Alcohol withdrawal syndrome (AWS) symptoms include hyperexcitability, anxiety, and sleep disorders. Chronic intermittent ethanol (CIE) treatment of rats with subsequent withdrawal of ethanol (EtOH) reproduced AWS symptoms in behavioral assays, which included tolerance to the sleep-inducing effect of acute EtOH and its maintained anxiolytic effect. Electrophysiological assays demonstrated a CIE-induced long-term loss of extrasynaptic GABA_A receptor (GABAAR) responsiveness and a gain of synaptic GABAAR responsiveness of CA1 pyramidal and dentate granule neurons to EtOH that we were able to relate to behavioral effects. After CIE treatment, the α4 subunit-prefering GABAAR ligands 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, La3+, and Ro15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate) exerted decreased effects on extrasynaptic currents but had increased effects on synaptic currents. Electron microscopy revealed an increase in central synaptic localization of α4 but not δ subunits within GABAergic synapses on the dentate granule cells of CIE rats. Recordings in dentate granule cells from δ subunit-deficient mice revealed that this subunit is not required for synaptic GABAAR sensitivity to low [EtOH]. The profound alterations in EtOH sensitivity and α4 subunit localization at hippocampal GABA_ARs of CIE rats suggest that such changes in these and other relevant brain circuits may contribute to the development of tolerance to the sleep-inducing effects and long-term dependence on alcohol.

**Key words:** subunit composition; anxiety; sleep; electron microscopy; alcoholism; inhibitory neurotransmission, GABA_A receptor

### Introduction

Alcohol abuse represents a significant problem in our society with an estimated 14 million people in the United States being dependent on alcohol (McGinnis and Foege, 1999). The alcohol withdrawal syndrome (AWS) is a particularly severe manifestation of alcohol abuse, presenting with a variety of symptoms such as anxiety, insomnia, agitation, and seizures. Clinical literature abounds with evidence that alcohol dependence is a chronic relapsing disorder in which alcoholics go through cycles of intense ethanol (EtOH) intoxication alternating with periods of abstinence; this results in a withdrawal syndrome, the severity of which is positively correlated to the number of intoxication and withdrawal cycles (Brown et al., 1988; Booth and Blow, 1993). Laboratory studies in rodents fully support these clinical findings (McCown and Breese, 1990; Becker and Hale, 1993; Kokka et al., 1993; Veatch and Gonzalez, 1996). Such studies have demonstrated the importance of intermittent EtOH administration in producing long-term alterations in the function of both GABA_A receptors (GABA_ARs) and NMDA receptors (NMDARs) (Hu and Ticku, 1997; Becker et al., 1998). EtOH withdrawal-induced increases in the extracellular glutamate levels are also positively correlated to the number of withdrawals (Dahchour and De Witte, 2003). Other studies showed that repeated EtOH treatment and withdrawal lead to higher alcohol intake and preference than continuous EtOH treatment (Rimondini et al., 2003; O’Dell et al., 2004).

GABA_ARs represent one of the several important pharmacological targets of EtOH (Allan and Harris, 1986; Franks and Lieb, 1987; Mehta and Ticku, 1988; Weiner et al., 1994; Lovinger, 1997; Harris, 1999; Ariwodola et al., 2003; Aroor and Shukla, 2004). A family of heteropentameric GABA_A isoforms of different subunit composition accounts for variable sensitivity to other modulatory drugs such as benzodiazepines, barbiturates, neurosteroids, and general anesthetics (Olsen and Homanics, 2000; Whiting et al., 2000). GABA_A receptor function and expression are altered after chronic administration of EtOH (Morrow et al., 1988; Mhatre et al., 1993; Kumar et al., 2004). EtOH tolerance and dependence also appear to be attributable, at least in part, to changes in the function of GABA_ARs, possibly involving alterations in native GABA_A subunit composition and trafficking (Kumar et al., 2004). In the chronic intermittent ethanol (CIE) model of alcohol withdrawal and dependence, rats are exposed to intermittent episodes (≥60 doses) of EtOH intoxication and...
withdrawal (approximating binge drinking episodes in humans), leading to behavioral hyperexcitability that includes decreased seizure thresholds and increased anxiety (Kokka et al., 1993). This is accompanied by presumably causal changes in GABA<sub>R</sub> expression and physiology including: (1) persistent decreases in hippocampal GABA<sub>R</sub>-mediated paired-pulse inhibition (Kang et al., 1996), (2) changes in levels of several GABA<sub>R</sub> subunits (Mahmoudi et al., 1997; Cagetti et al., 2003), and (3) remarkable alterations in the effectiveness of several clinically important drug classes that act through allosteric modulation of GABA<sub>R</sub> function (Kang et al., 1998; Cagetti et al., 2003; Liang et al., 2004). For example, there is a loss of sleep-inducing actions of the benzodiazepine flurazepam and the neuroactive steroid anesthetic alphaxalone. These changes are paralleled by the loss of synaptic and extrasynaptic GABA<sub>R</sub>-mediated responses to such compounds in hippocampal CA1 neurons (Cagetti et al., 2003; Liang et al., 2004).

Recent studies have suggested that GABA<sub>R</sub> Rs containing α<sub>4</sub>βδ or α<sub>6</sub>βδ subunit combinations, which are normally found outside or at the edges of GABAergic synapses (Nusser et al., 1998; Wei et al., 2003), are particularly sensitive to low millimolar concentrations of EtOH (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004). We hypothesized that the demonstrated alterations in the hippocampal levels of α<sub>1</sub>, α<sub>4</sub>, and δ subunits after CIE treatment are responsible for the altered function and pharmacological sensitivity of hippocampal GABA<sub>R</sub>Rs (Cagetti et al., 2003; Liang et al., 2004). Therefore, we evaluated the effects of CIE treatment and withdrawal on re-exposure to EtOH in anxiety and sleep assays and related these to changes in the effects of acute EtOH application on hippocampal GABA<sub>R</sub>-mediated synaptic and extrasynaptic currents. Then, we used electron microscopic immunogold labeling techniques to examine possible alterations in the localization of α<sub>4</sub> and δ subunits after CIE treatment. Our results are consistent with a net loss of extrasynaptic and a gain of synaptic GABA<sub>R</sub> responsiveness to EtOH, which are concomitant with increased α<sub>4</sub> but not δ subunit localization within hippocampal GABAergic synapses of CIE rats.

Materials and Methods

Production of CIE rats. The Institutional Animal Care and Use Commit-tee approved all animal experiments. Male Sprague Dawley rats (170-190 g) were housed in the vivarium under a 12 h light/dark cycle and had ad libitum access to food and water. Intoxicating doses of EtOH (Pharmco Products, Brookfield, CT) were administered by oral intuba-tion on a chronic regimen: for the first five doses, rats received 5 g/kg of body weight as a 25% (v/v) solution in saline once every other day, and for the following 55 doses, they received 6 g/kg of 30% (v/v) EtOH once every day. The control group received saline (20 ml/kg of body weight). With this EtOH regimen, rats experience multiple cycles of intoxication and withdrawal phases leading to a kindling-like state with a persistent decrease in pentyleneetetrazol seizure threshold (Kokka et al., 1993) and reduced hippocampal GABA<sub>R</sub>-mediated inhibition (Kang et al., 1996). After the treatment and 2 or 40 d of withdrawal, rats were subjected to behavioral experiments, or they were killed and tissues prepared for experiments.

Sleep-time assay. The hypnotic effect of EtOH (3 g/kg, i.p.) was tested on saline- and CIE-treated rats. EtOH (DSP-CT-18; Pharmco Products) was diluted to 17.8% in 0.9% saline. Injection volume was 2 ml/kg. Sleep time was determined as follows: after drug injection and loss of righting reflex, rats were placed on their backs in a V-shaped trough, and a timer was started. The sleep time period ended when animals were able to flip over three times in 30 s after being repeatedly placed on their backs.

Elevated plus maze assay. CIE- and saline-treated rats were tested for the anxiolytic effect of EtOH (0.5 g/kg) on the elevated plus maze (Cagetti et al., 2003). Rats were randomly divided into four groups: saline controls treated with vehicle (n = 7) or with EtOH (n = 7) and CIE rats treated with vehicle (n = 6) or EtOH (n = 7). EtOH was diluted in saline (0.9%) and administered to rats via gavage 30 min before testing. Rats were placed on the central area of the maze, tested for 5 min, and videotaped. The following measures were scored: number of entries into open arms, closed arms, or center platform and time spent in each of these areas. To measure the locomotor activity, the number of total entries was measured for each rat. Data are reported as percentage of number of entries in arms, percentage of time spent in different entries, and number of total entries. Statistical differences were determined using ANOVA.

Production of δ ~/~/ mice. Mice with a targeted disruption of the δ subunit gene were produced and genotyped as described previously (Mi-halek et al., 1999). All mice were of a mixed C57BL/6J × 129Sv/SvJ genetic background and were derived from heterozygote matings. Experiments were performed on male mice at 6 months of age.

Electrophysiological recordings. Transverse slices (400 μm thick) of rat or mouse dorsal hippocampus were obtained using standard techniques (Kang et al., 1996; Spigelman et al., 2003). Whole-cell patch-clamp recordings were obtained from cells located in the CA1 pyramidal or dentate granule (DG) cell layers at 34 ± 0.5°C during perfusion with artificial CSF (ACSF) composed of the following (in mM): 125 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, and 10 ionic glucose. The ACSF was bubbled continuously with a 95%/5% mixture of O<sub>2</sub>/CO<sub>2</sub> to ensure adequate oxygenation of slices and a pH of 7.4. Patch pipettes contained the following (in mM): 135 cesium gluconate, 2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 11 ethylene glycol-bis(β-aminoethoxy)ether-N,N,N',N”-tetraacetic acid (EGTA), 10 N-Methyl-D-aspartate (NMDA), 4 L-glutamate, and 5 4-aminopyridine. The following entry buffer solutions were used: (in mM): 100 Cs<sub>2</sub>SO<sub>4</sub>, 2 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 2 NaHPO<sub>4</sub>, and 10 ionic glucose. The ACSF was adjusted to pH 7.25 with NaOH. GABA<sub>R</sub>-mediated miniature IPSCs (mIPSCs) were pharmacologically isolated by adding tetrodotoxin (TTX; 0.5 μM), α-(3-aminopropyl)phosphonoxyanoate (40 μM), 6-cyano-7-nitroquinolaxine-2,3-dione (CNQX; 10 μM), and CGP 54626 (5,7,8,9-tetrahydro-5-hydroxy-6H-benzocyclohepten-6-ylideneacetic acid) (1 μM) to the ACSF from stock solutions. Stock solutions of CNQX were made with pure dimethyl sulfoxide (DMSO). Final concentration of DMSO did not exceed 42 μM in the recording chamber. Signals were recorded in voltage-clamp mode with an amplifier (Axoclamp 2B; Mo-lecular Devices, Union City, CA). Whole-cell access resistances were in the range of 8–20 MΩ before electrical compensation by ~90%. During voltage-clamp recordings, access resistance was monitored by measuring the size of the capacitative transient in response to a 5 mV step command, and experiments were abandoned if changes >20% were encountered. At least 10 min was allowed for equilibration of the pipette solution with the intracellular milieu before commencing recordings. Data were acquired with pClamp 8 software (Molecular Devices), digitized at 20 kHz (Axoclamp 2B; Molecular Devices), and analyzed using Clampfit soft-ware (Molecular Devices) and the Mini Analysis Program (versions 5.2.2 and 5.4.8; Synaptosoft, Decatur, GA).

Detection and analysis of mIPSCs and tonic currents. The recordings were low-pass filtered off-line (Clampfit software) at 2 kHz. The mIPSCs were detected (Mini Analysis Program) with threshold criteria of: amplitude, 5 pA and area, 20 pA ms. Frequency of mIPSCs was determined from all automatically detected events in a given 100 s recording period. For kinetic analysis, only single-event mIPSCs with a stable baseline, sharp rising phase, and exponential decay were chosen during visual inspection of the recording trace. Double and multiple peak mIPSCs were excluded. The mIPSC kinetics was obtained from analysis of the averaged chosen single events (>120 events per 100 s recording period) aligned with half rise time in each cell. Decay time constants were obtained by fitting a double exponential to the falling phase of the averaged mIPSCs. The tonic current magnitudes were obtained from the mean baseline current during the 100 s recording periods. The investigator performing the recordings and mIPSC analysis was blind to the treat-ment (saline or CIE) that the rats received.

Tissue preparation for electron microscopy. In preparation for postem-bryonic immunogold labeling for the α<sub>4</sub> and δ subunits of GABA<sub>R</sub> receptors, four CIE- and four saline-treated rats were anesthetized deeply with sodium pentobarbital (90 mg/kg, i.p.) and perfused through the ascending aorta with a fixative solution of 4% paraformaldehyde and
Cryoprotected sections were frozen at and then cryoprotected in 10, 20, and 30% glycerol in PB for 2 h each. These sections. Specimens were immersed in 5% sucrose in PB, pH 7.4, and then cryoprotected in 10, 20, and 30% glycerol in PB for 2 h each. Cryoprotected sections were frozen at −190°C in a cryofixation unit (EM CPC; Leica, Vienna, Austria) and then transferred to a cryosubstitution unit (EM AF5; Leica), which was programmed for all subsequent steps (Wei et al., 2003). Specimens were immersed in 4% uranyl acetate (Electron Microscopy Sciences, Fort Washington, PA), dissolved in anhydrous methanol for 24 h at −90°C, rinsed in methanol at −45°C, and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences) for 48 h at −45°C. The resin was polymerized with ultraviolet light (360 nm) for 24 h at −45°C and then warmed in 4°C steps to 0°C.

**Immunogold labeling for electron microscopy.** Ultrathin sections were cut on a microtome (Reichert-Jung, Vienna, Austria), picked up on nickel mesh grids, and processed for immunogold labeling with previously described methods (Wei et al., 2003). Briefly, ultrathin sections were treated with 0.2% NaN₃ in distilled water for 5 min and then with 0.1% NaBH₄ in 0.01 M Tris-buffered saline (TBS), pH 7.4, for 10 min and incubated in 2% human serum albumin (HSA) (Sigma, St. Louis, MO) and 0.05 M glycine in TBS containing 0.1% Triton X-100 for 10 min and 7 min, respectively. Sections were blocked in 2% HSA in TBS for 1.5 h and then incubated in the primary antiserum, rabbit anti-ø4 subunit (1:300; ABS457; Chemicon, Temecula, CA), or rabbit anti-β2 subunit (1:100; gift from Dr. Werner Sieghart, Medical University of Vienna, Vienna, Austria) in TBS containing 2% HSA for 18–24 h at room temperature. After a rinse with 0.05 M Tris-HCl buffer (TBS) containing polyethylene glycol (50 mg/ml), sections were incubated for 2.5 h in an appropriate secondary antiserum conjugated to 10 nm colloidal gold particles, diluted 1:30 in 0.05 M TBS, pH 8.0, containing 2% HSA. The secondary antiserum was either goat anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ) or goat anti-rabbit IgG (Aurion; distributed by Electron Microscopy Sciences). After labeling, sections were stained with uranyl acetate for 40 min and lead citrate for 4 min.

**Quantitative analysis.** Randomly selected series of ø4 or δ subunit-labeled synaptic profiles within the molecular layer of the dentate gyrus were studied and photographed with a Jeol (Peabody, MA) 100CX II electron microscope at a primary magnification of 19,000×. The localization of colloidal gold particles was determined for each symmetric synapse that exhibited immunogold labeling in these photomicrographs. Symmetric synaptic contacts were operationally defined as regions with close apposition between an axon terminal and putative granule cell dendrite at which the presynaptic and postsynaptic membranes were precisely parallel. Such contacts generally included a thin postsynaptic density and some electron-dense material in the cleft between the membranes. The quantitative analyses included 126 and 140 ø4 subunit-labeled synapses from saline- (n = 3) and CIE-treated (n = 3) rats, respectively.

Gold particle positioning along the synaptic membranes was operationally defined as either (1) perisynaptic or (2) synaptic. Labeling was classified as perisynaptic if the gold particles were located either directly at the ends of the synaptic contact, within 30 nm of the ends of the synaptic contact, or along the extrasyaptic membranes that extended up to 100 nm beyond the end of the synaptic contact. Gold particles that were located farther than 100 nm from the ends of a synaptic contact were not included in this analysis. Labeling was classified as synaptic if gold particles were located directly at synaptic contacts, excluding the perisynaptic sites indicated above. Such labeling was frequently concentrated near the center of the synapse.

**Results**

**CIE rats show tolerance to the hypnotic effect of EtOH**  Using a sleep-time assay, we compared the hypnotic effect of EtOH (0.5 g/kg, i.p.) in CIE- and saline-treated controls. CIE rats had a profoundly shorter sleep duration (3.5 ± 2.9 min; n = 14) compared with controls (37.2 ± 5.4 min; n = 12) after administration of EtOH, representing a significant reduction (89%; p < 0.001) in sleep time. These results indicated that CIE rats become tolerant to the sleep-inducing effect of EtOH.

**CIE rats retain the anxiolytic effect of EtOH**  The anxiolytic effect of EtOH (0.5 g/kg, p.o.) was tested in CIE- and saline-treated rats on the elevated plus maze. As shown previously using the same test (Cagetti et al., 2003), vehicle-treated CIE rats showed increased anxiety, and they did not enter the open arms (Fig. 1A). EtOH significantly increased the number of open entries in control (p = 0.03) and CIE (p = 0.02) rats. EtOH also increased the time CIE rats spent in the open arms (p = 0.027). Thus, despite showing tolerance to the sleep-inducing effect of EtOH, CIE rats are still sensitive to the anxiolytic effect of EtOH.

**EtOH potentiates extrasynaptic but not synaptic GABAₐR currents in CA1 neurons from normal rats**  To explore the possible mechanisms of EtOH actions, we recorded pharmacologically isolated GABAₐR currents in CA1 pyramidal and dentate granule cells in hippocampal slices from saline- and CIE-treated rats. In these and all subsequent whole-cell patch recordings, membrane voltage was clamped at 0 mV, and the initial extracellular solution containing TTX, ionotropic glutamate, and GABAₐ receptor blockers was applied for at least 10 min. Bath application of EtOH produced increases in the holding current (I_h) in CA1 neurons from saline-treated rats. We and others previously showed I_h to be mediated by extrasynaptic GABAₐR, because during selective blockade of synaptic GABAₐR currents by 1 μM gabazine, this tonic current is enhanced by various GABAₐR agonists and blocked by 50 μM picrotoxin (Bai et al., 2001; Liang et al., 2004). In CA1 neurons, increases in I_h were observed at 50 mM EtOH but became statistically significant only at 100 mM (Fig. 2). In contrast, the kinetics and frequency of mIPSCs were unaffected even by 100 mM EtOH (Fig. 2A, B).

**CIE-induced switch of EtOH actions from extrasynaptic to synaptic GABAₐR currents in CA1 neurons**  The profile of EtOH action was quite different in CA1 neurons from CIE-treated rats. In these recordings, EtOH (10–100 mM) no longer had an effect on the tonic GABAₐR current, whereas the mIPSCs became very sensitive to EtOH, such that the total charge transfer (area of averaged mIPSCs) was significantly increased from predrug condition by 10 mM EtOH. After application of 50 and 100 mM EtOH, the total charge transfer of mIPSCs surpassed that of mIPSCs from saline-treated rats (Fig. 2B). It is noteworthy that the total charge transfer of mIPSCs was significantly smaller in CIE rats without acute EtOH application, compared with saline controls. This was because of faster mIPSC τ_i and τ_2 decay time constants, as described previously (Cagetti et al., 2003; Liang et al., 2004). The baseline mIPSC frequency was also significantly reduced from 12.1 ± 0.4 to 9.7 ± 0.4 Hz after CIE treatment and remained unaffected by acute EtOH application. In contrast, no differences were seen in the magnitude of I_h between CA1 neurons from saline- and CIE-treated rats.
Both synaptic and extrasynaptic GABAARs of dentate granule cells from control rats are sensitive to EtOH

Differences in subunit combinations of synaptic and extrasynaptic GABAARs are known to profoundly affect both the kinetics of GABAAR activation and their response to various GABAergic drugs (Olsen and Homanics, 2000; Whiting et al., 2000). Recent studies have suggested that certain subunit combinations, namely those GABAARs containing α4β3 subunits, are highly sensitive to EtOH and the partial GABA agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) (Brown et al., 2002; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Hanschar et al., 2005). Increases in hippocampal α4 subunit levels were reported after chronic ethanol treatment (Mahmoudi et al., 1997; Matthews et al., 1998), and we recently used preferential benzodiazepine ligands [bretazenil and ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-3-carboxylate (Ro15-4513)] at α4 subunit-containing GABAARs to provide electrophysiological support for these data in CA1 neurons (Cagetti et al., 2003; Liang et al., 2004). Because the dentate gyrus is particularly enriched in α4 and δ subunits (Pickel et al., 2000; Peng et al., 2002), we hypothesized that GABAARs of DG cells would exhibit greater sensitivity to EtOH than GABAARs of CA1 neurons. Recordings in slices from saline-treated rats revealed this to be the case. Thus, potentiation of both mIPSCs and $I_{\text{hold}}$ was detectable at 10 mM EtOH and significantly potentiated at 50 mM EtOH in DG cells (Fig. 3A, B).

CIE-induced alterations in EtOH sensitivity of extrasynaptic and synaptic GABAARs in DG cells

Analogous to the effects in CA1 neurons, EtOH (10–100 mM) no longer significantly potentiated the tonic GABA current in DG cells after CIE treatment (Fig. 3). Furthermore, the sensitivity of mIPSCs in DG cells to EtOH was increased, such that 10 mM EtOH application produced significant increases in total charge transfer of averaged mIPSCs (Fig. 3B). Under baseline conditions (without acute EtOH application), faster decay accounted for the significantly smaller total charge transfer of averaged mIPSCs in DG neurons from CIE rats, compared with saline controls. The baseline frequency of DG neuron mIPSCs appeared to be slightly reduced after CIE treatment; inclusion of additional baseline recordings revealed significantly smaller mIPSC frequency of CIE rats ($11.0 \pm 0.3 \text{ Hz;} n = 23$ neurons, 8 rats) compared with saline controls ($12.4 \pm 0.4 \text{ Hz;} n = 19$ neurons, 5 rats).

CIE treatment leads to enhanced mIPSC responsiveness to THIP, La$^{3+}$, and Ro15-4513

To obtain further insight into the GABAAR subunit composition changes that may be present after CIE treatment, we first compared the effects of a partial GABA agonist THIP and the antagonist LaCl$_3$ on mIPSCs and tonic currents in DG cells from saline- and CIE-treated rats. In recombinant receptor studies, these compounds were shown to have a particularly high affinity for the α4β3δ GABAAR subunit combination (Brown et al., 2002). Based on this, we hypothesized that similar to EtOH, both THIP and La$^{3+}$, should have reduced effectiveness at extrasynaptic and increased effectiveness at synaptic GABAARs after CIE treatment. Application of THIP (1 μM) produced large increases in $I_{\text{hold}}$ and a small but significant prolongation of the mIPSC decay time in DG cells from saline-treated rats. Subsequent addition of LaCl$_3$ (100 μM) produced a significant reduction in the THIP-induced tonic current, without affecting mIPSC decay (Fig. 4A, B). In DG cells from CIE rats, THIP produced smaller increases in $I_{\text{hold}}$ but proportionately greater potentiation of mIPSCs. However, subsequent addition of LaCl$_3$ (100 μM) had no effect on $I_{\text{hold}}$ but decreased the THIP-induced mIPSC potentiation.

Next, we examined the effect of CIE treatment on responses to Ro15-4513, a partial inverse agonist at the benzodiazepine site of α1- and α2-containing GABAARs, which was also shown to bind with high affinity at α4-containing GABAARs (Knoflach et al., 1996). Importantly, Ro15-4513 has agonist activity at α4β3γ2.

Figure 1. Increased anxiety of CIE rats is alleviated by EtOH. The anxiolytic effect of EtOH (0.5 g/kg, p.o.) was tested on the elevated plus maze. Rats were divided in four groups: vehicle- and EtOH-treated controls and vehicle- and EtOH-treated CIE rats. Data are reported as mean ± SEM of percentage of entries in open arms and number of total entries (A) and percentage of time in different arms (B). EtOH significantly increased the number of open arms entries in CIE ($p = 0.02$) and in controls ($p = 0.03$) and for CIE rats, the time spent in open arms ($p = 0.027$).

(Fig. 2B). The above results were both qualitatively and quantitatively similar to those obtained in slices from saline- and CIE-treated rats at 40 d of withdrawal (Fig. 2B), indicating long-term alterations.

Both synaptic and extrasynaptic GABAARs of dentate granule cells from control rats are sensitive to EtOH
GABA<sub>AR</sub>s but does not modulate α4β3δ GABA<sub>AR</sub>s (Brown et al., 2002). In DG cells from saline-treated rats, Ro15-4513 (0.3 μM) slightly but significantly potentiated both mIPSCs and $I_{\text{hold}}$ (Fig. 5A, B). After CIE treatment, mIPSC potentiation by Ro15-4513 was markedly increased, whereas $I_{\text{hold}}$ was now inhibited by the drug.

α4 but not δ subunits assume a more central synaptic location after CIE treatment

To provide additional evidence in support of the hypothesis that GABA<sub>AR</sub> subunit alterations mediate the switch in responsiveness of synaptic and extrasynaptic GABA<sub>AR</sub>s to EtOH after CIE treatment, we examined the subcellular location of α4 and δ.
subunits with electron microscopic immunogold labeling. In the
dendrites of DG cells from saline-treated rats, the labeling for
both α4 and γ2 subunits was found predominantly in regions
immediately adjacent to or outside symmetric GABAergic syn-
apses, as demonstrated previously for the γ2 subunit in mice (Wei
et al., 2003). Quantitative analysis demonstrated a perisynaptic
location of the α4 subunit in 93% of α4-labeled synapses (117 of
126 synaptic profiles; n = 3 rats) (Fig. 6A, C). However, in EM
sections from CIE-treated rats, immunogold labeling of α4 sub-
units was detected predominantly near the center of postsynaptic

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**Figure 4.** Altered synaptic and extrasynaptic sensitivity to THIP and LiCl after CIE treatment. A. α4β3γ2-prefering agonist (THIP) and antagonist (Li3+) preferentially affect DG cell tonic current in saline-treated rats and synaptic currents in CIE-treated rats, respectively. Note the loss of Li3+ effect on Ihold and increased THIP effect on mIPSCs in CIE rats. B. Summary graphs of total charge transfer of averaged mIPSCs (top graph) and Ihold (bottom graph) before and after THIP and Li3+ application in saline- and CIE-treated rats. Each point is a mean ± SEM value from six neurons (1 rat per group). *p < 0.05 between saline and CIE groups; †p < 0.05 from pre-THIP value (two-way repeated-measures ANOVA).

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**Figure 5.** Altered synaptic and extrasynaptic sensitivity to Ro15-4513 after CIE treatment. A. The α4β2γ2-prefering partial inverse agonist Ro15-4513 potentiates both mIPSCs and tonic current in DG neurons from saline-treated rats. Note the greater potentiation of mIPSCs and a reduction in Ihold by Ro15-4513 after CIE treatment. B. Summary graphs of total charge transfer of averaged mIPSCs (top graph) and Ihold (bottom graph) before and after Ro15-4513 application in saline- and CIE-treated rats (points are mean ± SEM values from 5–6 neurons; 2–3 rats per group). *p < 0.05 between saline and CIE groups; †p < 0.05 from pre-Ro15-4513 value (two-way repeated-measures ANOVA).
densities of symmetric synapses (78% of α4-labeled synapses; 109 of 140 synaptic profiles; n = 3 rats) (Fig. 6 B, D). Statistical analysis revealed significant (p < 0.001; ANOVA) increases in α4 subunit labeling at the center of symmetric synapses (Fig. 6 E). Although the incidence of δ subunit immunogold labeling in CIE rats was quite low (consistent with decreased levels of the δ subunit in these animals (Cagetti et al., 2003)), labeling was detected predominantly at perisynaptic locations on DG cell dendrites from CIE rats (91% of δ-labeled synapses; 32 of 35 labeled profiles; n = 3) (Fig. 6 F).

δ Subunit is not required for synaptic GABA<sub>A</sub> Rs sensitivity to low [EtOH]

One implication of these findings is that sensitivity of synaptic GABA<sub>A</sub> Rs to low [EtOH] may not require the presence of the δ subunit. To test this, we compared EtOH sensitivity of synaptic and extrasynaptic GABA<sub>A</sub>R-mediated currents in DG cells from δ subunit null mice and their wild-type counterparts. Although the magnitude of the tonic current was similar between the two groups, its potentiation by EtOH was greatly reduced in δ −/− mice (Fig. 7). Consistent with our previous report (Spigelman et al., 2003), the total charge transfer of averaged mIPSCs from δ −/− mice was smaller compared with that of δ +/+ mice (Fig. 7). However, the EtOH potentiation of mIPSCs from δ −/− mice was actually increased compared with δ +/+ mice (Fig. 7).

Discussion

Relationship between sleep-inducing effects of EtOH and tonic inhibitory currents

In this study, we demonstrate profound tolerance to the sleep-inducing but not anxiolytic effects of EtOH in rats after withdrawal from CIE treatment. Alcoholics also exhibit tolerance to the sleep-inducing effects of EtOH (Allen et al., 1977; Brower, 2001; Roehrs and Roth, 2001) as well as cross-tolerance to barbiturate and benzodiazepine sedative hypnotic drugs (Miller, 1995). Tolerance to the actions of EtOH and other GABAergic drugs in CIE rats has previously been related to alterations in the pharmacological sensitivity and subunit composition of GABA<sub>A</sub> Rs (Mahmoudi et al., 1997; Kang et al., 1998; Cagetti et al., 2003; Liang et al., 2004). In hippocampal homogenates from CIE rats, these changes include increases in α4 and γ2 and reductions in α1 and δ subunit protein levels (Cagetti et al., 2003). The mechanisms by which the relative abundance and localization of specific GABA<sub>A</sub> <sub>R</sub> subunits are altered by CIE treatment are not known. However, selective endocytosis and recycling of receptors based on subunit composition, distinct assembly signals, and sensitivity to protein kinase C phosphorylation may be involved (for review, see Brandon et al., 2000; Kumar et al., 2004). Tolerance to the sleep-inducing effects of EtOH in CIE rats coincides with tolerance to acute EtOH potentiation of tonic GABA<sub>A</sub> R currents. A similar relationship between decreased sleep time and decreased tonic current enhancement has been observed for alphaxalone and THIP in CIE rats (Cagetti et al., 2003; Liang et al., 2004). Together, these data suggest that the sleep-inducing effects of GABAergic drugs may be mediated primarily by potentiation of extrasynaptic GABA<sub>A</sub> Rs whose sensitivity for these drugs is decreased after CIE treatment. It has also been shown that the general anesthetics propofol and thiopental exert most of their inhibitory effects on intrinsic excitability of hippocampal neu-
rons by potentiation of the tonic, and not phasic, GABA_{A}R-mediated currents (Bieda and Maclver, 2004). Similarly, low concentrations of the volatile anesthetic isoflurane selectively enhance tonic GABAergic currents in CA1 neurons (Caraiscos et al., 2004), whereas the sedative hypnotic THIP selectively enhances tonic currents but not mIPSCs in CA1 neurons (Liang et al., 2004) and in ventrobasal thalamocortical neurons (Belelli et al., 2005; Cope et al., 2005), at concentrations that enhance slow-wave sleep activity (Belelli et al., 2005).

Relationship between altered mIPSC kinetics and hyperexcitability after CIE treatment
Consistent with our previous reports (Cagetti et al., 2003; Liang et al., 2004), CIE treatment results in decreased total charge transfer of mIPSCs in both CA1 and DG neurons, in the absence of significant changes in baseline tonic currents (Figs. 2–5). In addition, small but significant decreases in the frequency of mIPSCs are observed. Such decreases in synaptic inhibition may account for the decreased paired-pulse inhibition (Kang et al., 1996) and abnormal discharges (Veatch and Gonzalez, 1996) recorded in the CA1 region of the hippocampus after CIE treatment. Analogous decreases in synaptic inhibition of other affected circuits (e.g., amygdala) are likely to contribute to the hyperexcitability and anxiety exhibited by CIE rats.

Synaptic GABA_{A}R subunit composition and EtOH sensitivity
This report provides pharmacological and electron microscopic evidence for increases in the α4- but not δ-containing GABA_{A}Rs at synaptic locations. Because of the relatively low sensitivity of immunogold labeling methods (Somogyi et al., 1996), the current labeling presumably identified sites with the highest densities of each subunit. Therefore, our findings do not preclude the presence of low densities of either subunit at additional synaptic and extrasynaptic locations. With this caveat in mind, our results suggest that after CIE treatment there is a net shift of α4 subunits from perisynaptic to synaptic locations at GABAergic synapses, without concomitant changes in δ subunit localization. The α4β6 GABA_{A}Rs are particularly sensitive to low [EtOH] (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Hanchar et al., 2005), which under normal conditions, preferentially potentiates extrasynaptic receptors that mediate tonic inhibition (Wei et al., 2003, 2004). Recently, changes in the levels of δ subunit protein were linked to cyclic changes in tonic inhibition during the ovarian cycle of mice (Maguire et al., 2005). Analogous increases in α4β2δ subunit combinations were proposed to underlie the behavioral and physiological changes induced by withdrawal from progesterone treatment, a model of premenstrual syndrome (Smith et al., 1998; Sundstrom-Poromaa et al., 2002). In the CIE model of alcohol tolerance and dependence, there is a loss of tonic current potentiation by EtOH but a large increase in synaptic GABA_{A}R responsiveness to EtOH. This is concomitant with a decrease in δ subunit protein levels (Cagetti et al., 2003), without electron microscopic evidence of a δ subunit shift to synaptic locations (Fig. 6). Therefore, our data suggest that CIE treatment and withdrawal lead to the formation of α4 subunit-containing synaptic GABA_{A}Rs that do not possess the δ subunit. The enhanced EtOH sensitivity of mIPSCs in δ subunit knock-out mice provides further support to the notion that δ subunit presence is not an absolute requirement for sensitivity of GABA_{A}Rs to low [EtOH]. The discriminative stimulus effects of EtOH are also preserved in δ−/− mice (Shannon et al., 2004).

Relationship of altered EtOH sensitivity of GABA_{A}Rs to alcoholism
The development of alcohol dependence is thought to involve an incremental neuroadaptation to the presence of alcohol (Robinson and Berridge, 1993; Koob and Le Moal, 1997). In CIE rats, the development of alcohol dependence, as measured by the decrease in seizure threshold, is gradual, requiring multiple cycles of EtOH intoxication and withdrawal to produce long-lasting changes (Kokka et al., 1993). Recent studies have confirmed the existence of a temporal threshold of a persistent alcoholic state, measured as a long-lasting increase in voluntary EtOH consumption after intermittent intoxication and withdrawal (Rimondini et al., 2003). It was also shown that intermittent exposure to EtOH is much more effective than continuous EtOH exposure to produce enhanced EtOH self-administration after withdrawal (Rimondini et al., 2003; Spanagel, 2003; O’Dell et al., 2004).

On the basis of many studies in rodents, nonhuman primates, and humans, it has been proposed that the negative affective state produced by alcohol deprivation can contribute to craving and subsequent relapse behavior after re-exposure to alcohol (Higley et al., 1991; Anton, 1999; Breese et al., 2005). Considerable evidence implicates an imbalance between excitatory and inhibitory neurotransmission among mechanisms that lead to the development of the alcohol withdrawal-induced negative affective state (De Witte, 2004). This includes the demonstrated increases in release of glutamate in the hippocampus (Dahchour and De Witte, 2003), amygdala (Roberto et al., 2004), and nucleus accumbens (Dahchour and De Witte, 2006) after chronic EtOH treatment and withdrawal. Chronic continuous EtOH and/or CIE treatment with subsequent withdrawal also increase the binding levels and activation of NMDARs (Nie et al., 1994; Hu and Ticku, 1995; Hu and Ticku, 1997), presumably as a result of increased expression of various NMDAR subunits (Trevisan et al., 1994; Follesa and Ticku, 1995; Nelson et al., 2005; Roberto et al., 2006). The resultant increases in glutamatergic transmission are exacerbated by the decrements in synaptic GABA_{A}R function (Cagetti et al., 2003), together contributing to the early withdrawal symptoms, as well as the long-term dependence and craving for alcohol.

Our demonstration of the persistent switch in EtOH actions from hippocampal extrasynaptic to synaptic GABA_{A}Rs in CIE rats suggests a possible mechanism by which alcohol dependence may be maintained. In CIE rats, hyperexcitability and anxiety symptoms likely brought on by the demonstrated decreases in the function of synaptic GABA_{A}Rs are effectively relieved by low doses of EtOH, despite tolerance to its sleep-inducing actions. We suggest that the CIE-induced switch of EtOH actions to synaptic GABA_{A}Rs in the appropriate circuits may represent the physiological substrate of its anxiolytic effects, which in vivo leads to enhanced preference for alcohol consumption. The enhanced EtOH action at inhibitory synapses of CIE rats is likely to be further accentuated by the increased EtOH responsiveness at excitatory synapses. Acute EtOH is well known to inhibit NMDARs (Hoffman et al., 1989; Lovinger et al., 1989). After chronic EtOH treatment and withdrawal, the responsiveness of NMDAR-mediated synaptic currents to acute EtOH challenges is increased (Nelson et al., 2005; Roberto et al., 2006). This dual increase in the effectiveness of EtOH at excitatory and inhibitory synapses should have potent physiological effects.

Although the hippocampus plays a role in anxiety (Gray, 1982; Ferreira et al., 1999), we view it as a model circuit for studying alcohol-induced alterations. Clearly, future studies will need to determine whether mechanisms analogous to those de-
scribed here for hippocampal synapses occur in other brain areas, such as amygdala, nucleus accumbens, and prefrontal cortex, which are thought to play key roles in the modulation of stress and drug-seeking behavior (Kooj and Le Moal, 1997; Nestler, 2001; Stevenson and Gratton, 2003).

In summary, our data provide an explanation for the apparent discrepancies between studies that demonstrated an absence of tolerance to acute EtOH potentiation of evoked inhibitory synaptic potentials in brain slice recordings (Kang et al., 1998; Sigmore and Yeh, 2000) and the marked tolerance to the sedative–hypnotic (Khanna et al., 1991), but not anxiolytic (Fig. 1), effects of acute EtOH after chronic EtOH treatment. It also underscores the importance of subunit composition of native receptors in determining the contribution of synaptic or extrasynaptic GABAA receptors to EtOH actions.

References
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