Mechanisms of Reversible GABA_A Receptor Plasticity after Ethanol Intoxication

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The time-dependent effects of ethanol (EtOH) intoxication on GABA_A receptor (GABA_AR) composition and function were studied in rats. A cross-linking assay and Western blot analysis of microdissected CA1 area of hippocampal slices obtained 1 h after EtOH intoxication (5 g/kg, gavage), revealed decreases in the cell-surface fraction of $\alpha 4$ and δ , but not $\alpha 1$, $\alpha 5$, or $\gamma 2$ GABA_AR subunits, without changes in their total content. This was accompanied (in CA1 neuron recordings) by decreased magnitude of the picrotoxin-sensitive tonic current (I_{tonic}), but not miniature IPSCs (mIPSCs), and by reduced enhancement of I_{tonic} by EtOH, but not by diazepam. By 48 h after EtOH dosing, cell-surface $\alpha 4$ (80%) and $\gamma 2$ (82%) subunit content increased, and cell-surface $\alpha 1$ (–50%) and δ (–79%) and overall content were decreased. This was paralleled by faster decay of mIPSCs, decreased diazepam enhancement of both mIPSCs and I_{tonic} , and paradoxically increased mIPSC responsiveness to EtOH (10–100 mM). Sensitivity to isoflurane- or diazepam-induced loss of righting reflex was decreased at 12 and 24 h after EtOH intoxication, respectively, suggesting functional GABA_AR tolerance. The plastic GABA_AR changes were gradually and fully reversible by 2 weeks after single EtOH dosing, but unexplainably persisted long after withdrawal from chronic intermittent ethanol treatment, which leads to signs of alcohol dependence. Our data suggest that early tolerance to EtOH may result from excessive activation and subsequent internalization of $\alpha 4\beta\delta$ extrasynaptic GABA_ARs. This leads to transcriptionally regulated increases in $\alpha 4$ and $\gamma 2$ and decreases in $\alpha 1$ subunits, with preferential insertion of the newly formed $\alpha 4\beta\gamma 2$ GABA_ARs at synapses.

Key words: alcoholism; inhibitory neurotransmission; subunit composition; GABA receptor; tolerance; dependence

Introduction

Numerous studies have demonstrated that acute ethanol (EtOH) potentiates GABA_A receptor (GABA_AR) function, whereas chronic intoxication leads to decreased GABA_AR function (for review, see Kumar et al., 2004). The acute potentiation by EtOH and other allosteric modulators of GABA_ARs is highly dependent on their subunit composition (Olsen and Homanics, 2000; Fritschy and Brunig, 2003). In particular, the $\alpha 4\beta\delta$ GABA_ARs (found predominantly at perisynaptic or extrasynaptic locations) have unusual properties distinct from the more common $\alpha 1$ and $\alpha 2$ subunit-containing GABA_ARs. These include high affinity for GABA, slow desensitization kinetics, benzodiazepine insensitivity and high sensitivity for EtOH, neuroactive steroids, and the partial GABA agonist 4,5,6,7 tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP; also known as gaboxadol) (Brown et al., 2002; Sundstrom-Poromaa et al., 2002; Stell et al., 2003; Wallner et al.,

2003; Wei et al., 2004); however, one group has not been able to reproduce the high EtOH sensitivity of the recombinant $\alpha 4\beta 3\delta$ GABA_ARs (Borghese et al., 2006). The kinetic properties of extrasynaptic $\alpha 4\beta\delta$ GABA_ARs make them particularly suitable for generating perpetual (tonic) inhibition in the presence of low extracellular [GABA] (Mody and Pearce, 2004; Semyanov et al., 2004; Farrant and Nusser, 2005).

Normally, the $\alpha 4$ and δ subunits are expressed at high levels mainly in the thalamus and dentate gyrus, with lower levels in cortex, striatum and other brain areas, including the hippocampal CA1 region (Pirker et al., 2000). Consistent with this distribution, tonic GABA_AR currents of dentate granule cells are more sensitive to potentiation by acute EtOH than similar currents in CA1 pyramidal neurons and this potentiation is lost in δ subunit knock-out mice (Wei et al., 2004; Liang et al., 2006). However, $\alpha 4$ mRNA and protein levels increase after short-term administration or withdrawal from progesterone (Smith et al., 1998; Gulinello et al., 2001), chronic EtOH treatment (Mahmoudi et al., 1997; Matthews et al., 1998), and after status epilepticus (Brooks-Kayal et al., 1998). There is evidence that such increases in $\alpha 4$ content and localization are observed not only in the dentate gyrus (Liang et al., 2006), but also in hippocampal CA1 region and cortex (Matthews et al., 1998; Cagetti et al., 2003; Liang et al., 2004), areas of normally low to moderate $\alpha 4$ content.

Previous studies demonstrated that the mRNA or protein levels of $\alpha 4$ subunits may be subject to very rapid regulation after administration of GABAergic drugs (Birzniece et al., 2006; Sekine

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et al., 2006). We were also intrigued by the possibility that the demonstrated development of rapid EtOH tolerance in rodents (Khanna et al., 1996; Wu et al., 1996; Ludvig et al., 2001) and in humans (Hurst and Bagley, 1972; Fillmore et al., 2005) may be mediated by functional alterations in GABA_ARs. Therefore, we set out to determine whether the function and subunit composition of GABA_ARs may be altered after a single intoxicating dose of EtOH and to examine some of the mechanisms of such alterations in the hippocampal CA1 and dentate gyrus regions.

Materials and Methods

The Institutional Animal Care and Use Committee approved all animal experiments. Male Sprague Dawley rats (190–220 g) were housed in the vivarium under a 12 h light/dark cycle and had *ad libitum* access to food and water. Rats were administered a single dose of EtOH (0.5–5 g/kg, as a 25% w/v solution in distilled water; Pharmco Products, Brookfield, CT) or distilled water (20 ml/kg) by oral intubation, followed by 2–14 d of withdrawal. Alternatively, a chronic intermittent EtOH (CIE) regimen was administered: for the first five doses rats received 5 g/kg, 25% (w/v) solution once every other day and, for the following 55 doses, 6 g/kg EtOH 30% (w/v) once every day. The CIE control group received water (20 ml/kg). At various intervals after EtOH treatment, rats were tested for responsiveness to GABAergic drugs or killed, and tissues prepared for experiments.

Blood [EtOH] assay. Naive rats were anesthetized with isoflurane (2.5–3 minimum alveolar concentration) and surgically implanted with a femoral artery catheter (polyethylene tubing, PE-90 1.27 outer diameter × 0.86 mm inner diameter; Warner Instruments, Hamden, CT). The tubing was exteriorized at the back of the rat's neck, back-filled with 0.1 ml of sterile heparinized saline and sealed. The femoral incision was closed in layers, a topical antibiotic cream (polymyxin B, bacitracin, and neomycin; Alpharma, Baltimore, MD) administered at the incision sites and the rat allowed to recover from anesthesia. Two hours later, rats were administered EtOH either by gavage (5 g/kg) or by intraperitoneal injection (3 g/kg) and blood samples (~70 μl) obtained from the exteriorized catheter at various time points after EtOH administration (2 min, 5 h). After collection of the last blood sample, rats were anesthetized with halothane (~33% via open-drop) and decapitated with a guillotine. Blood samples were centrifuged at 12,000 rpm for 3 min, the plasma supernatant (30 μl) placed in heparinized capillary tubes and stored at –80°C until analysis. The EtOH content of each blood sample was measured in duplicate along with EtOH standards using the alcohol oxidase reaction procedures (GM7 Micro-Stat; Analox Instruments, Lunenburg, MA).

LORR assay. The sedative effects of diazepam (10 mg/kg, i.p.) or isoflurane (5 ml, inhalation) were tested on vehicle and EtOH-treated rats. After diazepam administration, duration of loss of righting reflex (LORR) was noted and rats were placed on their backs in a V-shaped trough. LORR time ended when animals were able to flip over three times in 30 s after being repeatedly placed on their backs. Isoflurane (5 ml) was placed in a large desiccator containing the rat, the lid closed and the latency to LORR noted. Once completely anesthetized, the rat was decapitated and brain slices prepared for experiments.

Electrophysiological recordings. Transverse slices (400 μm thick) of rat dorsal hippocampus were obtained using standard techniques (Kang et al., 1996). Whole-cell patch-clamp recordings were obtained from cells located in the CA1 pyramidal or dentate granule cell layers at 34 ± 0.5°C during perfusion with artificial CSF (ACSF) composed of (in mM) 125 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, and 10 D-glucose. The ACSF was continuously bubbled with a 95/5% mixture of O₂/CO₂ to ensure adequate oxygenation of slices and a pH of 7.4. Patch pipettes contained (in mM) 135 cesium gluconate, 2 MgCl₂, 1 CaCl₂, 11 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 2 K₂ATP, and 0.2 Na₂GTP, pH adjusted to 7.25 with CsOH. GABA_AR-mediated mIPSCs were pharmacologically isolated by adding tetrodotoxin (TTX; 0.5 μM), D(-)-2-amino-5-phosphonopentanoate (APV; 40 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), and CGP 54626 (1 μM) to

the ACSF from stock solutions. Stock solutions of CGP 54626 were made with pure dimethyl sulfoxide (DMSO). Final [DMSO] did not exceed 42 μM in the recording chamber. Signals were recorded in voltage-clamp mode with an amplifier (Axoclamp 2B; Molecular Devices, Union City, CA). Whole-cell access resistances were in the range of 8–20 MΩ before electrical compensation by ~70%. During voltage-clamp recordings, access resistance was monitored by measuring the size of the capacitive 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 (Digidata 1200B, Molecular Devices), and analyzed using the Clampfit software (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: 5 pA, amplitude and 20 fC charge transfer. The 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 were obtained from analysis of the averaged chosen single events (>120 events/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 of a given recording period. The investigator performing the recordings and mIPSC analysis was blind to the treatment (vehicle, EtOH, or CIE) that the rats received.

Membrane preparation and Western blotting. Discrete brain regions were microdissected from individual 400-μm-thick brain sections, and incubated in a small volume chamber with or without the protein cross-linking reagent, bis(sulfosuccinimidyl)suberate (BS³) in ACSF at 4°C according to (Grosshans et al., 2002). BS³ is bifunctional and crosslinks all proteins exposed to the medium (i.e., cell-surface proteins). These large complexes do not enter the gel and are retained at the top; thus, the band of protein at the identified molecular weight corresponds to that fraction that is intracellular only. The difference between that value and the amount from an equivalent adjacent slice, untreated, and thus total, represents the surface pool. After incubation of brain sections in the absence (total) or presence (intracellular) of BS³, sections were washed three times with Tris wash buffer, pH 7.6, to wash away free BS³. The washed sections were homogenized in a buffer composed of 1% SDS, 1 mM EDTA, and 10 mM Tris, pH 8.0. Protein content was measured by the Bio-Rad (Hercules, CA) D_C protein assay system. Protein aliquots (40 μg) from samples were separated on 10% SDS-PAGE under reducing conditions using the Bio-Rad Mini-Protein 3 cell system. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (immuno-Blot PVDF membrane, 0.2 mm; Bio-Rad) with Transblot SD semidry transfer cell system (Bio-Rad). Blots were probed with anti-peptide α1 (amino acids 1–9), α2 (amino acids 416–424), α4 (amino acids 379–421), γ2 (amino acids 319–366), or δ (amino acids 331–430; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies (all at 1 mg/ml), followed by HRP-conjugated anti-rabbit or anti-mouse secondary antibody (Ab) (1:5000 dilution) and bands detected by ECL detection kit (GE Healthcare, Little Chalfont, UK), apposed to x-ray film under nonsaturating conditions. W. Sieghart and colleagues (Medical University of Vienna, Vienna, Austria) kindly provided the α1, α2, α4, α5 and γ2 GABA_AR subunit antibodies. A β-actin Ab (Sigma, St. Louis, MO) was used as a loading control in all experiments. The investigator performing the Western blots and analysis was blind to the treatment (vehicle, CIE, or single dose EtOH) that the rats received. Bands from different samples corresponding to the appropriate subunit were analyzed and absorbance values compared by densitometry using C-Imaging analysis systems (Complex, Cramberry Township, PA). Group differences were evaluated by *t* test. *p* < 0.05 was considered statistically significant.

Figure 1. Decreased potentiation of the GABA_AR-mediated tonic current and enhanced potentiation of mIPSCs by acute EtOH 2 d after withdrawal from a single intoxicating dose of EtOH. **A**, Examples of individual CA1 neuron recordings from vehicle-treated (top) and EtOH (5 g/kg)-treated rats (bottom traces). The I_{hold} needed to clamp the voltage at 0 mV before EtOH application is indicated by a dashed line. In a control recording, the kinetics of mIPSCs (top traces) averaged over the indicated 100 s periods during continuous recordings (bottom trace) are unaffected by 100 mM EtOH, whereas I_{hold} is visibly potentiated. Subsequent application of picrotoxin (50 μ M) reveals the GABA_AR-mediated tonic current component (I_{tonic}). Two days after EtOH intoxication there is a loss of I_{hold} potentiation (bottom trace), whereas mIPSCs are visibly potentiated even by 10 mM EtOH. **B**, I_{hold} is significantly potentiated by acute application of 50 and 100 mM EtOH from vehicle-treated rats. Each point represents a mean $\pm$ SEM value from three to seven neurons (2–3 rats/group). **C**, From the same recordings as in **B**, mIPSCs from vehicle-treated rats are relatively insensitive to EtOH, whereas after EtOH intoxication mIPSCs are greatly potentiated by 10–100 mM EtOH. * $p < 0.05$ between vehicle and EtOH groups; † $p < 0.05$ from pre-EtOH value (two-way repeated measures ANOVA).

Results

Altered EtOH responsiveness of synaptic and tonic GABA_AR currents after EtOH intoxication

We first measured the mIPSCs and tonic currents and their responsiveness to acute EtOH (10–100 mM) in CA1 neurons after vehicle treatment or after withdrawal from a single intoxicating dose of EtOH (5 g/kg, by gavage) (Fig. 1). We also used picrotoxin application to evaluate the magnitudes of tonic GABA_AR currents during these recordings. In these and all subsequent experiments, membrane voltage was clamped at 0 mV and the initial extracellular solution containing TTX and glutamate and GABA_B receptor blockers was applied for at least 10 min. Acute EtOH (50–100 mM) significantly increased the picrotoxin-sensitive holding current (I_{hold}) by 7–33% in CA1 neurons from vehicle-treated rats (Fig. 1B). This potentiation was absent in CA1 neurons from rats at 2 d withdrawal after a single EtOH dose (5 g/kg). In contrast, the mIPSCs of vehicle-treated rats were only slightly (6%) potentiated by 100 mM EtOH, whereas after 2 d withdrawal

from EtOH-intoxication we observed 53–101% potentiation of mIPSCs by 10–100 mM acute EtOH (Fig. 1C).

Subsequent recordings revealed that mIPSC sensitivity to EtOH remained minimal at 1 h after EtOH intoxication yet was maximal by 12 h to 2 d later, with recovery to control levels after 2 weeks (Fig. 2A,B). In contrast, the I_{hold} potentiation by EtOH (100 mM) was lost as early as 1 h after EtOH intoxication, but gradually recovered to control levels by 2 weeks after the single EtOH dose (Fig. 2C). Importantly, the sensitivity of both mIPSCs and I_{hold} to EtOH remained altered long after withdrawal from CIE treatment (Fig. 2).

Faster mIPSC decay and decreased tonic current magnitude after EtOH intoxication

Analysis of mIPSC kinetics revealed faster mIPSC decay in CA1 neurons from rats by 12 h (but not at 1 h) after the single EtOH dose (Fig. 3A). This resulted in significantly ($p < 0.05$) reduced total charge transfer of averaged mIPSCs from EtOH-treated rats. The decrease was maximal at 2 d and thereafter gradually recovered to control values by 14 d after EtOH dosing. The magnitude of the picrotoxin-sensitive tonic current was decreased by ~45% at 1 h after EtOH intoxication, remained decreased for 4 d, but recovered to control levels 7–14 d later (Fig. 3B). This contrasted with the persistent decrement in I_{tonic} magnitude in CA1 neurons from rats exposed to CIE treatment and 120 d of withdrawal (Fig. 3B).

Altered cell-surface and intracellular levels of GABA_AR subunits after EtOH intoxication

Based on the similarity of electrophysiological changes after single dose EtOH or CIE treatment, we suspected that GABA_AR subunit composition may also be similarly affected (Cagetti et al., 2003). Because different hippocampal regions express different levels of various GABA_AR subunits (Pirker et al., 2000), we measured changes in subunit proteins selectively from the microdissected CA1 (Fig. 4) and dentate gyrus regions (supplemental Fig. 4E, available at www.jneurosci.org as supplemental material) of hippocampal slices. By comparing blots of microdissected slices incubated with or without the membrane-impermeable cross-linking reagent BS³, we were able to identify the intracellular and, indirectly, the cell surface pools of GABA_AR subunits (Grosshans et al., 2002). Cell surface proteins form high molecular weight aggregates with BS³, such that they do not reliably enter the gel (Fig. 4B). In contrast, intracellular proteins are not accessed by the membrane-impermeant reagent and thus can be quantified through Western blot analysis. Subtraction of the intracellular protein from the total protein estimates the cell surface protein content. Analysis revealed a clear increase in the intracellular $\alpha 4$ subunit protein fraction at 1 h after EtOH admin-

istration (Fig. 4C,D). These increases occurred at the expense of the cell-surface $\alpha 4$ subunit content because total content was unchanged at 1 h after EtOH intoxication. At 2 d after EtOH, there was a large increase in the surface $\alpha 4$ subunit protein which gradually returned to near control levels at 7 d (Fig. 4C,D). In contrast, intracellular and total $\alpha 1$ subunit content was unchanged at 1 h after EtOH, but at 2 d after single-dose EtOH intoxication, both intracellular and surface content were significantly reduced, with recovery to control levels by 14 d (Fig. 4C,D). Unlike the reversible changes observed after single dose EtOH, the $\alpha 4$ subunit levels remained elevated, whereas the $\alpha 1$ subunit levels remained decreased in rats withdrawn from CIE treatment for 40 d (Fig. 4D). Similar alterations in $\alpha 4$ and $\alpha 1$ GABA_AR subunits were observed in the microdissected dentate gyrus (supplemental Fig. 4E, available at www.jneurosci.org as supplemental material).

The mechanisms by which the relative abundance and localization of specific GABA_AR subunits are altered by EtOH treatment are not known. However, selective endocytosis and/or recycling of receptors based on subunit composition and sensitivity to protein kinase C phosphorylation may be involved (Ali and Olsen, 2001; Kumar et al., 2003; Kittler et al., 2005). We hypothesized that the rapid development of tolerance to acute EtOH potentiation of the tonic current in CA1 neurons may be attributable to the internalization of the $\alpha 4\beta\delta$ extrasynaptic GABA_ARs, which are sensitive to low [EtOH] (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). This was in agreement with the increased intracellular $\alpha 4$ subunit levels observed at 1 h after EtOH intoxication (Fig. 4C,D). Therefore, we also measured the alterations in δ subunit protein, a substantial fraction of which normally coassembles with the $\alpha 4$ subunits to form extrasynaptic or perisynaptic receptors (Wei et al., 2003, 2004; Sun et al., 2004; Jia et al., 2005; Liang et al., 2006). A large increase in the intracellular fraction for the δ subunit was observed at 1 h after EtOH compared with vehicle controls (Fig. 5). In the absence of changes in the total δ fraction this corresponds to a 60% decrease in cell surface δ subunits (Fig. 5B). At 2 d after EtOH, the surface δ subunit fraction was further reduced to 17% of control values, with return to control levels measured at 14 d after single dose EtOH (Fig. 5B).

We next reasoned that when δ subunit levels are diminished, the most likely partner for the transcriptionally upregulated $\alpha 4$ subunit would be the $\gamma 2$ subunit. This subunit was shown previously to be increased after genetic deletion or seizure-induced decreases in the δ subunit (Peng et al., 2002, 2004). Therefore, we measured the changes in $\gamma 2$ subunit levels after EtOH intoxication. At 1 h after EtOH, we could not detect significant changes in the intracellular or total $\gamma 2$ subunit fractions (Fig. 5B). However, at 2 d after EtOH administration there was a large increase (82%) in the total, as well as cell surface $\gamma 2$ subunit fractions, with return to control levels measured at 14 d. These data suggested that shortly after EtOH intoxication, the $\alpha 4\beta\delta$ extrasynaptic

Figure 2. Changes in acute EtOH sensitivity of synaptic and tonic GABA_AR currents after EtOH intoxication. **A**, Traces are superimposed averages of mIPSCs obtained from analysis of 100 s recording segments from CA1 neurons under basal conditions and after acute application of different [EtOH]. The numbers denote applied [EtOH] (mM). Note the faster decay of mIPSCs and the appearance of EtOH sensitivity 2 d after a single EtOH (5 g/kg) dose. Sensitivity to EtOH disappears 14 d later. In contrast, mIPSCs from a CIE-treated rat decay faster and maintain EtOH sensitivity even after long-term withdrawal. **B**, Graph of acute EtOH effect (100 mM) on mIPSC charge transfer in hippocampal slices from rats treated with a single dose of saline, EtOH, or CIE. Data are mean $\pm$ SEM of values from three to eight neurons obtained at 1 h, 12 h, and 2, 4, 7, 14, and 120 d after respective treatments. Note the lack of mIPSC potentiation by EtOH after saline treatment and the significant ($p < 0.05$, one-way ANOVA) reversible potentiation first observed at 12 h, but not at 1 h after a single intoxicating EtOH dose. **C**, Graph of acute EtOH effect on the tonic I_{hold} from the same recordings as in **B**. Note the reversible loss of I_{hold} responsiveness to acute EtOH first observed at 1 h after a single dose EtOH treatment. Tolerance to the I_{hold} potentiation by acute EtOH persists after long-term withdrawal from CIE treatment.

GABA_ARs are internalized, followed within several hours by reversible increases in $\alpha 4\beta\gamma 2$ GABA_ARs and concomitant decreases in the $\alpha 1$ -containing GABA_ARs.

In CA1 pyramidal neurons, tonic currents have been proposed to be mediated primarily by $\alpha 5$ subunit-containing GABA_ARs (Caraiscos et al., 2004). To examine the involvement of these subunits in EtOH-induced GABA_AR plasticity, we measured the levels of $\alpha 5$ subunit protein in Western blots in microdissected CA1 area sections at 1 h, and 2 and 14 d after EtOH (5 g/kg) intoxication. Very small increases in the cell surface levels of $\alpha 5$ subunits were obtained as a consequence of small decreases in the intracellular fractions, without changes in total content (Fig. 6). Given these minor changes in $\alpha 5$ subunit protein and the previously demonstrated lack of $\alpha 5$ mRNA alterations at 2 d after CIE treatment (Mahmoudi et al., 1997), CIE specimens after 40 d of withdrawal were not examined.

Diazepam tolerance of GABA_AR currents after EtOH intoxication

To provide pharmacological evidence for the specificity of the EtOH-induced GABA_AR subunit plasticity we tested the mIPSC and I_{tonic} responsiveness to acute diazepam (DZ; 3 μ M) application in CA1 neurons from rats at various times after single dose EtOH (5 g/kg) administration. Because DZ has no agonist activity at $\alpha 4$ -containing GABA_ARs but potentiates other $\alpha\beta\gamma 2$ GABA_ARs (Möhler et al., 2000), we expected that increased $\alpha 4$ or decreased $\alpha 1$ subunit levels would result in diminished potentiation of currents mediated by either synaptic or extrasynaptic GABA_ARs. In slices from rats taken at 1 h after EtOH administration, DZ increased the charge transfer of mIPSCs by prolonging

Figure 3. Time course of changes in mIPSC charge transfer and tonic current magnitude after EtOH intoxication. **A**, Graph of the mIPSC charge transfer recorded in the absence of added allosteric modulators at various times after EtOH intoxication. Data are mean $\pm$ SEM of values from three to 10 neurons obtained at 1 h, 12 h, and 2, 4, 7, 14, and 120 d after respective treatments. $*p < 0.05$ from vehicle-treated controls (one-way ANOVA). Note the progressive decrease in mIPSC charge transfer after EtOH intoxication, which peaks at 2 d and recovers by 14 d, unlike mIPSCs from CIE rats. **B**, Graph of time-dependent changes in the picrotoxin-sensitive tonic current (I_{tonic}) after EtOH intoxication. Data are mean $\pm$ SEM of values from three to nine neurons obtained at 1 h, 12 h, and 2, 4, 7, 14, and 120 d after respective treatments. $*p < 0.05$ from vehicle-treated controls (one-way ANOVA). Note that I_{tonic} is already reduced at 1 h after EtOH intoxication. I_{tonic} remains diminished after long-term withdrawal from CIE treatment.

their decay to the same extent ($\sim 60\%$) as in those from vehicle-treated controls (Fig. 7*A,B*). In contrast, at 2 d after EtOH administration, mIPSC potentiation by DZ was reduced to $<10\%$, with gradual recovery to control levels by 14 d after EtOH administration. In recordings from rats after long-term (40 d) withdrawal from CIE treatment, DZ had no effect on mIPSCs (Fig. 7*A,B*).

Diazepam potentiation of tonic currents was also altered by EtOH intoxication. Maximal potentiation of I_{tonic} was observed in recordings from vehicle-treated controls (Fig. 7*C*). At 1 h after a single EtOH dose, DZ potentiation of I_{tonic} was unaffected, but maximal decreases occurred at 2 d after EtOH with gradual recovery to control levels by 14 d after EtOH. In contrast, DZ did not potentiate I_{tonic} in CA1 neurons from rats withdrawn for 40 d from CIE treatment (Fig. 7*C*).

Dose dependence of EtOH-induced tolerance to sedative/anesthetic effects of diazepam

To further explore the consequences of the altered GABA_AR subunit composition and function we wanted to test diazepam in the LORR assay because we expected that the EtOH-induced $\alpha 1$ and $\alpha 4$ subunit alterations would be relevant to behavioral sensitivity to diazepam, similar to its effects on GABA_AR-mediated mIPSCs and tonic currents. We also wanted to determine the dose dependence of EtOH-induced tolerance to diazepam. To that end, we first measured plasma [EtOH] in naive rats at different times after

the intoxicating 5 g/kg gavage EtOH dose. For comparison, we also measured plasma [EtOH] after the 3 g/kg intraperitoneal injection, previously used in the LORR assay to study EtOH tolerance after CIE treatment (Liang et al., 2006). High peak [EtOH] was observed within 2 min of the 3 g/kg dose (Fig. 8*A*) because intraperitoneal injection bypasses the initial gastric and hepatic metabolism by alcohol dehydrogenase (Pastino and Conolly, 2000). In contrast, peak [EtOH] of ~ 60 mM was reached only by 1 h after gavage administration thereafter declining at the same rate as the 3 g/kg intraperitoneal dose. Interestingly, LORR lasts for ~ 37 min after a 3 g/kg intraperitoneal EtOH dose (Liang et al., 2006), but LORR was not observed after the 5 g/kg gavage dose, although rats do appear sedated (Mahmoudi et al., 1997).

Next, we treated groups of rats with different doses of EtOH (0.5–5 g/kg, gavage) followed 24 h later by the LORR test with diazepam (10 mg/kg, i.p.). There was a clear dose dependence to the diazepam tolerance in that pretreatment with EtOH doses lower than 2.5 g/kg had no discernible effects on reducing LORR duration. The 5 g/kg EtOH dose resulted in the most profound decrease in duration of LORR compared with vehicle-treated controls (Fig. 8*B*).

Reversible tolerance to diazepam and isoflurane after single dose EtOH intoxication

We next measured the time dependence of diazepam tolerance after single dose EtOH (5 g/kg) intoxication. The results showed that there was a relatively rapid recovery from diazepam tolerance, such that by 4 d after EtOH the duration of diazepam-induced LORR was similar to that of vehicle-treated controls, with full recovery evident by 14 d (Fig. 9*A*). In contrast, significant tolerance to diazepam persisted after 40 d of withdrawal from CIE treatment.

Next we examined the effects of EtOH intoxication on the sensitivity of rats to the volatile general anesthetic, isoflurane, in the LORR assay. As expected, rats were slightly more sensitive to isoflurane exposure at 1 h after the single EtOH dose (Fig. 9*B*). However, a significant increase in the latency to LORR could be seen at 12 h after EtOH exposure. This tolerance to isoflurane induction of LORR was quickly reversible, returning to control levels by 2 d after single dose EtOH intoxication. In contrast, rats withdrawn from CIE treatment for 40 d exhibited significantly longer latencies to LORR for isoflurane than the vehicle-treated controls.

Discussion

This study demonstrates for the first time that a single intoxicating EtOH dose produces major changes in GABA_AR subunit composition and function, which are accompanied by altered behavioral and *in vitro* pharmacological sensitivity to diazepam, isoflurane and EtOH itself. Importantly, these changes are fully reversible by 2 weeks or less after intoxication, unlike the persistent alterations induced by CIE treatment. Acute tolerance to EtOH enhancement of GABA_AR-mediated I_{tonic} is observed within 1 h after *in vivo* EtOH exposure, suggesting that this physiological mechanism may underlie the acute behavioral tolerance to EtOH observed in animals and humans, (Khanna et al., 1996; Fillmore et al., 2005).

Tonic inhibitory currents are thought to be mediated by extrasynaptic GABA_ARs activated by low ambient [GABA] as well as by GABA spillover from presynaptic sites (Mody and Pearce, 2004; Semyanov et al., 2004; Farrant and Nusser, 2005). Evidence indicates that the relative densities and subunit composition of extrasynaptic GABA_ARs is quite different from that of synaptic

GABA_ARs (Nusser et al., 1995; Devor et al., 2001). Functionally, extrasynaptic GABA_ARs activate at lower [GABA] and desensitize more slowly than the synaptic GABA_ARs (Brickley et al., 1999; Bai et al., 2001). Noteworthy, tonic current detection is clearly influenced by the brain region under investigation, as well as the recording conditions. For example, some investigators detect I_{tonic} in CA1 neurons only after artificially increasing ambient [GABA] (cf. Semyanov et al., 2004), whereas we and others routinely record pharmacologically isolated tonic currents in these neurons without increasing ambient [GABA] (Bai et al., 2001; Bieda and MacIver, 2004; Liang et al., 2004, 2006).

The acute EtOH tolerance of extrasynaptic GABA_ARs could be mediated by various mechanisms common to ionotropic receptors. In our recording conditions of maintained ion gradients, I_{tonic} desensitization was not observed during acute (8–30 min) application of EtOH, diazepam or other allosteric modulators in the current or previous studies (Liang et al., 2004, 2006). However, at 1 h after EtOH intoxication, decreased magnitude of I_{tonic} and its potentiation by EtOH were accompanied by selective decreases in the surface content of $\alpha 4$ and δ subunits. Given that the $\alpha 4\beta\delta$ GABA_ARs are particularly sensitive to EtOH (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), we propose that both the reduced magnitude and the rapid tolerance to acute EtOH potentiation of I_{tonic} are mediated by the selective internalization of $\alpha 4\beta\delta$ GABA_ARs. This is reinforced by the demonstration that the cell-surface and intracellular levels of the abundantly expressed $\alpha 1$, $\alpha 5$, and $\gamma 2$ subunits are unaltered at this time. Moreover, at 1 h after EtOH intoxication, the remaining I_{tonic} shows no tolerance to diazepam. We suspect that this remaining I_{tonic} is partly mediated by the $\alpha 5$ -containing extrasynaptic GABA_ARs, which contribute to I_{tonic} in CA1 pyramidal neurons (Caraiscos et al., 2004), and whose expression appears unaltered by single dose EtOH intoxication (this study) or CIE treatment (Mahmoudi et al., 1997). By 2 d after EtOH intoxication, consistent with maximal upregulation of the diazepam-insensitive $\alpha 4$ and maximal decreases in diazepam-sensitive $\alpha 1$ subunits (Möhler et al., 2000); diazepam potentiation of I_{tonic} is also maximally reduced. Collectively and in agreement with our previous reports (Cagetti et al., 2003; Liang et al., 2004, 2006) these results indicate that some I_{tonic} in CA1 pyramidal cells is normally carried by GABA_ARs containing subunits other than $\alpha 5$, such as $\alpha 1$ and $\alpha 4$. Single-channel studies in native tissues with select phar-

Figure 4. Reversible changes in $\alpha 4$ and $\alpha 1$ subunit proteins in microdissected CA1 region after single EtOH dosing. **A**, Schematic of slice microdissection procedures. Dashed lines represent microblade cuts. Arrow points to the hippocampal fissure. **B**, Examples of gels from the microdissected CA1 region incubated with ACSF or with the BS³ cross-linking reagent. BS³-linked cell-surface $\alpha 4$ protein is present as high molecular weight aggregates (arrow) that do not reliably enter the gel. In contrast, gel migration of the intracellular protein β -actin is unaffected. **C**, Western blots of $\alpha 4$ and $\alpha 1$ subunit protein at 1 h and 2 and 14 d after a single dose of vehicle or EtOH (5 g/kg; gavage). β -Actin was used as a loading control. Tot, Total protein (incubation with ACSF); Int, intracellular protein fraction (incubation with BS³). Note the increased $\alpha 4$ intracellular signal at 1 h after EtOH. At 2 d after EtOH, the Tot lane $\alpha 4$ increases and $\alpha 1$ signal decreases compared with vehicle. These differences are not seen at 14 d. **D**, Summary graph of changes in cell-surface $\alpha 4$ and $\alpha 1$ subunit content after single dose EtOH and CIE treatments relative to vehicle-treated controls (dashed line). Data are mean $\pm$ SEM from vehicle, single dose EtOH, or CIE treatments ($n = 4$ –5 rats/treatment). * $p < 0.05$ (t test) compared with vehicle-treated controls. Note the persistence of changes after long-term withdrawal from CIE treatment.

macological agents (Lindquist and Birnir, 2006), as well as studies with $\alpha 4$ knock-out mice (Chandra et al., 2006) should help resolve these issues.

The development of faster mIPSC decay and acute EtOH sen-

Figure 5. Reversible changes in δ and $\gamma 2$ subunit proteins in microdissected CA1 region after single EtOH dosing. **A**, Examples of Western blots of δ and $\gamma 2$ subunits at 1 h and 2 and 14 d after a single dose of vehicle or EtOH (5 g/kg, gavage). β -Actin was used as a loading control. Tot, Total protein (incubation with ACSF); Int, intracellular protein fraction (incubation with BS³). Note the large increases in the intracellular δ signal at 1 h and 2 d after EtOH. In contrast, a large increase in the total $\gamma 2$ signal is seen at 2 d after EtOH compared with vehicle. These differences are not seen at 14 d. **B**, Summary graph of changes in cell-surface δ and $\gamma 2$ subunit content after single dose EtOH and CIE treatments relative to vehicle-treated controls (dashed line). Data are mean $\pm$ SEM from vehicle, single dose EtOH, or CIE treatments ($n = 4$ –5 rats/treatment). * $p < 0.05$ (t test) compared with vehicle-treated controls. Note that the subunit alterations are maintained long after withdrawal from CIE treatment.

Figure 6. Minor changes in $\alpha 5$ subunit protein in microdissected CA1 region after single EtOH dosing. Examples of $\alpha 5$ subunit Western blots at 1 h and 2 and 14 d after a single dose of vehicle or EtOH (5 g/kg, gavage) are shown. β -Actin was used as a loading control. Tot, Total protein (incubation with ACSF); Int, intracellular protein fraction (incubation with BS³). Calculated cell surface $\alpha 5$ subunit levels were at 1 h ($117 \pm 4\%$; n.s., $n = 4$), at 2 d ($127 \pm 4\%$; $p < 0.05$, $n = 4$), and at 14 d ($116 \pm 2\%$; n.s., $n = 4$) of vehicle controls. Note the absence of differences in the Tot lane optical densities, whereas small decreases in the intracellular protein fractions account for the changes in cell-surface subunit content after EtOH intoxication.

sitivity of mIPSCs after CIE treatment is thought to involve insertion of “new” $\alpha 4$ -containing GABA_ARs into synaptic locations (Liang et al., 2006). This also leads to the loss of mIPSC potentiation by diazepam, which has no agonist activity at $\alpha 4$ -containing GABA_ARs (Möhler et al., 2000), and enhanced potentiation by Ro15–4513 (Cagetti et al., 2003), which is a positive modulator of $\alpha 4\beta\gamma 2$, but not $\alpha 4\beta\delta$ GABA_ARs (Brown et al., 2002). Here, we demonstrate that the alterations in mIPSC kinet-

Figure 7. Time course of changes in diazepam sensitivity of synaptic and tonic GABA_A currents after EtOH treatment. **A**, Traces are superimposed averages of mIPSCs obtained from analysis of 100 s recording segments from CA1 neurons under basal conditions and after acute application of DZ (3 μ M). The numbers denote applied [DZ] (μ M). Note the faster decay of mIPSCs and the loss of DZ sensitivity at 2 d, but not 1 h, after a single EtOH (5 g/kg) dose. Sensitivity to DZ is fully restored 14 d later. In contrast, mIPSCs from a CIE-treated rat decay faster and remain DZ-insensitive even after long-term withdrawal. **B**, Graph of acute DZ effect on mIPSC charge transfer in hippocampal slices from rats treated with a single dose of vehicle, EtOH, or CIE. Data are mean $\pm$ SEM of values from three to ten neurons obtained at 1 h and 2, 4, 7, 14, and 40 d after respective treatments. Note the reversible reduction of mIPSC potentiation by DZ at 2 d, but not at 1 h after a single intoxicating EtOH dose. **C**, Graph of DZ effect on the picrotoxin-sensitive tonic current (I_{tonic}) from the same recordings as in **B**. Note the maintained I_{tonic} responsiveness to DZ at 1 h, followed by a large decrease at 2 d and gradual recovery by 14 d after single dose EtOH intoxication. Tolerance to the I_{tonic} potentiation by DZ persists long after withdrawal from CIE treatment. * $p < 0.05$ (one-way ANOVA or t test) compared with vehicle-treated controls.

Figure 8. EtOH dose dependence of diazepam cross-tolerance. **A**, **B**, Plasma [EtOH] after intragastric (5 g/kg, open circles) or intraperitoneal (3 g/kg, closed triangles) administration. Data are shown from 1 rat/dose for clarity. Note the high peak plasma [EtOH] achieved within 2 min after intraperitoneal injection compared with the slow LORR duration (**B**; mean $\pm$ SEM) induced by diazepam (10 mg/kg, i.p.) in rats ($n = 5$ –7/point) treated 24 h previously with different doses of EtOH (0–5 g/kg, gavage). Note the complete lack of tolerance to diazepam with EtOH doses ≤ 2.0 g/kg. * $p < 0.05$ (one-way ANOVA or t test) compared with vehicle-treated controls.

ics and pharmacology occur gradually (over 12–48 h) after EtOH intoxication, are temporally separable from the early decreases in I_{tonic} (visible at 1 h), and coincide with the increased cell-surface expression of $\alpha 4$ and $\gamma 2$ and decreased expression of $\alpha 1$ subunits. This suggests that $\alpha 4\beta\gamma 2$ are the newly inserted synaptic GABA_ARs.

If EtOH intoxication results in $\alpha 4\beta\delta$ replacement by extrasynaptic $\alpha 4\beta\gamma 2$, which then moves into synapses where it becomes highly sensitive to EtOH, it begs the question of why this subunit combination gains EtOH-sensitivity at synaptic but not extrasynaptic sites. One possibility is that synaptic anchoring proteins

Figure 9. *A, B*, Time course of diazepam (*A*) and isoflurane (*B*) tolerance after single dose EtOH intoxication. *A*, Each point is mean $\pm$ SEM of LORR duration values obtained from rats ($n = 5$ – 12 /point) after a single dose of vehicle, EtOH (5 g/kg, gavage) or CIE treatments followed at 1, 2, 4, 7, 14, or 40 d by diazepam (10 mg/kg, i.p.). Note the profound tolerance to diazepam at 1 d after EtOH intoxication with gradual recovery by 2 weeks. Significant tolerance to DZ persists long after withdrawal from CIE treatment. *B*, Each point is mean $\pm$ SEM of time to LORR values obtained from rats ($n = 4$ – 5 /group) exposed to isoflurane at 1, 2, 4, 7, or 14 d after a single dose of vehicle or EtOH (5 g/kg, gavage), or 40 d after CIE treatment. * $p < 0.05$ (one-way ANOVA or t test) compared with vehicle-treated controls.

confer EtOH sensitivity only to synaptic $\alpha 4 \beta \gamma 2$ GABA_ARs. If so, disrupting the anchoring protein-GABA_AR interaction might decrease synaptic EtOH sensitivity. Another possibility is that the allosteric potentiation of synaptic GABA_ARs by EtOH is dependent on the applied [GABA], becoming apparent only at the high [GABA] experienced by synaptic GABA_ARs. This possibility may be tested with recombinant $\alpha 4 \beta \gamma 2$ GABA_ARs by examining their EtOH sensitivity in the presence of high [GABA] (e.g., 10–100 μ M).

The mechanisms by which the relative abundance and localization of specific GABA_AR subunits are altered by EtOH intoxication are unknown. However, phosphorylation (protein kinase C)-dependent subunit-selective receptor endocytosis and/or recycling may be involved (Ali and Olsen, 2001; Kumar et al., 2003; Kittler et al., 2005). GABA_AR assembly may be partly based on subunit availability, especially in the unnatural environment of recombinant expression in heterologous cells; however, distinct assembly signals in certain subunits appear to drive formation of preferred receptor subunit compositions (Bollan et al., 2003). One possibility for the current observations is that decreased $\alpha 1$ subunit content after EtOH intoxication and withdrawal permits $\beta 3$ to assemble with $\alpha 4$ and $\gamma 2$ or δ subunits. However, gene-targeted $\alpha 1$ subunit deletion in mice does not affect expression of $\alpha 4$ or δ , but increases $\alpha 2$ and $\alpha 3$ subunit expression (Sur et al., 2001; Kralic et al., 2002). Also, deletion of the δ subunit increases $\gamma 2$, but decreases $\alpha 4$ subunit levels in mice (Korpi et al., 2002; Peng et al., 2002). Based on such data, the upregulation of $\alpha 4$ subunits after EtOH intoxication and withdrawal is unlikely to

Figure 10. Schematic of hypothesized mechanism of EtOH-induced GABA_AR plasticity. High [EtOH] exposure leads to internalization of overactivated extrasynaptic receptors (i.e., $\alpha 4 \beta 3 \delta$, green) which may explain the acute functional tolerance to EtOH and the decreased magnitude of tonic currents. Compensation is by insertion of the readily inducible $\alpha 4 \beta \gamma 2$ GABA_ARs (red) from intracellular stores. The increased surface $\alpha 4 \beta \gamma 2$ inserted exocytotically at extrasynaptic sites, quickly moves into the synaptic membrane by mass action, changing the kinetics and pharmacology of mIPSCs. The $\alpha 1 \beta \gamma 2$ pentamers (purple) are removed from the surface synaptic, and also to some extent extrasynaptic, pools because of crowding out by $\alpha 4 \beta \gamma 2$. This switch from $\alpha 1$ - to $\alpha 4$ -containing GABA_ARs results in cross-tolerance to benzodiazepines (BZ) and alters mIPSC kinetics. Thus, the GABA_AR $\alpha 4$ participates in a critical compensation, serving as an emergency brake, but the new GABA_ARs have physiological properties different from normal and are “less functional” under certain conditions.

occur simply from the downregulation of $\alpha 1$ or δ subunits. Instead, we suspect that it is the insertion of the readily inducible $\alpha 4 \beta \gamma 2$ GABA_ARs which crowds out the $\alpha 1 \beta \gamma 2$ GABA_ARs from the surface, synaptic and also to some extent, extrasynaptic pools. Figure 10 illustrates the proposed scheme of subunit reorganization after EtOH intoxication.

Withdrawal from chronic EtOH or CIE treatment in rats is characterized by behavioral hyperexcitability which includes heightened anxiety (Cagetti et al., 2003), decreased seizure thresholds (Hunter et al., 1973; Kokka et al., 1993), and sleep disorders (Mendelson et al., 1978; Ehlers and Slawewski, 2000). Analogous symptoms are exhibited by alcoholics during withdrawal (Roehrs and Roth, 2001; Bayard et al., 2004). The similarity in GABA_AR alterations after chronic or single dose EtOH suggests that withdrawal symptoms should be detectable after single dose EtOH. Indeed, transient decreases in seizure thresholds (Goldstein, 1972; Mucha and Pinel, 1979) and heightened anxiety (Doremus et al., 2003; Zhang et al., 2007) have been documented after single-dose EtOH intoxication in rodents. Although we are unaware of published reports, it seems reasonable to suggest that transient sleep disturbances should also be detectable after a single intoxicating EtOH dose.

Development of cross-tolerance to barbiturates and benzodiazepines after chronic EtOH treatment is well documented (Khanna et al., 1998). Cross-tolerance to diazepam and isoflurane is detectable 12–24 h after single EtOH dosing (Fig. 8). Moreover, cross-tolerance is clearly dose dependent; only EtOH

doses >2 g/kg produced detectable cross-tolerance to diazepam. After an intragastric dose of 2 g/kg in the adult rat, blood [EtOH] slowly peaks at ~20 mM (Bielawski and Abel, 2002), whereas 5 g/kg produces ~60 mM peak plasma [EtOH] (Fig. 8A). The brain experiences [EtOH] similar to that in plasma (Sunahara et al., 1978). In electrophysiological recordings, the δ -containing extrasynaptic GABA_ARs are activated by 10–30 mM EtOH (Wei et al., 2004; Hancher et al., 2005). We suggest that exposure to [EtOH] above this range is necessary for the overstimulation and subsequent internalization of $\alpha 4\beta\delta$ GABA_ARs, whereas lower doses do not result in GABA_AR plasticity and hence do not produce diazepam cross-tolerance.

Whereas our studies cannot define which brain regions, circuits, and receptors mediate the behavioral drug effects, it is likely that similar GABA_AR plasticity also occurs in the relevant brain circuits. Although hippocampal GABA_ARs do participate in sedative/anesthetic drug action (Ma et al., 2002), other brain regions such as certain brainstem, thalamic, and hypothalamic nuclei may be more relevant to the GABAergic mechanisms of sedative-hypnotic and anesthetic drug action (Kim et al., 1997; Saper et al., 2005; Sukhotinsky et al., 2007), whereas the cerebellum appears to be particularly important for the motor coordination impairment effects of EtOH (Dar, 1995; Carta et al., 2004; Hancher et al., 2005). Cross-tolerance to diazepam recovers faster than GABA_AR function and subunit plasticity in the CA1 or dentate gyrus. This temporal discrepancy is likely caused by the greater importance of other brain areas in the sedative/anesthetic effects of diazepam and isoflurane compared with hippocampus.

After the EtOH-induced GABA_AR plasticity, synaptic GABA_ARs become sensitive to low [EtOH]. After CIE treatment, such changes appear to become permanent. The novel EtOH sensitivity at GABAergic synapses was proposed to represent the physiological substrate of its maintained anxiolytic effects, which *in vivo* leads to enhanced preference for EtOH consumption (Liang et al., 2006). Similar models of repeated EtOH treatment and withdrawal in rodents lead to high EtOH intake and preference (Spanagel, 2003). Because anxiety is a major withdrawal symptom and low dose EtOH (0.5 g/kg) readily relieves anxiety in CIE rats (Liang et al., 2006), this could be a potent impetus to seek “self-medication” in rodent models of repeated EtOH exposure and withdrawal, as well as in human alcoholics.

Considerable evidence points to the importance of GABA_ARs in mesolimbic circuits (in which the hippocampus participates) in mediating such behaviors as EtOH discrimination (Besheer et al., 2003), reinforcement (Samson et al., 1993), and reward (Koob, 2004). EtOH-induced changes within mesolimbic circuits include region-specific changes in GABA_AR $\alpha 1$ and $\alpha 4$ subunit peptide expression and receptor function in the amygdala, nucleus accumbens, ventral tegmental area (Papadeas et al., 2001; McCool et al., 2003), the hippocampal CA1 and dentate gyrus (present study) (Matthews et al., 1998), and cortex (Mhatre et al., 1993; Matthews et al., 1998). Interestingly, a single EtOH dose was also demonstrated to produce increases in GABAergic synaptic transmission via presynaptic mechanisms in the ventral tegmental area, with recovery observed by 2 weeks after EtOH withdrawal (Melis et al., 2002). Given the importance of GABA_AR function and expression in the control of reward behaviors (Koob, 2004), unraveling the mechanisms by which repeated EtOH administration produces the persistent GABA_AR alterations observed in CIE-treated rats should help understand the chronic aspects of alcohol addiction.

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