCs, a higher concentration of allopregnanolone (100 nM) that has direct actions on the GABA_A receptor (Callachan et al., 1987; Shu et al., 2004) is similarly effective in epileptic DGCs (Mtchedlishvili et al., 2001; Sun et al., 2007). Reduced neurosteroid modulation is likely to diminish the gating function of DGCs and therefore increase the susceptibility to seizures. In addition, neurosteroid levels in the hippocampus fluctuate and allow neurons to respond to a changing hormonal milieu. A recent study demonstrated that neurosteroids controlled seizures in epileptic animals (Lawrence et al, 2010). Preventing metabolism of progesterone to allopregnanolone using finasteride markedly enhanced the frequency of spontaneous seizures in female epileptic rats that had either basal or artificially elevated progesterone levels suggesting that diminished neurosteroid sensitivity enhanced seizure susceptibility.

Reduced affinity for GABA and neurosteroids in receptors mediating tonic inhibition of DGCs likely contribute to seizure susceptibility of the hippocampus. Persistently open GABA_A receptors contribute significantly to inhibition of DGCs, and absence of this conductance is likely to contribute to the disinhibition of these neurons. In addition, because the $\alpha 4\gamma 2$ subunit-containing receptors desensitize faster and extensively compared to even those receptors containing the $\alpha 1\gamma 2$ subunits during prolonged exposure to GABA (Lagrange et al., 2007), it is likely that the contribution of such receptors to maintenance of tonic inhibition is limited. It may also be likely that receptors composed of other subunits such as $\alpha\beta$ that are found in hippocampal pyramidal neurons (Mortensen and Smart, 2006) or those containing the neurosteroid insensitive ε subunit (Belelli et al., 2002) could participate in the tonic inhibition of granule cells and compensate for the loss of the δ subunit containing receptors in the epileptic DGCs. Loss of neurosteroid modulation is likely to further diminish inhibition of DGCs. Other studies suggest that loss of specific subsets of GABAergic interneurons can modestly reduce inhibition of DGCs (Sloviter, 1987; Obenaus et al., 1993; Kobayashi and Buckmaster, 2003; Sun et al., 2007). A combination of reduced tonic inhibition, reduced neurosteroid sensitivity of tonic inhibition, and diminished synaptic inhibition is likely to diminish the gating function of DGCs.

The $\alpha 4\beta\gamma 2$ receptors represent unique therapeutic target for antiepileptic drugs. The pharmacological properties of $\alpha 4\beta\gamma 2$ receptors are distinct from the $\alpha 1\beta\gamma 2$ receptors (Wafford et al, 1996; Sieghart, 2006). Although these receptors are mostly benzodiazepine insensitive, selective benzodiazepines such as flumazenil and Ro15–4530 act as partial agonists. Hence a benzodiazepine with selective action against the $\alpha 4\gamma 2$ subunit-containing receptors may have minimal sedative effects but powerful anticonvulsant properties and can be used as therapeutic tool.

Research Highlight

- GABA_A receptor-mediated tonic currents recorded from hippocampal granule cells are enhanced by neurosteroids in health.
- Neurosteroid sensitivity of tonic currents recorded from granule cells of experimental animals with temporal lobe epilepsy is diminished.

- Diminished surface expression of the δ subunit-containing GABA_A receptors and the assembly of novel $\alpha 4\gamma 2$ subunit-containing receptors could explain diminished neurosteroid sensitivity observed in epilepsy.

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Figure 1.

Modulation of tonic inhibition by allopregnanolone is diminished in epileptic DGCs. **A. (a)** Representative current traces recorded from DGCs of control and epileptic animals before and after application of 30 nM allopregnanolone. **(b)** Decimated traces of (a) for better temporal resolution. In this and all subsequent traces, arrowheads represent the beginning of drug application. The straight dotted lines in these and subsequent traces indicate the mean holding current for the respective recordings. **(c)** Quantitative data of mean shift in holding current (I_{Δ}) in recordings from control and epileptic DGCs following application of 30 nM allopregnanolone. **B. (a)** Representative current traces recorded from DGCs of control and epileptic animals before and after application of 60 nM allopregnanolone. **(b)** Decimated traces of (i) for better temporal resolution. **(c)** Quantification of the mean I_{Δ} in recordings from control and epileptic DGCs following application of 60 nM allopregnanolone. All recordings were performed in the presence of 10 μ M NO-711 and 3 μ M GABA. * p <0.05, ** p <0.01 compared to control, unpaired t test.

Figure 2.

Surface expression of the δ subunit was reduced in chronic epileptic animals and more of it was retained in the ER. **A.** Surface expression of δ subunit was studied in control (lane C) and epileptic (lane E) animals by a biotinylation assay (lanes marked "Surface"). Expression of δ subunit was also studied using total cell lysates for normalization (lanes marked "Total"). The same blots were used for expression of β -actin used as a marker for cytoplasmic proteins. Absence of a β -actin signal in surface samples indicates specificity of the biotinylation assay. **B.** The total microsomal fraction was isolated from control (lane C) and epileptic (lane E) animals and blotted for expression of δ subunit (lanes marked "Microsomal Fraction"). Expression in total cell lysates was studied for normalization (lanes marked "Total"). A stronger signal in the microsomal fraction from epileptic animals indicates ER retention of δ subunit. Blots were also reprobbed with the ER markers calnexin and Grp78/Bip to confirm equal loading of ER membranes.

Figure 3.

The magnitude of tonic inhibition recorded in the presence or absence of 10 μ M NO-711 and 3 μ M GABA in control and epileptic DGCs was similar. **(a)** Representative current traces recorded from DGCs of control and epileptic animals before and after application of 100 μ M bicuculline in slices bathed in ACSF with uptake blocker NO-711 and GABA. Bicuculline application is shown by same bar as in control. **(b)** Quantification of the mean I_{Δ} in recordings from control and epileptic DGCs following application of bicuculline. **(c)** Representative current traces recorded from DGCs of control and epileptic animals before and after application of 100 μ M bicuculline in slices bathed in ACSF without uptake blocker NO-711 and GABA. Bicuculline application is shown by the same bar as in control. **(d)** Cumulative frequency plot

of I_{rms} distribution before (solid line) and after (dotted line) application of bicuculline in control (upper panel) and epileptic (lower panel) DGC. Note the leftward shift of distribution plot in presence of bicuculline.

Figure 4.

Tonic inhibition in epileptic DGCs is not maintained by $\alpha 5$ subunit GABA_A receptors. **(a)** Representative current traces recorded from control and epileptic DGCs before and after application of the selective $\alpha 5$ subunit antagonist L-655708 (1 μ M). **(b)** Decimated traces of (a) for better temporal resolution. **(c)** Quantification of the mean I_{Δ} in recordings from control and epileptic DGCs following application of L-655708. All recordings were performed in the presence of 10 μ M NO-711 and 3 μ M GABA. * $p < 0.05$ compared to control, unpaired t test.

Figure 5.

Tonic inhibition in epileptic DGCs is mediated by GABA_A receptors assembled with $\alpha 4\gamma 2$ subunits. **A.** (a) Representative current traces recorded from control and epileptic DGCs before and after application of 100 μ M lanthanum (LaCl_3). (b) Decimated traces of (a) for better temporal resolution. (c) Quantitative analysis of the mean shift in holding current (I_{Δ}) in recordings from control and epileptic DGCs following application of lanthanum. **B.** (a) Representative current traces recorded from control and epileptic DGCs before and after application of Ro-154513 (300 nM), a partial inverse agonist against non- $\alpha 4$ subunit-containing GABA_A receptors. (b) Decimated traces of (i) for better temporal resolution. (c) Quantification of the mean I_{Δ} in recordings from control and epileptic DGCs following application of Ro-154513. All recordings were performed in the presence of 10 μ M NO-711 and 3 μ M GABA. * $p < 0.05$ compared to control, unpaired t test.

Figure 6.

Low concentration of GABA (1 μ M) enhanced tonic inhibition in control but not epileptic DGCs. **A.** Representative current traces recorded from control and epileptic DGCs (TLE) before and after application of 1 μ M GABA in the absence of an uptake blocker or prior introduction of exogenous GABA. **B.** Decimated traces of (A) for better temporal resolution. **C.** Slope conductance of the RMS noise measured at various holding potentials before and after application of 1 μ M GABA in control and epileptic DGCs. The solid line represents the regression line through observations made during baseline in control DGCs, and the dashed line represents observations made after application of GABA.

Figure 7.

Open channel blocker penicillin inhibits persistently open GABA_A receptors in control DGCs but not in epileptic DGCs. In all traces I_{rms} is shown by arrows between solid and dashed lines. **A.** Representative current trace recorded from control DGC before (baseline upper trace) and after (lower trace) application of 300 μ M penicillin. **B.** Representative current traces recorded from an epileptic DGC, before (baseline top) and after (bottom) application of penicillin. **C.** Cumulative frequency plot of I_{rms} during 60 30 sec epochs during before (solid line) and after (dotted line) application of penicillin from a control DGC. Note the left shift of the distribution. **D.** Cumulative frequency plot of I_{rms} distribution before (solid line) and after (dotted line)

application of penicillin in epileptic DGC. Note the minimal shift compared to that of control DGC.

Figure 8.

Surface expression of the $\alpha 4$ subunit increased in epileptic animals and its association with the $\gamma 2$ subunit was higher. **A.** Surface expression of the $\alpha 4$ subunit was studied from surface proteins isolated from naïve (lane C) and epileptic (lane E) animals using a biotinylation assay (panel "Surface"). Total expression of the $\alpha 4$ subunit was also studied for normalization (panel "Total"). **B.** Association between the $\alpha 4$ and $\gamma 2$ subunits was studied in control and epileptic animals. Using an anti- $\gamma 2$ antibody, the $\gamma 2$ subunit and associated proteins were separated from hippocampi of control (lane C) and epileptic (lane E) animals. Expression of the $\gamma 2$, $\alpha 4$, and $\alpha 1$ subunits was studied by Western blotting. Expression of these subunits was also studied from total proteins for normalization (panel total). To confirm specific pull-down of the $\gamma 2$ and

associated proteins using anti- $\gamma 2$ IgG, normal rabbit IgG was also used to pull-down proteins (lane IgG).