

Withdrawal from Chronic Intermittent Ethanol Treatment Changes Subunit Composition, Reduces Synaptic Function, and Decreases Behavioral Responses to Positive Allosteric Modulators of GABA_A Receptors

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ABSTRACT

One of the pharmacological targets of ethanol is the GABA_A receptor (GABAR), whose function and expression are altered after chronic administration of ethanol. The details of the changes differ between experimental models. In the chronic intermittent ethanol (CIE) model for alcohol dependence, rats are exposed to intermittent episodes of intoxicating ethanol and withdrawal, leading to a kindling-like state of behavioral excitability. This is accompanied by presumably causal changes in GABAR expression and physiology. The present study investigates further the effect of CIE on GABAR function and expression. CIE is validated as a model for human alcohol withdrawal syndrome (AWS) by demonstrating increased level of anxiety; diazepam improved performance in the test. In addition, CIE rats showed remarkably reduced hypnotic response to a benzodiazepine and a steroid anesthetic, reduced sensitivity to a barbiturate, but not propofol. Immunoblotting

revealed decrease in $\alpha 1$ and δ expression and increase in $\gamma 2$ and $\alpha 4$ subunits in hippocampus of CIE rats, confirmed by an increase in diazepam-insensitive binding for ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5- α)(1,4)benzodiazepine-3-carboxylate (Ro15-4513). Elevated mRNA levels were shown for the $\gamma 2S$ and $\gamma 1$ subunits. Recordings in hippocampal slices from CIE rats revealed that the decay time of GABAR-mediated miniature inhibitory postsynaptic currents (mIPSCs) in CA1 pyramidal cells was decreased, and potentiation of mIPSCs by positive modulators of GABAR was also reduced compared with control rats. However, mIPSC potentiation by the $\alpha 4$ -preferring benzodiazepine ligands bretazenil and Ro15-4513 was maintained, and increased, respectively. These data suggest that specific alterations in GABAR occur after CIE and may underlie the development of hyperexcitability and ethanol dependence.

The molecular mechanisms involved in ethanol dependence and tolerance are poorly understood. Although a specific binding site has not been established for ethanol, several studies have shown that short- and long-term effects of ethanol involve an enhancement or a reduction of inhibitory synaptic transmission at the level of GABA_A receptors (GABAR) function (Allan and Harris, 1987; Mehta and Ticku, 1988; Morrow et al., 1990; Wafford et al., 1991; Kang et al., 1996; 1998). A family of heteropentameric GABAR isoforms of different subunit composition accounts for variable sensitivity to modulatory drugs such as benzodiazepines, barbiturates, neurosteroids, alcohol, and general anesthetics. Ethanol tolerance and dependence

seem to be caused by changes in the function of GABAR (Morrow et al., 1990; Kang et al., 1996; 1998), possibly involving alterations in native GABAR subunit assembly. During long-term ethanol consumption and withdrawal, GABAR subunit expression is altered in several brain regions at the mRNA and protein levels (Mhatre et al., 1993; Devaud et al., 1997; Becker, 1998; Matthews et al., 1998). These adaptations to long-term treatment with ethanol may be responsible for CNS hyperexcitability in ethanol dependence and withdrawal. Long-term treatment with ethanol increased the binding for the benzodiazepine partial inverse agonist [³H]Ro15-4513; in the cerebellum, this is caused by elevation of the levels of the $\alpha 6$ subunit (Mhatre et al., 1988; Petrie et al., 2001). Cerebellar changes in $\alpha 6$ subunit are not likely to explain all the behavioral adapta-

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ABBREVIATIONS: GABAR, GABA_A receptor(s); CNS, central nervous system; CIE, chronic intermittent ethanol; AWS, alcohol withdrawal syndrome; aa, amino acid(s); RT-PCR, reverse transcription-polymerase chain reaction; DZ, diazepam; ANOVA, analysis of variance; ACSF, artificial cerebrospinal fluid; mIPSC, miniature inhibitory postsynaptic current; CGP 54626, [S-(R*,R*)]-3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl[(cyclohexylmethyl) phosphinic acid]; Ro15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5- α)(1,4)benzodiazepine-3-carboxylate.

tions to long-term treatment with ethanol, so other areas are of interest.

The chronic intermittent ethanol (CIE) treatment is a good model for human alcohol dependence; the alternating-day schedule of ethanol administration, in which rats experience intoxicating doses of ethanol and repeated withdrawals, leads to a persistent hypoinhibition and reduced seizure threshold, which we refer to as 'kindling-like' (Kokka et al., 1993; Becker, 1998). Importantly, this persistent withdrawal state occurs only after the multiple withdrawal paradigm of CIE. Despite the well known development of tolerance to ethanol's actions after long-term administration (Le et al., 1986; Allan and Harris, 1987), CIE rats, tested 2 days or longer after ethanol cessation, did not exhibit tolerance to the temperature-lowering or antimetrazole activity of ethanol or benzodiazepines (Kokka et al., 1993). Hyperexcitability in CIE rats is accompanied by a reduction of GABAR-mediated inhibition in the hippocampus (Kang et al., 1996). Reduced GABAR function in CIE rats is accompanied by a significant elevation in mRNA levels of the GABAR $\alpha 4$ subunit in the hippocampal formation (Mahmoudi et al., 1997). The $\alpha 4$ subunit is subject to plastic changes under a variety of conditions besides long-term treatment with ethanol (e.g., Matthews et al., 1998); it is elevated after long-term treatment with progesterone (Smith et al., 1998a,b; Sundstrom-Poromaa et al., 2002) or in models of epileptic seizures (Brooks-Kayal et al., 1998). In CIE, we also observed pharmacological changes in hippocampal slice recordings: *increased* sensitivity to short-term treatment with ethanol and the benzodiazepine inverse agonist 6,7-dimethoxy-methyl- β -carboline-3-carboxylate. Furthermore, modulation of benzodiazepine binding by neurosteroids was increased (Kang et al., 1998), and the GABAR subunit $\gamma 2L/S$ splice variant ratio was decreased in CIE rats (Petrie et al., 2001).

This study further delineates the changes in GABAR subunit expression and pharmacology in the hippocampus. Furthermore, it relates these to the behavioral plasticity seen in CIE. Our results further validate the CIE model by observing increased anxiety in CIE rats, as observed in humans during alcohol withdrawal syndrome (AWS). We show remarkably reduced sensitivity to positive modulators of GABAR-mediated inhibitory synaptic transmission *in vitro* and also sedative-hypnotic effects *in vivo*. Tolerance to the hypnotic action of benzodiazepines, neuroactive steroids, and barbiturates is produced in CIE, but no tolerance to their antianxiety effects is observed. Many of the changes are consistent with altered GABAR subunit composition in the hippocampus.

Materials and Methods

Animals and Neurochemistry

Production of CIE Rats. The Institutional Animal Care and Use Committee approved all animal experiments. Male Sprague-Dawley rats (170–190 g) were housed in the vivarium under a 12-h/12-h light/dark cycle and had free access to food and water. Intoxicating doses of ethanol (Pharmco Products, Brookfield, CT) were administered by oral intubation on a long-term regimen: for the first five doses, rats received 5 g/kg of body weight as a 25% (w/v) solution in saline once every other day and, for the following 55 doses, 6 g/kg of ethanol 30% (w/v) once every day. The control group received saline (20 ml/kg of body weight). This ethanol regimen led rats to experience multiple cycles of intoxication and withdrawal phases. It has been shown previously that CIE treatment led to a kindling-like state with a persistent decrease in pentylenetetrazol seizure thresh-

old (Kokka et al., 1993). After the treatment and 2 days of withdrawal, rats were euthanized and tissues prepared for experiments.

Membrane Preparation and Western Blot. Individual hippocampi were dissected on ice from each rat brain and P2 membrane fractions were prepared by homogenization, low-speed centrifugation in 0.32 M sucrose, and centrifugation of the supernatant at 12,000g for 20 min. The pellet was resuspended and washed in 20 volumes of phosphate-buffered saline (150 mM NaCl, 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.4). The final pellet was resuspended in 5 volumes of phosphate-buffered saline and protein concentration was detected by bicinchoninic acid protein assay system (Pierce, Rockford, IL). Aliquots of 40 μg of protein from each sample were separated on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions using the Mini-Protein 3 Cell electrophoresis system (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences, Piscataway, NJ) with LKB2117 Multiphor II Electrophoresis system (Pharmacia LKB Biotechnology, Uppsala, Sweden). That identical amounts of proteins were loaded was demonstrated by Ponceau staining. Blots were probed with anti-peptide $\alpha 1$ (aa 1–9), or $\alpha 4$ (aa 379–421), or $\gamma 2$ (aa 319–366), or δ (aa 1–44) antibodies (Sperk et al., 1997; Matthews et al., 1998), 1 $\mu\text{g}/\text{ml}$ final concentration each antibody, followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies, and bands were detected by enhanced chemiluminescence detection kit (Amersham) and exposed to X-ray film under nonsaturating conditions. W. Sieghart and colleagues (Vienna, Austria) kindly provided all antibodies. The bands from different control rats ($n = 10$) and treated rats ($n = 12$) corresponding to the appropriate molecular weight for each subunit were analyzed and absorbance values compared by densitometric measurements using C.IMAGING image analysis systems (Complix Inc., Cranberry Township, PA) and Simple 32 software. An antibody to actin (Sigma, St. Louis MO) was used to prove the equal amount of proteins loaded on the gel; amounts of endogenous actin did not change between control and CIE rats. Data analysis was conducted by *t* test and the difference was expressed as percentage of control peptide levels \pm S.E.M. *P* values < 0.05 were considered statistically significant.

RT-PCR Quantification. Quantification of relative mRNA expression of $\alpha 2$, $\gamma 2L$, $\gamma 2S$, and $\gamma 1$ subunit, and glyceraldehyde-3-phosphate dehydrogenase as an internal control, was done using the method of Horikoshi and Sakakibara (2000). Hippocampus total RNA from different saline-treated control rats ($n = 8$) and CIE-treated rats ($n = 13$) was isolated using acid guanidinium/phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) and 3.6 μg of total RNA from each animal was reverse-transcribed to cDNA using SuperScript First-Strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) using oligo-dT primer. The nucleotide sequences and expected RT-PCR product sizes from primer sets for glyceraldehyde-3-phosphate dehydrogenase, GABAR- $\gamma 2L$, GABAR- $\gamma 2S$, GABAR- $\gamma 1$, and GABAR- $\alpha 2$ are given in Table 1. The identity of each fragment was confirmed by direct sequencing of the PCR product (T7 Sequenase version 2.0 DNA sequencing kit; USB Corp., Cleveland OH). Each PCR reaction was carried out in a volume of 25 μl using bulk master mixes except template cDNA prepared from multiple reactions. The concentration of starting cDNA to be amplified for each subunit was determined by building a standard curve for each gene plotting the density of PCR product against the amount of template cDNA. There was a linear region in which the density of PCR was directly proportional to the amount of template cDNA. Two points of the standard curve (1 ng and 500 pg of starting cDNA) were chosen to compare the relative expression of each gene in control rats and CIE rats. To relate the expression of the gene of interest to that of the endogenous reference gene, a ratio was determined between the amount of PCR product within the linear amplification range of the target gene and the endogenous reference gene; this ratio, compared among different cDNAs, provided a relative gene expression level. PCR cycle (single hot start at 94°, 8.5 min at 94°, 30 s at 50°, and 45 s at 72°) was conducted for 30 cycles; the series of PCR

TABLE 1

Primer sequences for selective amplification of GABA_A subunit receptors by RT-PCR

Target mRNA Bases	Primer Sequence	Product Size	Accession No.
		<i>base pairs</i>	
$\alpha 2$ 131–530	5'–CAGCATTACTGAAGTCTTC–3' 5'–TAAGTGACTTCCGAGGTCG–3'	399	L08491
$\gamma 1$ 687–1257	5'–TATGGATGAACATTCTGTGTC–3' 5'–AGTAGATCCAGCATGGAGAC–3'	570	X57514
$\gamma 2L$ 1168–1534	5'–CTTCTTCGGATGTTTCTCTCAAG–3' 5'–CATAGGGTATTAGATCGTTGGACT–3'	390	M55563
$\gamma 2S$ 1156–1492	5'–AAGAAAAACCCTGCCCTACCATT–3' 5'–TTCGTGAGATTGAGCGAATAAGAC–3'	336	L08497
GAPDH 1608–1847	5'–TGATGACATCAAGAAGGTGGTGAAG–3' 5'–TCCTTGAGGCCATGTAGGCCAT–3'	239	AF106860

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

amplifications from which the relative gene expression level was calculated were always prepared from the same master mix, cDNA stock. Each PCR reaction contains 1.25 units of AmpliTaq Gold, 2.5 mM MgCl₂, 1× PCR buffer, 200 μ M dNTP mix (Applied Biosystems, Foster City, CA), 12.5 pmol for each primer (Genosys, Sigma, Woodlands, TX). After PCR amplification, reactions were run on 2.5% agarose gel in 1× Tris/acetate/EDTA buffer stained with 0.5 μ g/ml ethidium bromide and then densitometric analysis of bands for specific gene and internal control were done with AlphaEase software (Alpha Innotech Corporation, San Leandro, CA). Data are presented as mean \pm S.E.M. Statistic differences were assessed by *t* test and *P* values <0.05 were considered statistically significant.

Autoradiography: [³H]Ro15-4513 Binding to Benzodiazepine-Insensitive Sites. The diazepam-insensitive site in CIE rat hippocampus was measured using [³H]Ro15-4513 (23.06 Ci/mmol; PerkinElmer Life Sciences, Boston, MA; Petrie et al., 2001). CIE rats (*n* = 5) and saline-treated control rats (*n* = 4) were decapitated and the brains were rapidly removed and frozen in 2-methylbutane (Aldrich, Milwaukee, WI) on dry ice. Four coronal sections (14 μ m) per slide from each hippocampus area were cut and thaw-mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at –70°C until performing binding assays. The day of the experiment, brain sections were thawed and preincubated in assay buffer (0.1 M KCl and 20 mM KHPO₄, pH 7.5) on ice for 30 min. Sections were then incubated for 90 min on ice with 5 nM [³H]Ro15-4513 plus diazepam 10 μ M (total DZ-insensitive binding) in assay buffer. The nonspecific binding was estimated by parallel assay, including 10 μ M cold Ro15-4513 (Hoffmann-La Roche, Nutley, NJ) and was negligible. After a 2-min rinse in chilled buffer and a quick rinse in water, slides were dried and exposed to Biomax MS Kodak film (Eastman Kodak, Rochester, NY) for 3 months at –70°C to generate autoradiograms (Kang et al., 1998). Computer-assisted micro-densitometry was performed with a diode camera/image analyzer (TCID, St. Catherine's, ON, Canada) and quantification made by comparison to radioactive micro-scale standards (Amersham Biosciences). Each measurement was the mean of multiple sections per treatment group and both brain hemispheres were analyzed. The hippocampal regions examined were dentate gyrus and CA1 region. The results are expressed as mean \pm S.E.M. Statistical significance was calculated by using the Student's *t* test.

Behavioral Analysis

Basic Anxiety on Elevated-Plus Maze. After 60 doses of ethanol and 2 days of withdrawal, rats were tested for basic anxiety on the elevated-plus maze. Rats were brought to the procedure room 2 h before testing. The plus-maze was constructed as described previously (Pellow et al., 1985). Each rat was tested for 5 min on the maze and videotaped; a rat was placed on the central platform of the maze,

facing an open arm. Saline-treated control rats (*n* = 11) were compared with CIE rats (*n* = 12). The following measures were scored: number of entries into open arm, closed arm, or center platform and time spent in open arms, closed arms, or center platform. An entry was defined as the entry of all four feet into one arm. Significance differences between number of entries into the open and into the closed arms and time spent in the closed and in the open arm for the two experimental groups were evaluated by Student's *t* test. *P* values <0.05 were considered significant.

Anxiolytic Effect of Diazepam. Diazepam (2 mg/kg) (Sigma Chemical Co., St. Louis, MO) anxiolytic effect was tested on the elevated plus-maze. Rats were randomly divided into four groups: saline-treated control rats treated with vehicle (*n* = 8) or with diazepam (*n* = 8) and CIE rats treated with vehicle (*n* = 8) or diazepam (*n* = 9). Rats were injected intraperitoneally 30 min before testing; injection volume was 2 ml/kg. Diazepam was dissolved in distilled water with a drop of Tween 20. Rats were tested for 5 min each and videotaped. Data were analyzed by ANOVA, with the percentage of open-arm entries or time spent in the open arms with drug treatment as a factor.

Sleep Time Assay. Alphaxalone (10 mg/kg i.v.; Sigma), flurazepam (40 mg/kg i.p.; Sigma), pentobarbital (35 mg/kg i.p.; Sigma), and propofol (10 mg/kg i.v.; Abbott Laboratories, North Chicago, IL) were tested on CIE rats and control rats. Alphaxalone was dissolved in 22.5% (w/v) solution of 2-hydroxypropyl- β -cyclodextrin (Sigma) and sonicated, flurazepam, and pentobarbital were dissolved in 0.9% saline. Injection volumes were 0.8 μ l/g of body weight i.v., 2 ml/kg for i.p. injection. Sleep times were 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 the animal was able to flip over three times in 30 s. Statistical significance was calculated by Student's *t* test.

Electrophysiological Recordings

Hippocampal Slices. Transverse slices (400 μ m thick) of dorsal hippocampus were obtained using standard techniques (Kang et al., 1998). Recordings were obtained from cells located in CA1 layer at 34 \pm 5°C during perfusion with artificial cerebrospinal fluid (ACSF) composed of 124 mM NaCl, 3.5 mM KCl, 1.25 mM NaH₂PO₄, 2.0 mM CaCl₂, 2.0 mM MgCl₂, 26 mM NaHCO₃, and 10 mM dextrose. 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 135 mM cesium gluconate, 5 mM NaCl, 1.1 mM CaCl₂, 11 mM EGTA, 10 mM HEPES, 2 mM MgATP, and 0.2 Na₂GTP; pH was adjusted to 7.25 with CsOH. GABAR-mediated mIPSCs were pharmacologically isolated by adding tetrodotoxin (0.5 μ M), D-(–)-2-amino-5-phosphonopentanoate (40 μ M), 6-cyano-7-nitroquinoxaline-2,3-dione (10 μ M), and CGP 54626 (1 μ M) to the ACSF from stock

solutions. Stock solutions of 6-cyano-7-nitroquinoxaline-2,3-dione, alphaxalone, bretazenil, diazepam, and Ro15-4513 were made with pure dimethyl sulfoxide. Final concentration of dimethyl sulfoxide did not exceed $42 \mu\text{M}$ in the recording chamber. Signals were recorded in voltage-clamp mode with an amplifier (Axoclamp 2B; Axon Instruments, Union City, CA). Whole-cell access resistances were in the range of 2.5 to 15 M Ω before electrical compensation by about 90%. 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 mIPSC recordings. All mIPSC recordings were of 100-s duration in the continuous voltage-clamp mode. Data were acquired with pClamp 8 software (Axon Instruments), digitized at 20 kHz (Digidata 1200B; Axon Instruments) and analyzed using the Clampfit software (Axon Instruments) and the Mini Analysis Program (versions 5.2.2 and 5.4.8; Synaptosoft, Decatur, GA).

Detection and Analysis of mIPSCs. The recordings were band-pass filtered off-line (Clampfit software) at 1 kHz. The mIPSCs were detected (Mini Analysis Program) with threshold criteria of 5 pA amplitude and $20 \text{ pA} \times \text{ms}$. The location of baseline average before a peak was set to 12 ms. Time to peak was set to 10 ms. Time to decay was set to 10 ms. Decay percentage was set to 30%. Frequency of mIPSCs was determined from all automatically detected events in the 100-s recording period. Undetected events and false positives were corrected by visual inspection of the recording trace. Only single events were chosen as mIPSCs during visual inspection. The mIPSC kinetics were obtained from the averages of 64 to 453 chosen single events in each cell. Decay time constants were obtained by fitting a double exponential to the falling phase of the averaged mIPSC in each neuron. The investigator

performing the recordings and mIPSC analysis was blind to the treatment (saline or CIE) that the rats received. All comparisons of group differences in mIPSC kinetics and drug effects were made with ANOVA (Sigmaplot; SPSS Inc., Chicago, IL).

Results

Immunoblotting and RT-PCR Measurements Show Altered GABA_A Subunit Composition in CIE Rat Hippocampus. After CIE treatment and 2 days of withdrawal, levels for GABA_A receptor subunits in rat hippocampus were measured using specific antibodies raised against $\alpha 1$, $\alpha 4$, $\gamma 2$, and δ subunits. The $\alpha 1$ antibody recognized a 51-kDa band, the $\alpha 4$ antibody recognized a 67-kDa band, the $\gamma 2$ antibody recognized a 43- to 48-kDa band, and the δ antibody recognized a 54-kDa band. CIE treatment altered GABA_A receptor subunit expression in hippocampus, increasing the expression of $\alpha 4$ subunit ($+50 \pm 5\%$) and $\gamma 2$ ($+38 \pm 4\%$) and decreasing $\alpha 1$ ($-48 \pm 7\%$) and δ ($-52 \pm 5\%$) subunit expression, respectively, compared with saline-injected control rats (Fig. 1A). The protein product of the housekeeping gene actin is identical in control and CIE rats (Fig. 1B).

To understand which of the two splice-variants of the $\gamma 2$ subunit receptor $\gamma 2\text{L}$ and $\gamma 2\text{S}$ was primarily responsible for the protein increase, we measured the levels of mRNA for the two splice-variants in hippocampus of CIE rats. Only $\gamma 2\text{S}$ mRNA levels were significantly increased by $+48 \pm 8\%$. $\gamma 2\text{L}$ mRNA levels were slightly increased but not significantly. Also, $\gamma 1$ mRNA levels were significantly increased ($+80 \pm$

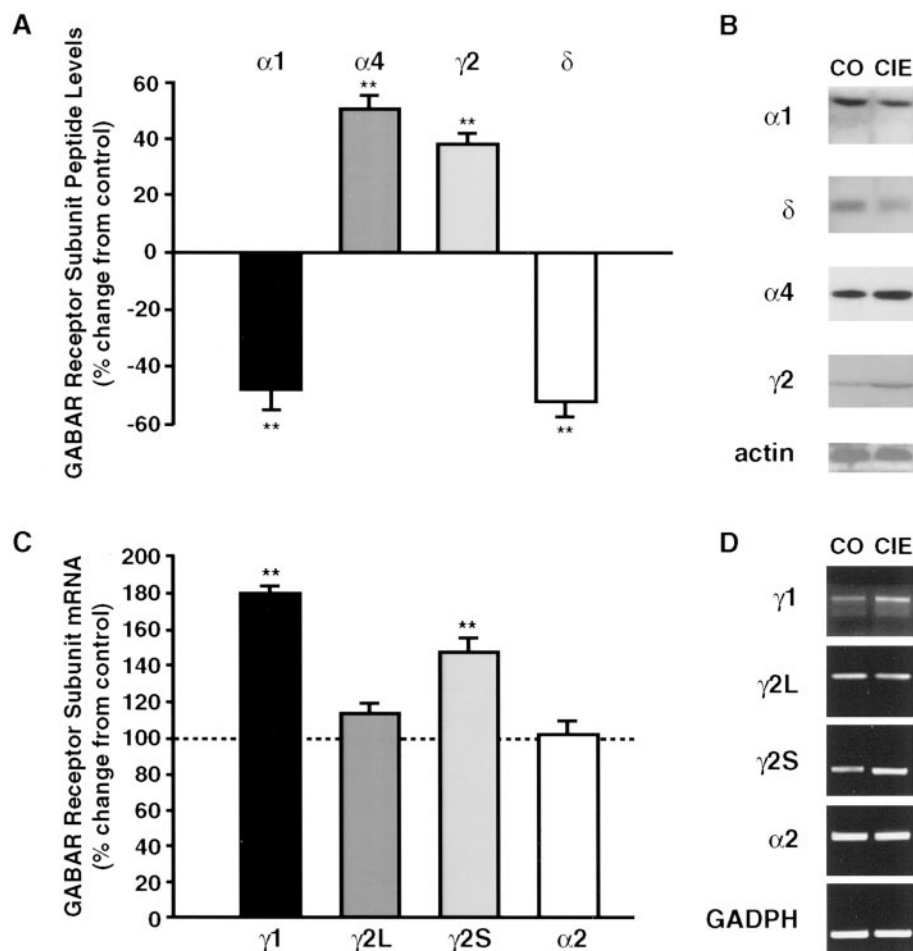


Fig. 1. A, Western blot analysis of hippocampal GABA_A subunit peptides after CIE compared with control rats. Equal amounts of membrane protein (40 μg) were separated by 10% SDS polyacrylamide gel electrophoresis; protein levels were detected with subunit-specific antibody. Data are presented as percentages of control peptide levels mean \pm S.E.M. For each subunit, results were obtained from 10 control rats and 12 CIE rats. Significant difference was analyzed by *t* test, **, $p < 0.01$. B, representative Western blot films for the GABA_A receptor subunit peptides and actin levels measured. Lane 1, control; lane 2, CIE. C, RT-PCR measurements of mRNAs for the two splice variants of GABA_A subunit receptor $\gamma 2$, $\gamma 2\text{L}$, $\gamma 2\text{S}$, $\gamma 1$, and $\alpha 2$. Total RNA was extracted from each hippocampus of CIE rats ($n = 13$) and control rats ($n = 8$) and reverse-transcribed into cDNA. Specific primers were used in each PCR reactions. All measurements were normalized against the level of an endogenous reference gene. Data are expressed as percentage of control group mean \pm S.E.M.; *t* test was performed for statistical difference. **, $p < 0.01$. D, representative gels for GABA_A receptor subunit mRNA levels measured. Left lane, control; right lane, CIE.

5%) after CIE treatment, and no change was found for the $\alpha 2$ subunit (Fig. 1C).

Diazepam-Insensitive Sites: Ro15-4513 Binding Is Elevated in CIE Rat Hippocampus. Ro15-4513 is a partial benzodiazepine inverse agonist that has been shown to antagonize some of the pharmacological and physiological actions of ethanol (Suzdak et al., 1986). It binds to two distinct populations of GABA_A receptor sites: one blocked by diazepam and the other not affected by diazepam (DZ-insensitive; Turner et al., 1991; Petrie et al., 2001). We have studied DZ-insensitive binding of Ro15-4513 using a high concentration of diazepam (10 μ M) to exclude benzodiazepine-sensitive sites. In this way, we were able to detect changes specifically in receptor subtypes containing the $\alpha 4$ subunit, which is known to be responsible for DZ-insensitive binding in the hippocampus (Wieland et al., 1992), where we detected an increase of $\alpha 4$ subunit by Western blot. Using autoradiography, we examined the abundance of Ro15-4513 DZ-insensitive sites in rat hippocampus after CIE treatment. Figure 2 shows a representative autoradiogram of binding in the hippocampus, the major area of change. DZ-insensitive sites were present and apparently unchanged in CIE in several regions, but were increased in CA1 and dentate gyrus. In both regions, after CIE treatment, the DZ-insensitive subset of Ro15-4513 binding was significantly increased, by 18% ($p = 0.002$) in CA1 and by 34% ($p < 0.01$) in dentate gyrus, compared with levels in saline-injected control rats (Table 2). The binding affinity K_D for [³H]Ro15-4513 was not changed (data not shown), as previously reported (Petrie et al., 2001), indicating that the change was caused by an increase in B_{max} .

CIE Rats Show Increased Anxiety on Elevated Plus-Maze: Effect of Diazepam. During AWS, humans experience a variety of symptoms, such as anxiety, insomnia, agitation, and seizures. Anxiety is a major symptom during AWS and is treated by the administration of benzodiazepines, such as diazepam. Therefore, behavioral analysis of anxiety in CIE rats should provide at least a partial validation of CIE as a model for human AWS. Using an elevated plus-maze assay, we have compared the basal anxiety of CIE rats with that of control rats by measuring the number of entries and the time spent in the open and closed arms. Generally, rats spend a significantly greater amount of time in the closed arms and enter them more frequently than the open arms. Results from the two groups are shown in Fig. 3. In both groups, the number of open-arm entries and the time spent in the open arms were significantly lower than the same measures taken in the closed arms, but control rats had more open entries and they spent more time in them com-

TABLE 2

Effect of chronic intermittent ethanol treatment on Ro15-4513 DZ-insensitive binding sites in hippocampus

All values are presented as mean \pm S.E.M.

Hippocampus Region	Saline	CIE
	<i>nCi/mg</i>	
CA1	5.3 \pm 0.2	6.2 \pm 0.1
Dentate gyrus	3.3 \pm 0.1	4.3 \pm 0.1

pared with CIE rats (Fig. 3A), validating the hypothesis that AWS occurs in this model for alcohol dependence. We also tested the effect of short-term treatment with diazepam (2 mg/kg) in CIE and control rats on elevated plus-maze compared with saline-treated rats, based on the fact that anxiolytics increase the time spent in and the number of entries into the open arms. The following groups were compared: saline versus diazepam in saline-treated control rats and saline versus diazepam in CIE rats. The number of open entries and the time spent in open arms were used as parameters to compare groups. ANOVA was used for statistical analysis. In both groups, short-term treatment with diazepam increased significantly the number of open entries compared with saline treated-control rats and saline-treated CIE rats; diazepam also increased the time spent in the open entries in control rats (Fig. 3B).

Changes in Sensitivity to Hypnotic/Anesthetics in CIE: Reduced Sleep Time. Using a sleep-time assay, we compared the response to alphaxalone, flurazepam, pentobarbital, and propofol between CIE rats and saline-treated control rats. Results are presented in Table 3. CIE rats ($n = 17$) had a 93% reduction in sleep-time duration compared with control rats ($n = 10$) after administration of alphaxalone (10 mg/kg i.v.). Also, the effect of a soporific dose of flurazepam (40 mg/kg i.p.) was changed in CIE rats ($n = 14$) compared with control rats ($n = 10$); CIE rats had 89% reduction in sleep time compared with control rats; the majority of CIE rats did not lose the righting reflex, although flurazepam had a sedative effect because rats were drowsy. Pentobarbital sleep time was reduced by 31% in CIE rats ($n = 17$) compared with control rats ($n = 14$). Propofol, however, had the same effect in CIE rats ($n = 7$) and control rats ($n = 7$). These results suggest that the pharmacology of GABA_A is changed and correlates with changes in subunit expression that occur in CIE rats.

CA1 Neurons from CIE Rats Exhibit Altered mIPSC Kinetics. Analysis of mIPSC kinetics in CA1 neurons from untreated and age-matched saline-treated rats revealed no significant differences between these two groups (Table 4). However, significant differences were observed between mIPSC kinetics in CIE-treated rats compared with both untreated and saline-treated rats. The amplitude of mIPSCs in CIE rats was slightly but significantly smaller than that of saline-treated rats. Also, the decay time constant (τ_1) was significantly smaller in CIE rats (Fig. 4 and Table 4); i.e., CIE rats have reduced inhibitory synaptic currents. In addition, the frequency of mIPSCs in CA1 neurons of CIE rats was significantly smaller than in untreated or saline-treated rats. These data suggest both presynaptic and postsynaptic decreases in GABAergic transmission in hippocampus of CIE rats.

CA1 Neurons from CIE Rats Are Insensitive to Alphaxalone. We next considered the possibility that in addi-

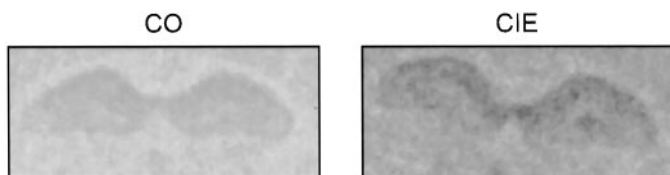


Fig. 2. Autoradiogram of DZ-insensitive binding of [³H]Ro15-4513 in hippocampal formation of CIE rats (A) compared with control rats (B) measured in the presence of an excess of diazepam (10 μ M). Binding density was measured and expressed as nanocuries per milligram of tissue based on tritiated standards coexposed with the sections analyzed by computer-assisted microdensitometry as described under *Materials and Methods*.

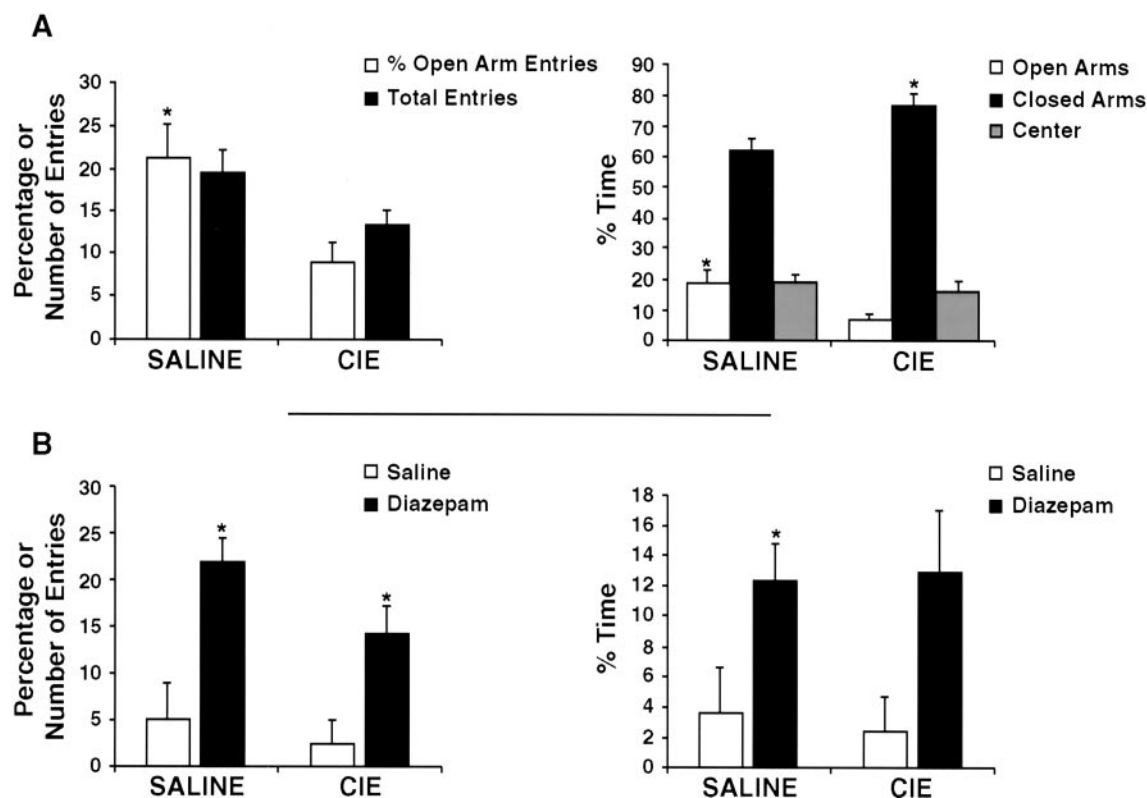


Fig. 3. Performance of CIE rats and control rats in the elevated plus maze. A, saline-treated control rats ($n = 11$) and CIE rats ($n = 12$) were tested for basic anxiety on elevated plus-maze. Each rat was tested for 5 min and videotaped. Data shown are mean \pm S.E.M values for percentage of entries into open arms (\square) or total entries (black bars) (left) and percentage of time spent in different entries (right). Statistical difference was determined by ANOVA. *, $p < 0.05$. B, anxiolytic effect of diazepam (2 mg/kg) on elevated plus-maze. Vehicle-treated animals were compared with diazepam-treated animals in both control and CIE groups. Data are presented as mean \pm S.E.M values for the percentage of open-arm entries (left) and time spent in the open arms (right). Statistical differences were analyzed using ANOVA, *, $p < 0.05$.

tion to kinetic differences in mIPSCs of CIE rats, there might be differences in the responses of CA1 neurons in CIE rats to allosteric modulators of GABAR. We found previously that neurosteroids such as alphaxalone ($\sim 1 \mu\text{M}$) enhanced GABAR synapses in CA1 neurons (Kang et al., 1998). Preliminary recordings in CA1 neurons from untreated rats revealed that bath application of $3 \mu\text{M}$ alphaxalone (20 min) gave a

robust increase in the amplitude and the decay time of mIPSCs ($n = 2$, data not shown). Based on these data, we used $3 \mu\text{M}$ alphaxalone for subsequent experiments. Analysis of mIPSC kinetics revealed that, similar to its effects in untreated rats, alphaxalone application produced significant increases in both amplitude and decay time in saline-treated rats (Fig. 5A). In contrast, similar application of alphaxalone to CA1 neurons from CIE rats had no significant effects on any of the measured mIPSC parameters (Fig. 5B).

CA1 Neurons from CIE Rats Are Insensitive to Diazepam. We next tested whether the mIPSCs of CIE rats would respond differently than mIPSCs in saline-treated rats to the application of diazepam. In this set of CA1 neuron recordings, again a test concentration was determined by testing, on slices from untreated rats, several concentrations (0.3 – $1 \mu\text{M}$) of diazepam previously reported in the literature to be effective in CA1 (e.g., Tietz et al., 1999). Diazepam (0.3

TABLE 3

Sleep time assays

Data are presented as mean \pm S.E.M.

Drug	Saline	CIE
<i>min</i>		
Alphaxalone (10 mg/kg)	30.4 \pm 5.4	2.5 \pm 1.2
Flurazepam (40 mg/kg)	52.5 \pm 12.1	5.4 \pm 3.1
Propofol (10 mg/kg)	9.9 \pm 0.4	10.1 \pm 0.3
Pentobarbital (35 mg/kg)	92.4 \pm 4.2	63.3 \pm 5.1

TABLE 4

CIE treatment alters the kinetics of GABAR mIPSCs in CA1 neurons

Rats were untreated, treated with 60 doses of 0.9% saline, or treated with 60 doses of 25 to 30% ethanol in saline. P values were determined by ANOVA.

Measures	Untreated	Saline	CIE	P Value
Frequency (Hz)	19.9 \pm 1.2	19.0 \pm 0.6	16.6 \pm 0.7*	<0.05
Rise time (ms)	0.64 \pm 0.06	0.52 \pm 0.03	0.47 \pm 0.02*	0.016
Amplitude (pA)	19.7 \pm 0.7	20.2 \pm 0.2	18.4 \pm 0.5†	<0.05
Decay τ_1 (ms)	5.0 \pm 0.3	5.5 \pm 0.2	3.9 \pm 0.2*†	<0.001
Decay τ_2 (ms)	29.0 \pm 6.9	39.8 \pm 7.5	26.7 \pm 2.9†	0.005
<i>n</i>	22	49	37	

* Significantly different from untreated group.

† Significantly different from saline-treated group

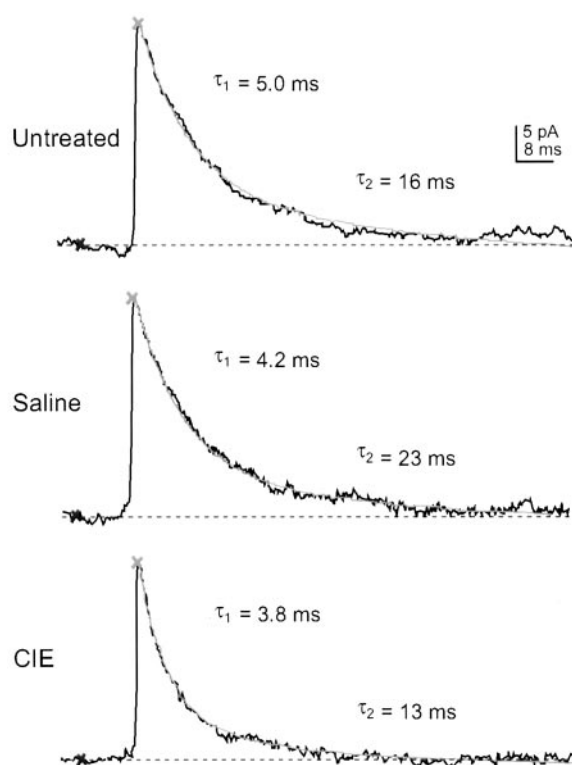


Fig. 4. Comparison of kinetics of GABA_A mIPSCs in CA1 neurons. The traces are mIPSCs from representative recordings in CA1 neurons from untreated, saline-treated, and CIE-treated rats. Each mIPSC trace is an average of 80 to 130 events. The decay of the averaged mIPSCs was fit by a double exponential. The decay time constant values for each averaged mIPSC are indicated. Note the faster decay of the averaged mIPSC from the CIE-treated rat.

μ M) application in slices from saline-treated rats prolonged both the rise time and decay time of mIPSCs without affecting their amplitude (Fig. 6A). In slices from CIE rats, diazepam had no significant effects on any of the measured mIPSC parameters (Fig. 6B).

CA1 Neurons from CIE Rats Respond to Bretazenil.

We next wanted to determine whether a benzodiazepine with agonist activity on $\alpha 4$ -containing GABAR (Knoflach et al., 1996) would have different effects on mIPSCs from saline-treated versus CIE-treated rats. The $\alpha 4$ -containing GABAR in the hippocampus and other brain regions normally are associated at least partially with the δ subunit (Whiting et al., 2000), which is localized extrasynaptically in the cerebellum (Nusser et al., 1998). If the switch to $\alpha 4$ subunits seen in CIE hippocampus leads to increased *synaptic* $\alpha 4$ -containing GABAR, the mIPSCs would retain sensitivity to bretazenil despite losing sensitivity to diazepam. As expected, bretazenil (0.3 μ M) prolonged the mIPSCs from untreated rats by 276%, whereas decay τ_2 was unaffected ($n = 6$, data not shown). The mIPSCs in both saline-treated and CIE-treated rats were potentiated to a similar extent by bretazenil (Fig. 7).

CA1 Neurons from CIE Rats Exhibit Positive Responses to Ro15-4513. Based on the above results, we decided to test another positive allosteric modulator of $\alpha 4$ subunit-containing GABAR, Ro15-4513, which is known to possess little effect, or perhaps slight inhibition, on $\alpha 1$ -containing GABAR (Whiting et al., 2000). In CA1 neurons from saline-treated rats, Ro15-4513 unexpectedly caused a slight

but significant increase in decay τ_1 and τ_2 , respectively, but other parameters were unchanged (Fig. 8A). In CIE rats, however, application of Ro15-4513 produced significantly greater potentiation of mIPSCs than in saline-treated rats (Fig. 8B).

Discussion

CIE Rats Show Heightened Anxiety

The observation of increased anxiety in the elevated plus maze, which remained for at least 2 days after ethanol cessation, helps to validate the CIE model to human AWS. Withdrawal signs to short-term treatment with alcohol are visible at 7 to 20 h but gone at >36 h (results not shown). The anxiety measurements were made with the rats in a familiar environment, videotaped with no people present. Elevated

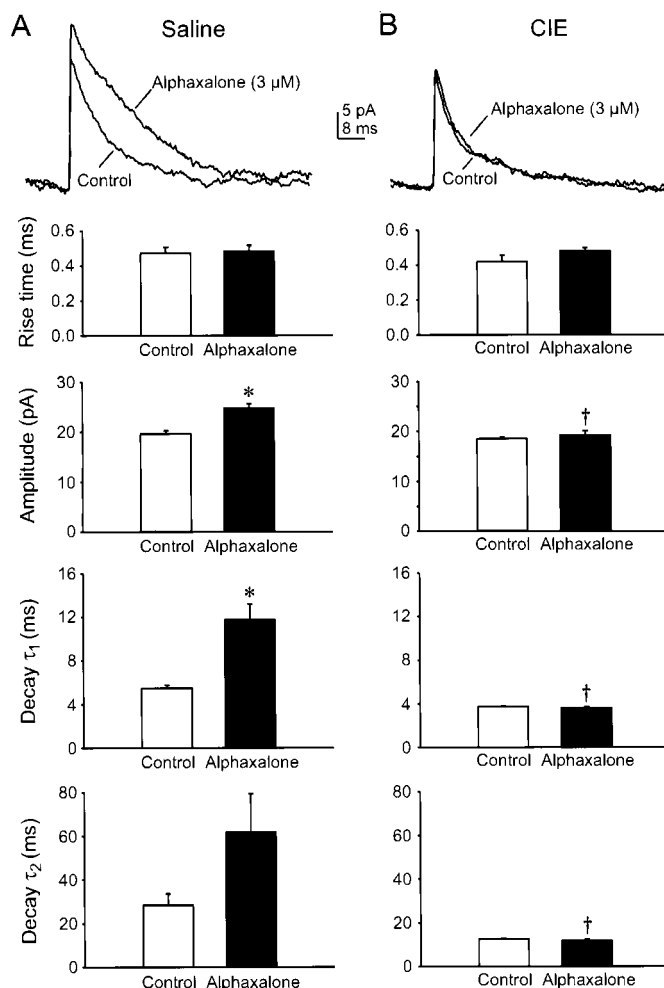


Fig. 5. Comparison of alphaxalone effects on mIPSCs in saline- and CIE-treated rats. A, superimposed averaged mIPSCs obtained from a representative CA1 pyramidal neuron recording from a saline-treated rat before (control) and 20 min after bath application of alphaxalone (3 μ M). The graphs below represent changes in rise time, amplitude, decay τ_1 , and decay τ_2 , respectively (mean \pm S.E.M., $n = 11$ cells, 3 rats). *, significantly different from control. B, superimposed averaged mIPSCs obtained from a representative CA1 neuron recording from a CIE-treated rat before and 20 min after application of alphaxalone. Graphs below correspond to the same parameters ($n = 6$ cells, 3 rats) as in A. †, statistically different from alphaxalone effect in saline-treated rats. For clarity, only the statistical differences between drug effect columns are indicated. Note the complete absence of alphaxalone effects measured on mIPSC parameters from CIE rats.

anxiety is consistent with our previous demonstration of increased seizure susceptibility (retained to 40 days after ethanol) and hyperactivity (Kokka et al., 1993; Kang et al., 1996; Petrie et al., 2001).

CIE Rats Show Altered GABAR Subunit Composition in the Hippocampal Formation

We showed previously that GABAR function was impaired, specifically in hippocampus (Kang et al., 1996; 1998), and that certain GABAR subunits were changed in hippocampus and cerebellum (Mahmoudi et al., 1997; Petrie et al., 2001). Variable results on subunit changes from several laboratories may be caused in part by different ethanol regimens, but we now present studies on microdissected hippocampus with significantly large numbers of animals and accuracy to establish the situation conclusively in the CIE model, at least

for those subunits previously shown to be changed. Western blots showed decreased levels of $\alpha 1$ and δ subunit polypeptides and increased levels of $\alpha 4$ and $\gamma 2$. We previously reported increased $\alpha 4$ but no change in $\alpha 5$ mRNA in hippocampal formation (Mahmoudi et al., 1997) and decreased $\gamma 2L/S$ ratio but no changes in several other subunits (Petrie et al., 2001). There have been previous reports of changes in $\gamma 2$ and δ with long-term treatment with ethanol, despite their importance to synaptic and extrasynaptic receptors, respectively (Nusser et al., 1998; Mihalek et al., 1999). RT-PCR is consistent with the Western blots (elevated $\gamma 2$ peptide), showing elevated $\gamma 2S$ mRNA, rather than decreased $\gamma 2L$ (antibodies are not available to distinguish the splice variants). We also found significantly increased levels of mRNA for the $\gamma 1$ subunit, and no change in $\alpha 2$, a 'control' α subunit. Consistent with increased levels of $\alpha 4$ and $\gamma 2$ subunits, a

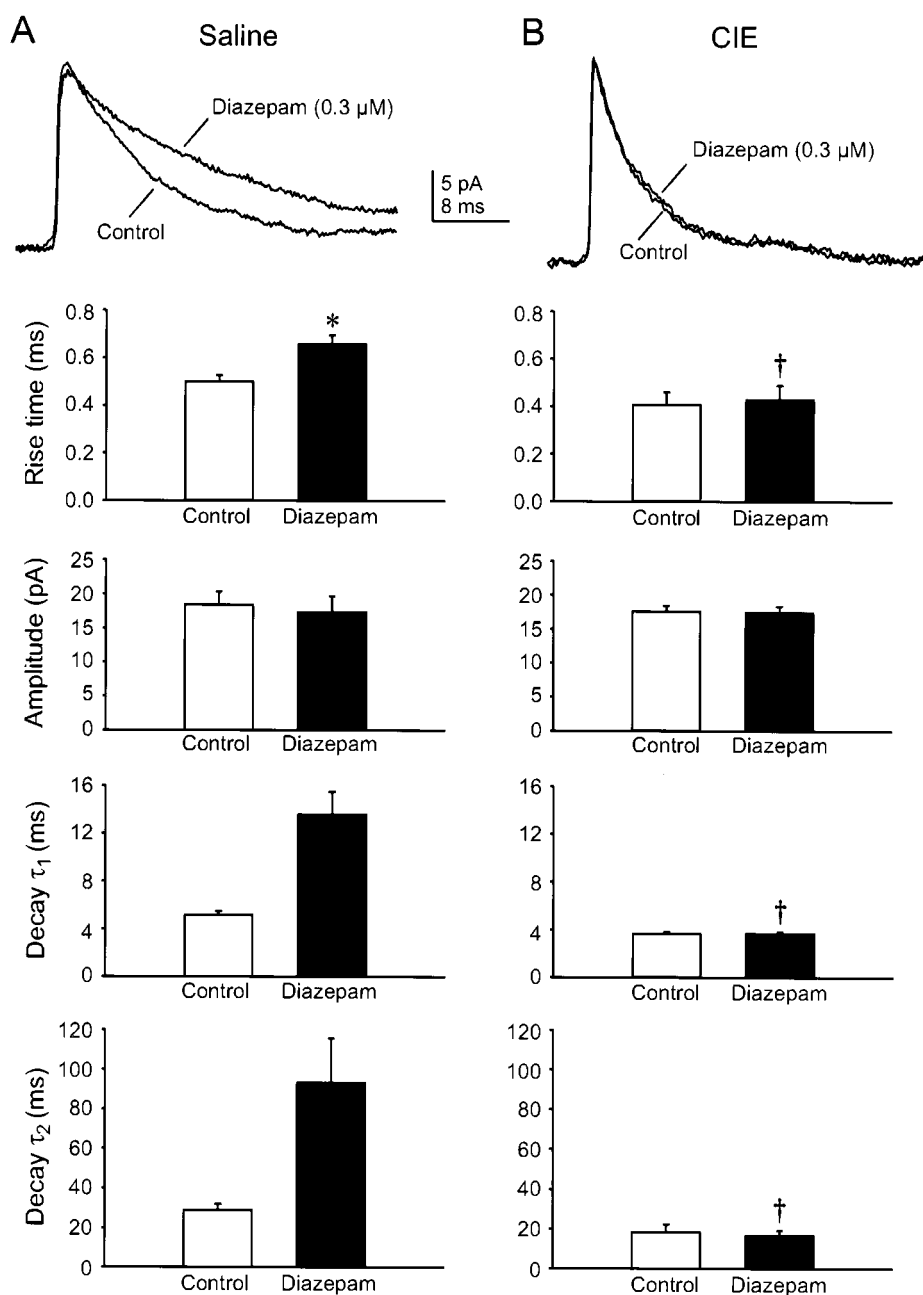


Fig. 6. Comparison of diazepam effects on mIPSCs in saline- and CIE-treated rats. **A**, superimposed averaged mIPSCs obtained from a representative CA1 neuron recording from a saline-treated rat before (control) and 20 min after bath application of diazepam (0.3 μ M). The graphs below represent changes in rise time, amplitude, decay τ_1 , and decay τ_2 , respectively. The bars represent parameters before (\square) and after (\blacksquare) 20 min of diazepam application. Data are presented as mean \pm S.E.M. ($n = 8$ cells, 6 rats). *, significantly different from control. **B**, superimposed averaged mIPSCs obtained from a representative CA1 neuron recording from a CIE-treated rat before (control) and 20 min after bath application of diazepam. Graphs below correspond to the same parameters ($n = 6$ cells, 6 CIE rats) as in **A**. Daggers represent statistical difference from diazepam effect in saline-treated rats. For clarity, only the statistical differences between drug effect columns are indicated. Note the complete absence of diazepam effects on the measured mIPSC parameters from CIE rats.

significant increase in DZ-insensitive binding sites for [3 H]Ro15-4513 (those not displaced by excess 10 μ M diazepam) was found in hippocampus using the autoradiography method to measure binding to brain sections. These binding sites are known to be produced by GABAR containing α 4 and γ 2 subunits (Whiting et al., 2000). The increased DZ-insensitive binding in cerebellum (Mhatre et al., 1988; Petrie et al., 2001) is caused by elevation of the α 6 subunit: α 6 is not expressed outside the cerebellum.

CIE rats Show Remarkably Reduced Behavioral Sensitivity to Hypnotic Effects of Positive GABAR Modulators

CIE rats became resistant to the sedative-hypnotic action of several positive allosteric modulators of GABAR. The hypnotic effect of flurazepam was reduced dramatically, measured with the loss of righting reflex, consistent with the subunit switch from α 1 to α 4. Furthermore, the hypnotic action of the neuroactive steroid (anesthetic) alphaxalone

was greatly reduced, that of pentobarbital was partially lost, whereas that of propofol was not affected. Thus, we conclude that the function of GABAR in major circuits involved in anesthesia/sedation/sleep is reduced in alcohol dependence and probably contributes in a major way to this condition.

In contrast to the reduced sedation by the benzodiazepine, no such reduction was seen for the anxiolytic effect of diazepam in the CIE rats. This indicates that the plastic changes in GABAR are pathway- and subunit-specific. This might reflect the drop in GABAR α 1 subunit observed, because the α 1 subunit has been shown to selectively mediate the sedative but not anxiolytic actions of the benzodiazepines (Rudolph et al., 1999; McKernan et al., 2000), which involve, more importantly, the α 2 subunit-containing GABAR (Low et al., 2000). It seems that CIE provides a phenocopy of the genetically engineered α 1 subunit point mutation H101R described in those reports.

Comparison of Changes Seen in CIE Rats versus Those with Continuous Ethanol Administration Regimens

The critical difference between a multiple withdrawal paradigm, as in CIE rats, versus other more continuous models of long-term treatment with ethanol are described in the introduction and in our previous publications on CIE (compare Becker, 1998). Some of these behavioral and biochemical changes seen in CIE, such as elevated subunits (α 4, α 6, γ 1, γ 2S) and reduced α 1 (Morrow et al., 1990; Mhatre et al., 1993; Devaud et al., 1997; Matthews et al., 1998) are also seen with regimens of long-term treatment with ethanol that do not involve such dramatic or frequent withdrawal episodes as does CIE. In those cases, unlike CIE, the changes do not persist more than 1 day after ethanol treatment. We tentatively conclude that the changes observed in both the continuous ethanol regimens (not persistent) and in CIE (persistent) are involved in withdrawal signs after short-term ethanol treatment and the persistent and exaggerated withdrawal signs seen in CIE. We propose that the persistent changes are highly relevant to the condition of alcohol dependence.

Equally important are those changes that differ between regimens. For example, neurosteroid modulation of GABAR and sleep time are drastically reduced in CIE, whereas the neurosteroid anticonvulsant effects are enhanced after long-term continuous ethanol (Devaud et al., 1996). Therefore, the reduced sensitivity to the hypnotic action of neurosteroids in CIE rats seems to be associated with the hyperexcitable state and possibly with the drug-dependent state.

Electrophysiological Recordings on CA1 Pyramidal Cells in Hippocampal Slices of CIE and Control Rats

Reduced Amplitude and Faster Decay of mIPSCs Validate Our Previous Report of Reduced GABAR Inhibition in Hippocampus. We previously assayed GABA-activated $^{36}\text{Cl}^-$ flux in brain slices and found reduced function specifically in hippocampus and not in three lobes of cortex, inferior colliculus, or cerebellum (Kang et al., 1996). Measures of paired-pulse inhibition using field potential recordings in CA1 found deficits in CIE rats that suggested impaired inhibitory synaptic transmission, possibly at the GABAR level (Kang et al., 1996). Depth recordings after multiple ethanol withdrawals are consistent with our observations, indicating spiking EEG records in rat hippocampus

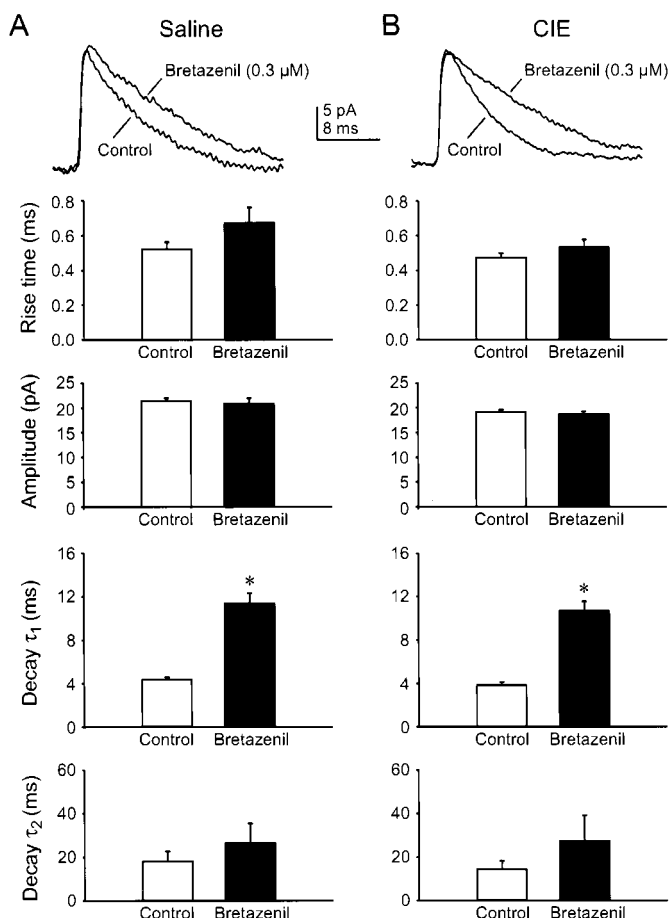


Fig. 7. Comparison of bretazenil effects on mIPSCs in saline- and CIE-treated rats. A, superimposed averaged mIPSCs obtained from a representative CA1 neuron recording from a saline-treated rat before (control) and 20 min after bath application of bretazenil (0.3 μ M). The graphs below represent changes in rise time, amplitude, decay τ_1 , and decay τ_2 , respectively. The bars represent parameters before (□) and after (■) 15 to 20 min of bretazenil application. Data are presented as mean \pm S.E.M. ($n = 9$ cells, 3 rats). *, significantly different from control. B, superimposed averaged mIPSCs obtained from a representative CA1 neuron recording from a CIE-treated rat before (control) and 20 min after bath application of bretazenil. Graphs below correspond to the same parameters ($n = 9$ cells, 2 CIE rats) as in A. Note the similar effects of bretazenil on the measured mIPSC parameters from CIE rats.

(Veatch and Gonzalez, 1996) and spike/wave spindling in mouse thalamocortical regions (Veatch et al., 1999), areas known to participate in sleep and seizure activities.

Reduced Inhibition in CA1 Is Apparently Caused by GABAR "Subunit Switch", Shown by Selective Changes in Benzodiazepine Pharmacology. The "switched" GABAR subunits contribute to synaptic transmission, because mIPSCs from CIE rats exhibit altered kinetics (smaller amplitude, faster decay). Also, modulation by the benzodiazepine diazepam of mIPSCs is lost in CIE rats, but modulation by the benzodiazepine bretazenil is not. The former drug is active on $\alpha 1\beta\gamma 2$ types of GABAR but not $\alpha 4\beta\gamma 2$, whereas the latter drug is active on both (Wieland et al., 1992; Knoflach et al., 1996). Furthermore, mIPSCs are enhanced only slightly by the benzodiazepine 'partial inverse

agonist' Ro15-4513 in saline-treated control rats, but become well enhanced by this drug in CIE rats. This is further consistent with the subunit switch from $\alpha 1$ to $\alpha 4$.

CIE Rats Show Dramatic Loss of Sensitivity to Diazepam and Neurosteroid Modulation of GABAR in CA1, Consistent with Reduced Sleep Time in Response to These Agents. The mIPSCs in CA1 principal cells lost positive allosteric modulation of GABAR by diazepam and the neuroactive steroid alfaxalone.

Role of GABAR in Alcohol Dependence

Studies on recombinant GABAR (Whiting et al., 2000; Olsen and Macdonald, 2002) do not suggest a simple correlation of subunits and behavior, but they are no doubt related intimately. Interestingly, as in CIE, a similar decrease in

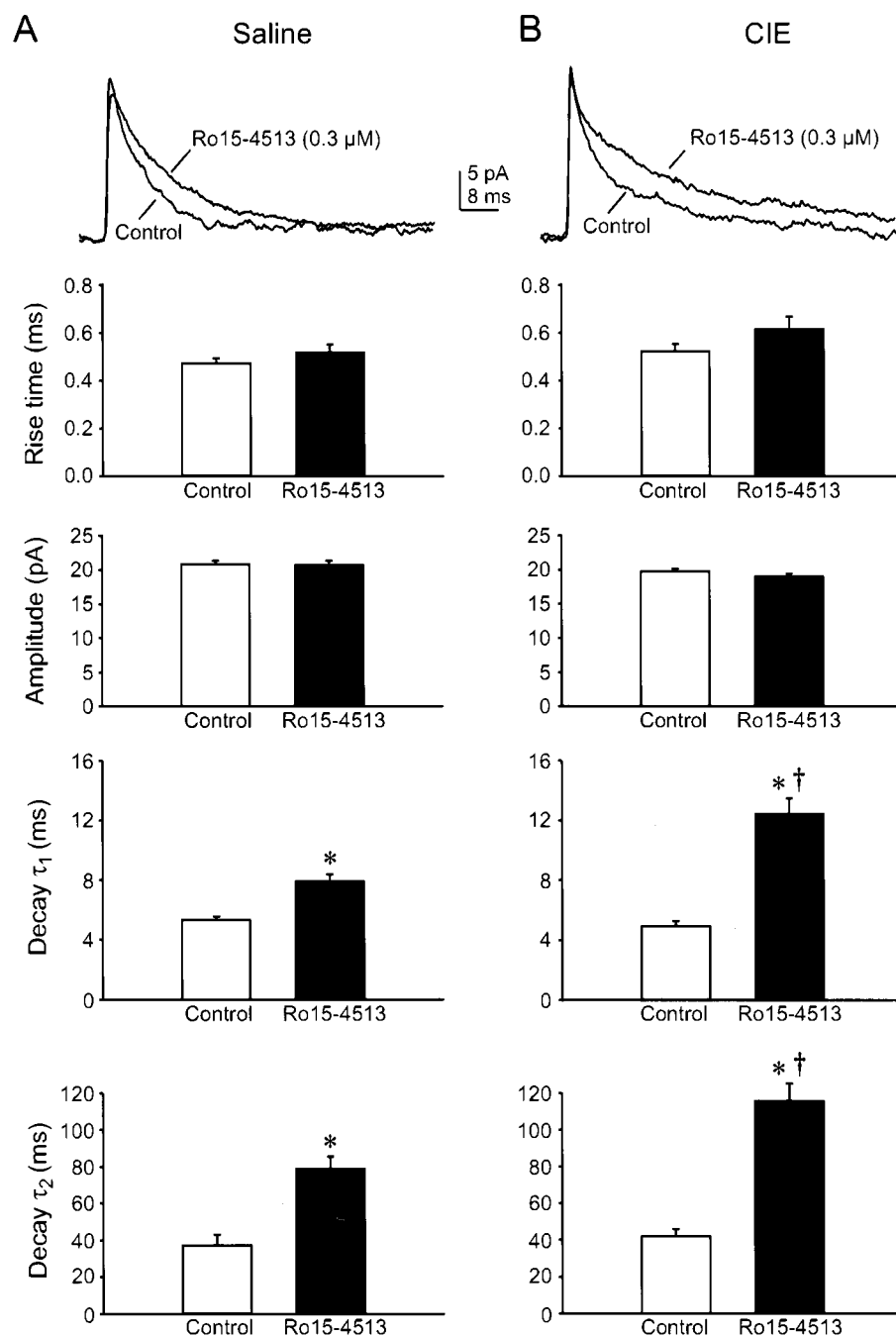


Fig. 8. Comparison of Ro 15-4513 effects on mIPSCs in saline- and CIE-treated rats. A, superimposed averaged mIPSCs obtained from a representative CA1 neuron recording from a saline-treated rat before (control) and 20 min after bath application of Ro15-4513 (0.3 μ M). The graphs below represent changes in rise time, amplitude, decay τ_1 , and decay τ_2 , respectively. The bars represent parameters before (\square) and after (\blacksquare) 15 to 20 min of Ro15-4513 application. Data are presented as mean \pm S.E.M. ($n = 9$ cells, 4 rats). *, significantly different from control. B, superimposed averaged mIPSCs obtained from a representative CA1 neuron recording from a CIE-treated rat before (control) and 20 min after bath application of Ro15-4513. Graphs below correspond to the same parameters ($n = 7$ cells, 4 CIE rats) as in A. †, statistically different from Ro15-4513 effect in saline-treated rats. Note the significantly larger increases in mIPSC decay time constants by Ro15-4513 in CA1 neurons from CIE rats.

mIPSC current because of increased decay rate and a loss of benzodiazepine sensitivity of GABAR recorded in CA1 neurons were observed in rats subjected to long-term exposure and withdrawal from the GABA-active steroid metabolite of progesterone, allopregnanolone; the changes were accompanied by elevated levels of the GABAR $\alpha 4$ subunit and reversed by administration of antisense RNA for $\alpha 4$ (Smith et al., 1998a,b). These steroid-withdrawal animals become tolerant to neurosteroids and cross-tolerant to benzodiazepines. CIE rats become tolerant to benzodiazepines and neurosteroids, at least in CA1, as shown by electrophysiology, and in hypnotic effects, as shown by behavior. The $\alpha 1$ subunit is known to be more sensitive to benzodiazepines than $\alpha 4$ (Wieland et al., 1992; Whiting et al., 2000), but in light of the current results, it also will be interesting to carefully examine the role of the $\alpha 1$ subunit in anesthetic action.

Reduced sensitivity to neurosteroids in particular is present in mice lacking the GABAR δ subunit (Mihalek et al., 1999), a subunit reduced in CIE. Studies on recombinant GABAR show that δ -containing $\alpha 4/6\beta\delta$ subtypes are more sensitive to neurosteroid modulation than $\alpha 4/6\beta\gamma 2$ (Brown et al., 2002; Wohlfarth et al., 2002). Thus, the δ receptors may be important steroid targets in vivo, and a δ -to- $\gamma 2$ subunit switch might account for reduced steroid sensitivity. Recently, the $\alpha 4\beta\delta$ combination has been shown to be highly sensitive to direct modulation by ethanol (Sundstrom-Poromaa et al., 2002); thus, reduced δ subunit could account for ethanol tolerance. The $\gamma 2$ and δ subunits can also be distinguished by sensitivity to zinc. We did not test zinc on the mIPSCs of CA1 neurons because receptors containing the δ subunits, which are more sensitive to zinc than those containing $\gamma 2$ subunits (Whiting et al., 2000; Olsen and Macdonald, 2002), are not expected to contribute to miniature synaptic currents caused by an exclusively extrasynaptic localization (Nusser et al., 1998). Conversely, the $\gamma 2L$ subunit was suggested to play a specific role in alcohol-GABAR pharmacology (Wafford et al., 1991), but its exact role remains in question (Olsen and Macdonald, 2002).

In summary, observed behavioral alterations (reduced sensitivity to hypnotics and neuroactive steroids, increased activity, anxiety, and seizure susceptibility) in the CIE rat are supportive of the relevance of this model to alcohol dependence in humans. We can explain much of the behavioral plastic changes in terms of altered function of GABAR-mediated inhibition, caused by a switch in subunit composition in critical anatomical circuits, one of which includes the hippocampus. Selective anatomic changes in GABAR produce selective behavioral alterations. Decreased functions of GABAR in alcohol dependence include a major effect on the state of the CNS regarding sleep/wakefulness and anxiety. Although the hippocampus is not known as a critical region for sleep control, it seems that the hyperexcitable hippocampus can impair normal sleep and effects of sedative-hypnotic drugs in the alcohol-dependent kindled subject. Further studies on areas implicated more directly in anxiety, such as mesolimbic structures, are needed to understand this plastic change. This demonstration of even partial selectivity is a large advance in understanding the role of specific gene products in CNS function and plasticity, especially in pathological conditions like such as drug dependence and alcoholism.

Acknowledgments

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