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Chronic Ethanol Administration Alters γ-Aminobutyric Acid$_A$ Receptor Gene Expression

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SUMMARY

Chronic ethanol (alcohol) administration has been associated with alterations in the binding and function of the γ-aminobutyric acid (GABA$_A$) receptor. To evaluate the mechanism underlying these changes, we measured the steady state levels of the mRNAs for the α_1, α_2, α_3, α_5, and α_6 subunits of the GABA$_A$ receptor after chronic ethanol administration to rats and ethanol withdrawal for 24 hr. The results indicated that chronic ethanol administration resulted in a 61% decline in the level of the GABA$_A$ receptor α_1 subunit mRNAs [3.8 and 4.3 kilobases (kb)] in the cerebral cortex in rats. The levels of the α_2 subunit mRNAs (6 and 3 kb) and the α_5 subunit mRNA (2.8 kb) were also reduced, by 61, 45, and 51%, respectively, whereas there was no change in the level of the α_3 subunit mRNA (3 kb). Furthermore, the ethanol-induced decrease in receptor mRNA levels persisted for 24 hr, after withdrawal of ethanol and returned to control values at 36 hr of withdrawal. α_1 mRNA levels in cerebellum also decreased by 28%. The level of the α_6 subunit mRNA, which selectively encodes Ro15–4513 binding sites, was found to be increased by ~76% in the cerebellum. Also, the photoaffinity labeling studies using [^{3}H]Ro15–4513 indicated an increase in the levels of various protein components of the GABA$_A$ receptor, in the cerebellum and the cerebral cortex (e.g., 50- and 55-kDa proteins in the cerebellum and 41- and 50-kDa proteins in the cortex), after chronic ethanol treatment. The increase in α_6 mRNA in the cerebellum might be related to the increased labeling of the 55-kDa (~56-kDa) protein and partially responsible for the increased binding, as reported previously by us. Because the α_6 subunit is not expressed in cortex, involvement of an as yet unknown subunit in this region cannot be ruled out. The effect of chronic ethanol treatment appears to be specific for GABA$_A$ receptor subunit mRNAs, because the same treatment did not alter the levels of glyceraldehyde-3-dehydrogenase mRNA or poly(A)$^+$ RNA. In summary, these data indicate that chronic ethanol treatment results in an alteration in the regulation of expression of GABA$_A$ receptor subunit-encoding mRNAs, which could be due to alterations in transcription or mRNA stability.

The GABA$_A$ receptor is an important site of drug action and modulation by benzodiazepines, barbiturates, steroids, and ethanol (alcohol) in the mammalian CNS (1–3). GABA, the major inhibitory neurotransmitter in the vertebrate CNS, binds to this receptor. Activation of GABA$_A$ receptors by GABA produces an influx (hyperpolarization) or efflux (depolarization) of Cl^-, resulting in presynaptic or postsynaptic inhibition, depending upon the Cl^- gradient (4). The GABA$_A$ receptor is a member of the ligand-gated ion channel family of receptors (5). Recent studies have indicated that ethanol affects the receptors belonging to this family, e.g., ethanol inhibits the response of the *N*-methyl-D-aspartate receptor and facilitates the responses of GABA$_A$ receptors to agonists (6). Several lines of behavioral, electrophysiological, and biochemical functional studies strongly implicate the involvement of GABAergic transmission in the action of ethanol in the CNS. Ethanol, like benzodiazepines, is known to potentiate GABA-induced $^{36}Cl^-$ flux in cultured embryonic spinal cord neurons (6, 7), rat cerebral cortical synaptoneurosomes (8), and microsacs (9, 10).

Chronic exposure to ethanol results in tolerance and physical dependence. Also, ethanol has a pharmacological profile very similar to that of benzodiazepines and barbiturates, and a partial inverse agonist of the benzodiazepine receptor, Ro15–4513, blocks the behavioral effects of ethanol (11, 12). Thus, it is reasonable to assume that the molecular and cellular mechanisms underlying the behavioral action of ethanol, including the development of tolerance and dependence, could be explained by an understanding of its unique interaction with the GABA$_A$-benzodiazepine receptor complex. Moreover, ethanol withdrawal symptoms can be treated with benzodiazepines. Our previous studies showed that chronic ethanol treatment produced an up-regulation of binding sites for the benzodiazepine inverse agonist and ethanol antagonist Ro15–4513, in the cerebral cortex and cerebellum of the rat brain and in embryonic

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ABBREVIATIONS: GABA, γ-aminobutyric acid; CNS, central nervous system; Ro15–4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate; DMCM, 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; kb, kilobases.

spinal cord neurons in primary culture, with no change in the binding of benzodiazepine agonists or antagonists (13, 14). Furthermore, chronic ethanol administration also decreased the efficacy of GABA-induced $^{36}Cl^-$ influx in synaptoneurosomes (15) and cultured spinal cord neurons.[1] The mechanism of this chronic ethanol-induced subsensitivity of the $GABA_A$ receptor has not yet been fully explored. It could be the consequence of a change in $GABA_A$ receptor gene transcription or altered post-translational events in the expression of receptor.

Recent cloning studies have indicated that the $GABA_A$-benzodiazepine receptor is a complex protein consisting of several homologous membrane-spanning glycoprotein subunits (α, β, γ, δ) (5, 16–18). The exact subunit structures and the stoichiometry needed has yet to be defined. However, the available evidence suggests that the $GABA_A$ receptor is a hetero-oligomer of 220–400 kDa, composed of two to four different polypeptides and a total of four or five subunits (3). Numerous studies have indicated structural as well as functional heterogeneity of $GABA_A$ receptors in different regions of the brain, which is related to differential expression of α variants within the CNS (19, 20). These α subunit mRNAs, in combination with the β subunit mRNA, express receptor subtypes in *Xenopus* oocytes, which can be distinguished due to their different pharmacological properties (17). In the present study, in order to investigate a molecular basis for chronic ethanol-induced changes in the $GABA_A$ receptor, we have evaluated steady state levels of mRNA for α_1, α_2, α_3, α_5, and α_6 subunits of the receptor. After chronic ethanol treatment, the mRNA levels for α_1, α_2, and α_5 subunits were found to be decreased in cerebral cortex, and these changes were found to persist after 24 hr of ethanol withdrawal, returning to control values by 36 hr of withdrawal. On the other hand, the same treatment resulted in a decrease in α_1 mRNA and an increase in the level of ethanol antagonist-specific α_6 subunit mRNA in the cerebellum. The photoaffinity labeling studies also revealed a marked increase in irreversible binding of the benzodiazepine inverse agonist Ro15–4513 in the cerebral cortex and cerebellum. These observations together provide evidence of chronic ethanol-induced mechanistic changes in $GABA_A$ receptors and may explain the depressed sensitivity of GABAergic transmission, as well as the development of tolerance and dependence, after chronic ethanol treatment.

Materials and Methods

Chronic ethanol treatment. Adult male Sprague-Dawley rats (130–170 g) were purchased from Harlan (Indianapolis, IN). Chronic ethanol was administered by an intragastric intubation method, as described earlier (21, 22). Briefly, a priming dose of 5 g/kg ethanol was administered to all animals. This was followed by adjustment of doses individually for each animal, according to the presence or absence of ataxia, loss of righting reflex, and motor activity, as described (22). Forty-eight animals were given 20% (v/v) ethanol, in normal saline, three times each day for 6 days. The control rats (n = 20) received equivalent amounts of saline. In the ethanol-maintained rats, brain regions were dissected 1 hr after the last dose of ethanol. The rats from the ethanol withdrawal group were sacrificed 24 hr (or, in some cases, 36 or 48 hr) after the last dose of ethanol. Of 48 animals, 40 animals survived after ethanol treatment, 21 in the ethanol-treated group and 19 in the withdrawal group.

[1] M. C. Mhatre and M. K. Ticku, unpublished observations.

cDNA probes. Rat $GABA_A$ receptor cDNA probes for α_1, α_2, α_5, and α_6 subunits used in this study were isolated from their respective clones in Bluescript plasmid vector, obtained as gifts from Dr. A. Tobin (UCLA, Los Angeles) and Dr. D. Pritchett (Children's Hospital, Philadelphia). The α_1 cDNA probe included an open reading frame of 455 codons, of which 27 were in a putative signal peptide (23). This probe was 540 base pairs long and was isolated from the vector by *Bam*HI and *Xho*I digestion. The α_2 cDNA was a 1.5-kb *Eco*RI fragment isolated by *Eco*RI digestion. The α_5 cDNA was 2.7 kb long and contained an internal *Eco*RI site (24) (also termed α_4 in Ref. 23). The 1.2- and 1.5-kb bands of α_5 cDNA were separated on 1% low melting point-agarose and pooled to make the cDNA probe. The α_6 cDNA probe was 180 base pairs long and was labeled by nick translation. The sequence of bovine α_3 oligonucleotide used was 5′-ACACCAGCAGTCCCGACCAA-GAAAACCAGCACCACTTTCAACATCGTGGGAACCACC-3′ (25). The α_3 probe was labeled at the 3′ end, using the oligonucleotide as a template for terminal deoxytransferase, in a 20-μl reaction mixture containing 20 units of terminal deoxytransferase (Bethesda Research Laboratories, Inc.), 100 mM potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM dithiothreitol, and 100 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham). All cDNA probes, except α_5, were gel-purified twice and labeled with ^{32}P by random hexamer priming. Specific activity for oligo-labeling and nick translation was approximately 1–3 × 10^9 cpm/μg of DNA and 2 × 10^8 cpm/μg of DNA, respectively.

RNA preparation, electrophoresis, and blotting. Total RNA was isolated from dissected rat brain tissue by homogenization in 4 M guanidine thiocyanate, followed by centrifugation through 5.7 M CsCl, according to the method of Chirgwin *et al.* (26). The RNA was purified by repeated ethanol precipitation, and its concentration was estimated from A_{260} values. Poly(A)$^+$ RNA was isolated by using two cycles of oligo(DT)-cellulose affinity chromatography (27). All RNA samples were stored at −80°.

The RNA was denatured in 50% deionized formamide, 6.5% formaldehyde, at 65° for 5 min and then size-fractionated by electrophoresis on a horizontal agarose gel (1.1%) containing 6.5% formaldehyde (28). The RNA was subsequently electrically transferred to nylon membrane (Nytran; Schleicher & Schuell) and immobilized by baking at 80° for 2 hr, under vacuum. Membranes were prehybridized for 6 hr at 42° in 5× SSC, 50% formamide, 0.5% SDS, 50 mM sodium phosphate, pH 6.5, containing 250 μg/ml denatured salmon sperm DNA, 5× Denhardt's solution, and 100 μg/ml polyadenylic acid (29). The ^{32}P-labeled cDNA probe (1 × 10^7 cpm/ml) was added to the prehybridization solution at approximately 4 ng/ml. After hybridization at 42° overnight, the membranes were washed twice for 30 min at room temperature in 2× SSC and twice for 30 min at 50° in 0.1× SSC (4.4% sodium chloride, 2.2% sodium citrate), 0.1% SDS. Membranes were exposed to X-ray film, with two intensifying screens, at −80°.

We quantified the poly(A)$^+$ RNA content in each total RNA sample by using a ^{32}P-labeled oligo(dT)12–18 probe (Pharmacia). Total RNA was dissolved in 10 μl of water and mixed with 50 μl of 20× SSC and 40 μl of 37% (v/v) formaldehyde, and the mixture was heated at 50° for 15 min. The denatured RNA samples were chilled on ice and then applied to a Nytran membrane mounted in a Bio-dot apparatus (Bio-Rad). Prehybridization and hybridization was done at 4°. Blots were washed successively in 6×, 4×, 2×, and 1× SSC and 0.5× SSC, 0.1% SDS, at room temperature, were exposed to X-ray film at room temperature, and were quantitated using IMAGE (Blot analyzer, Model 603, Betagene Corp.).

To quantify the α subunit mRNAs detected by Northern analysis, the absorbance of the corresponding hybridization signals was measured using IMAGE, an image-processing and analysis computer program for Macintosh II, as well as Betascopy. Images were captured according to the program directions, with a Philips S6473 digital CCD camera connected via a Quick Capture 2255 (Data Translation) frame-capture board. The linearity of densitometry was first established by plotting absorbance against exposure time. The densities of α subunit mRNAs in the Northern blot autoradiograph, which was loaded succes-

sively with samples containing 2–10 μg of poly(A)$^+$ RNA and was exposed for several time periods after blotting with ^{32}P-labeled α subunit cDNA probe (1.2×10^9 cpm/μg of DNA), were compared. The correlation coefficient of analysis was found to be >1, and analysis gave a linear curve.

GABA$_A$ receptor α subunit mRNA levels in cerebral cortex were measured in the poly(A)$^+$ fractions purified from individual animals in each experiment, and each group included 12 rats (number of experiments = 12). For cerebellar tissue, the poly(A)$^+$ fraction was purified from total RNA obtained from two rats per experiment (n = 8). Two gels, which gave identical results, were run for each experiment. The absorbance values were analyzed by Student's t test.

The length of the GABA$_A$ receptor subunit mRNAs was measured by plotting 28 S (5 kb) and 18 S (2 kb) RNAs against the distance travelled by these RNAs. GABA receptor subunit mRNA size was extrapolated from this, using R_F values of the respective transcript.

Photoaffinity labeling. Membrane ($P_2 + P_3$) suspension (21) from control and ethanol-treated rat cerebral cortex and cerebellum was incubated (in Krebs-15 mM Tris buffer, pH 7.4) with [^{3}H]flunitrazepam (10 nM 84.3; Ci/mmol) or [^{3}H]Ro15–4513 (10 nM; 24.3 Ci/mmol), in the absence or presence of 10 μM diazepam, at 0° in the dark for 90 min. The membrane suspension was irradiated with UV light (366 nm) for 30 min at 4°, is described (30), repeatedly washed with buffer to remove free and reversibly bound radioligands, solubilized, and subjected to SDS-polyacrylamide gel electrophoresis and fluorography (30).

Results

The experimental protocol used in this study for chronic ethanol treatment produced a peak blood ethanol concentration of 425 ± 65 mg/dl after the last injection (21). The intoxication produced by this treatment and the behavioral changes observed during withdrawal were consistent with the development of physical dependence in the rats, as described previously (21, 22).

The GABA$_A$ receptor α subunit mRNA levels were measured in the poly(A)$^+$ RNA fraction purified from samples of total RNA, individually isolated from cerebral cortex (n = 12) and cerebellum (n = 16) of ethanol-treated, ethanol-withdrawn (24 hr), and control rats. As described below, Northern blot analysis of rat cerebral cortex showed four α subunit mRNA species. They included two distinct α_1 mRNA transcripts (a doublet consisting of 3.8 and 4.3 kb), two α_2 mRNA transcripts (3 and 6 kb), the α_3 mRNA transcript (3 kb), and the α_5 mRNA transcript (2.8 kb), in agreement with previous studies (23–25); whereas in the cerebellum, only two α subunit mRNA species were expressed, i.e., α_1 (3.8 and 4.3 kb) and α_6 (2.7 kb). In both cerebral cortex and cerebellum, the α_1 subunit was the most abundant mRNA among various subunits of the GABA$_A$ receptor, and the α_6 subunit mRNA (2.7 kb) transcript could be detected only in rat cerebellum. The α subunit probes used did not show any cross-reactivity with various subunit mRNA transcripts for the GABA$_A$ receptor.

Chronic ethanol treatment decreases α subunit mRNA levels in the cortex. Absorbance measurements of the autoradiogram signals, corresponding to the GABA$_A$ receptor α subunits, were obtained using image analysis. Fig. 1A shows that both the α_1 subunit transcripts were decreased by 61% (Table 1) after chronic ethanol treatment ($p < 0.005$, Student's t test). There were two transcripts of α_2 mRNA, of 6 kb and 3 kb, in the cortex (Fig. 1B). An approximately 61% decrease in the absorbance of the 6-kb transcript was observed in the cortical RNA from ethanol-treated rats ($p < 0.005$), whereas

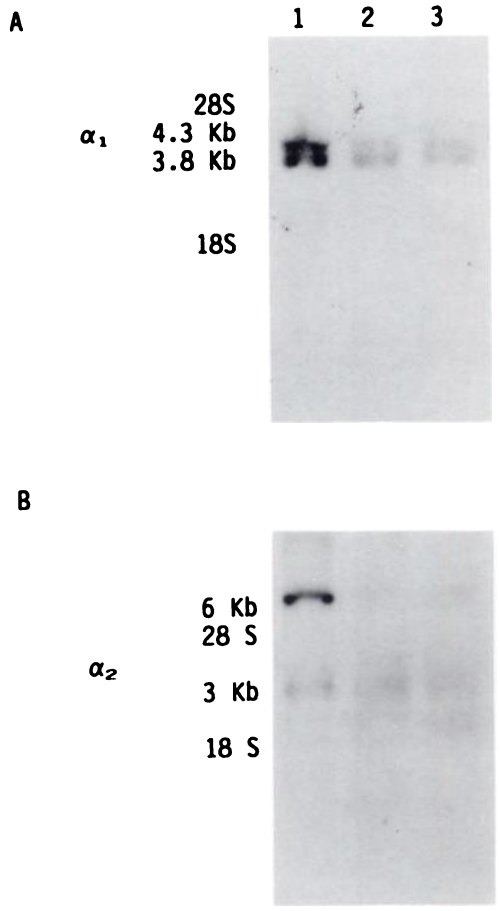

Fig. 1. A, Effect of chronic ethanol treatment and withdrawal on GABA$_A$ receptor α_1 subunit mRNA levels in rat cerebral cortex, analyzed by Northern blot analysis. Poly(A)$^+$ RNA isolated from control (*lane 1*), ethanol-treated (*lane 2*), and ethanol-withdrawn (*lane 3*) rat cerebral cortex was fractionated by electrophoresis, blotted onto nylon membrane, and hybridized with radiolabeled cDNA probe specific for the α_1 subunit of the GABA$_A$ receptor. Poly(A)$^+$ samples (10 μg) were loaded in each well. B, Effect of chronic ethanol treatment and withdrawal on GABA$_A$ receptor α_2 subunit mRNA levels in cerebral cortex. The results are summarized in Table 1.

the 3-kb transcript was decreased by 45% in ethanol-treated rats ($p < 0.005$). GABA$_A$ receptor α_1, α_2, and α_3 subunit cDNAs show about 70% homology (3). Poly(A)$^+$ RNA probed with α_3 subunit oligonucleotide showed one transcript of 3 kb, the level of which remained unchanged in ethanol-treated rats (Fig. 2). The rat α_5 amino acid sequence (as published in Ref. 24; also called α_4 in Ref. 23) also shows 70% identical residues, when compared with the α_1 sequence from rat (23). It is evident from Fig. 3 that there is a marked decrease in α_5 subunit mRNA level after ethanol treatment (51%) (Table 1). Thus, levels of all GABA$_A$ receptor α subunit mRNAs (except α_3 mRNA) were decreased in the cerebral cortex of rats continually exposed to ethanol.

α subunit mRNA levels remain decreased in the cerebral cortex after ethanol withdrawal for 24 hr. After 24 hr of withdrawal of the treatment, there was no ethanol detected in this group of animals. However, the mRNA level of the α_1 subunit remained decreased in the cortex of these animals (71% decline) (Fig. 1A). For the α_2 subunit mRNA, 6-kb as well as 3-kb transcripts were substantially reduced, by 61 and 43%, respectively, during withdrawal (Fig. 1B). The α_3

subunit mRNA level remained unchanged (Fig. 2; Table 1), whereas the α_5 subunit mRNA level decreased during withdrawal (45%) (Fig. 3). To gain more information regarding the duration of these changes, we measured the levels of a representative mRNA (α_2) at 36 and 48 hr of withdrawal, in the cerebral cortex. The α_2 subunit mRNA levels (6 kb and 3 kb) returned to control values at 36 hr of withdrawal. Similar values were observed for the 48-hr withdrawal group.

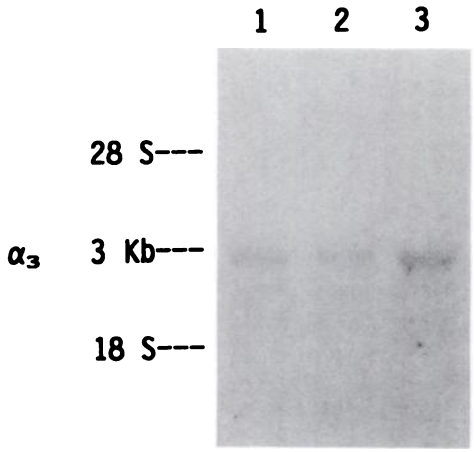

Fig. 2. Effect of chronic ethanol treatment and withdrawal on $GABA_A$ receptor α_3 subunit mRNA levels in cerebral cortex. The lanes represent control (*lane 1*), ethanol-treated (*lane 2*), and ethanol-withdrawn (*lane 3*) groups. The results are summarized in Table 1.

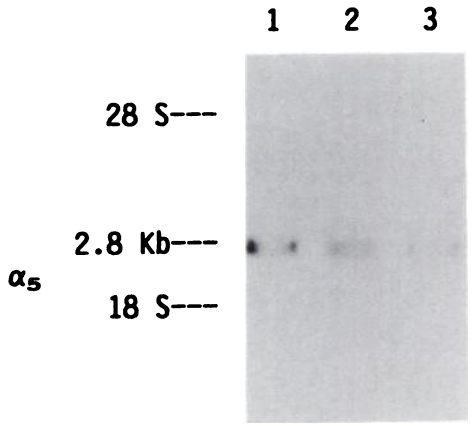

Fig. 3. Effect of chronic ethanol treatment and withdrawal on $GABA_A$ receptor α_5 subunit mRNA levels in cerebral cortex. The lanes represent control (*lane 1*), ethanol-treated (*lane 2*), and ethanol-withdrawn (*lane 3*) groups. The results are summarized in Table 1.

α_6 subunit mRNA encoding Ro15–4513 binding sites increases in the cerebellum. The α_6 subunit mRNA, in combination with β_2 and γ_2 subunits, selectively encodes the Ro15–4513 binding sites in cerebellum, which are insensitive to diazepam (31). Chronic ethanol treatment produced a ~76% increase in the α_6 subunit mRNA levels in the cerebellum (Fig. 4; Table 2). This observation is consistent with the previous report that Ro15–4513 binding sites increase after chronic ethanol treatment (13). Also, this dramatic change in mRNA level persisted at 24 hr after ethanol withdrawal (150% increase, $p < 0.005$). The α_1 subunit mRNA level decreased by 28% after ethanol treatment and by 34% after 24 hr of withdrawal, in the cerebellum (Fig. 5; Table 2). Both α_1 and α_6 mRNA levels returned to control values at 36 hr of withdrawal (Table 2). Similar values were observed for the 48-hr withdrawal group.

The chronic ethanol treatment did not change α_3 subunit mRNA levels (Fig. 2) or poly(A)$^+$ (Fig. 6) or glyceraldehyde-3-phosphate dehydrogenase mRNA levels (data not shown).

Photolabeling by [^{3}H]Ro15–4513 in the cerebral cortex and cerebellum increases after chronic ethanol treatment. The results shown in Fig. 7 indicate that [^{3}H]Ro15–4513 specifically and irreversibly labeled three different proteins, with apparent molecular masses of 41, 50, and 59 kDa (P_{41}, P_{50}, and P_{59}) in the cortical receptor preparation (Fig. 7, *lanes 3* and *4*). The levels of P_{41} and P_{50} irreversibly bound to [^{3}H]Ro15–4513 were found to be increased in the cortex from ethanol-treated rats (Table 3). Similarly, in the cerebellum, [^{3}H]Ro15–4513 irreversibly bound to 50-kDa and 55-kDa proteins (P_{50} and P_{55}), and there was an increase in the photolabeling of both these components. Also, in a similar experiment using [^{3}H]flunitrazepam as a ligand, there was no change in photolabeling in either the cortical or cerebellar receptor preparation after chronic ethanol treatment (Fig. 7). This indicates an involvement of other untested subunits, in addition to the α_6 subunit, after chronic ethanol treatment, because α_6 mRNA encodes only a 56-kDa component, a specific binding site for Ro15–4513.

Discussion

In the present studies, chronic ethanol treatment that produced physical dependence in the rats resulted in the reduction of α_1, α_2, and α_5 subunit mRNA levels in the cerebral cortex and α_1 subunit mRNA levels in the cerebellum (Fig. 1, 3, and

TABLE 1

Effect of chronic ethanol and its withdrawal on α_1, α_2, α_3, and α_5 subunit mRNA levels in cortex

Rats were treated with ethanol as described in the text. Results are in relative units. The values in parentheses represent percentage decreases in the mRNA levels. Results are the mean ± standard deviation of 8–12 experiments, except for the 36- and 48-hr withdrawal groups (three experiments).

Subunits	mRNA levels				
	Control	Ethanol-treated	Ethanol-withdrawn		
			24 hr	36 hr	48 hr
α_1 (3.8, 4.3 kb)	1.12 ± 0.06	0.44 ± 0.06[a] (−61%)	0.33 ± 0.02[a] (−71%)	ND[b]	ND
α_2 (6 kb)	1.61 ± 0.06	0.62 ± 0.08[a] (−61%)	0.63 ± 0.05[a] (−61%)	1.60 ± 0.20[c]	1.87 ± 0.10[c]
α_2 (3 kb)	0.92 ± 0.01	0.51 ± 0.01[a] (−45%)	0.52 ± 0.08[a] (−43%)	1.01 ± 0.10[c]	1.16 ± 0.10[c]
α_3 (3 kb)	0.63 ± 0.02	0.54 ± 0.01 (−14%)	0.62 ± 0.03[c]	ND	ND
α_5 (2.8 kb)	1.27 ± 0.15	0.62 ± 0.10[a] (−51%)	0.70 ± 0.20[a] (−45%)	ND	ND

[a] $p < 0.005$, compared with control.
[b] ND, not determined.
[c] Not significant, compared with control group.

TABLE 2

Effect of chronic ethanol and its withdrawal on α_1 and α_6 subunit mRNA levels in cerebellum

The α_2, α_3, and α_5 subunit mRNAs were not detectable in the cerebellum. The values in parentheses represent percentage changes in the mRNA levels. Results are the mean ± standard deviation of eight experiments, except for the 36- and 48-hr withdrawal groups (three experiments); each experiment utilized two rats.

	mRNA levels				
	Control	Ethanol-treated	Ethanol-withdrawn		
			24 hr	36 hr	48 hr
α_1 (3.8, 4.3 kb)	1.07 ± 0.03	0.78 ± 0.04 (−28%)	0.71 ± 0.08 (−34%)	1.13 ± 0.04	0.96 ± 0.10
α_6 (2.6 kb)	1.14 ± 0.12	2.01 ± 0.12[a] (+76%)	2.85 ± 0.30[a] (+150%)	1.13 ± 0.02	1.15 ± 0.01

[a] $p < 0.005$, compared with control.

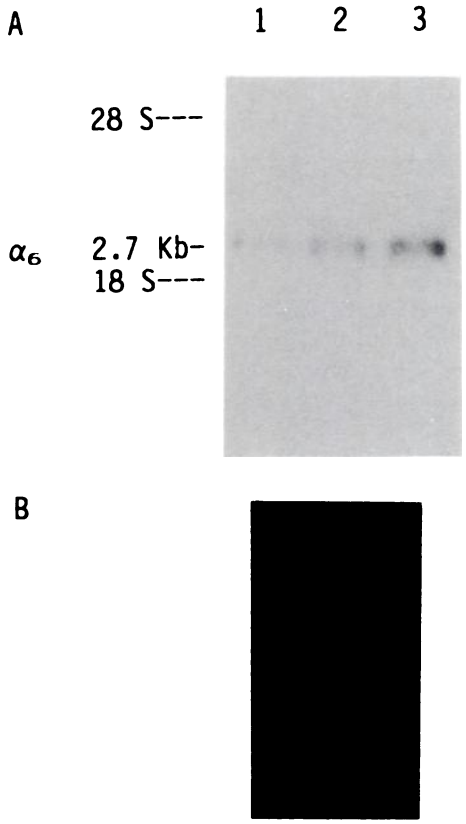

Fig. 4. A, Effect of chronic ethanol treatment and withdrawal on GABA$_A$ receptor α_6 subunit mRNA levels in rat cerebellum. Experiments were performed as described for Fig. 1. The lanes represent control (*lane 1*), ethanol-treated (*lane 2*), and ethanol-withdrawn (*lane 3*) groups. The results are summarized in Table 2. B, Photograph of the ethidium bromide-stained gel before transfer, to demonstrate normalized loading of the samples.

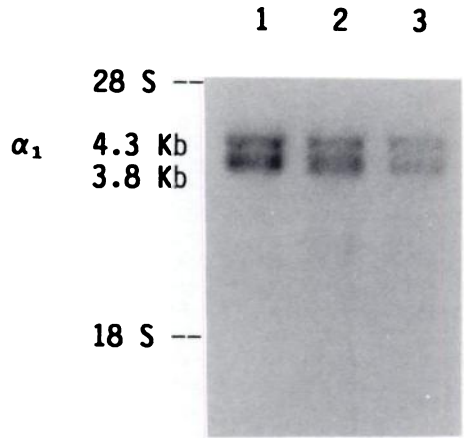

Fig. 5. Effect of chronic ethanol treatment and withdrawal on GABA$_A$ receptor α_1 subunit mRNA levels in cerebellum. Experiments were performed as described for Fig. 1. The lanes represent control (*lane 1*), ethanol-treated (*lane 2*), and ethanol-withdrawn (*lane 3*) groups. A representative Northern blot, which contained poly(A)$^+$ RNA, was hybridized with ^{32}P-labeled α_1 subunit cDNA probe. The results are summarized in Table 2.

5; Tables 1 and 2). Chronic ethanol treatment did not change G_3PDH mRNA or poly(A)$^+$ RNA levels in cortex or cerebellum (Fig. 6). Also, the α_3 subunit mRNA level remained unchanged. Thus, the changes observed appear to be specific to various GABA$_A$ receptor subunit mRNAs. The selectivity of these changes rules out, for the receptor disturbance, a generalized change in the mRNA turnover or in nuclear transcription as such. Montpied *et al.* (32, 33) have also observed a similar reduction in α_1 and α_2 subunit mRNA levels in the cerebral cortex, after chronic ethanol treatment by vapor inhalation method. This down-regulation of mRNA levels for α subunits could be associated with the subsensitivity of GABAergic transmission observed after chronic ethanol exposure (15, 34). The maximal response to GABA was significantly lowered in chronically ethanol-treated primary cultures of spinal cord neurons, compared with untreated neurons.[1] An ethanol-induced decrease in GABA$_A$ receptor sensitivity is also evident from the study of Buck and Harris (35). In that study, chronic ethanol exposure (50 mM) of *Xenopus* oocytes microinjected with mouse brain mRNA reduced the amplitude of current activated by muscimol. Thus, whether a decrease in α_1 subunit mRNA is related to the subsensitivity shown in particular regions of the brain still remains to be explored.

It is not clear why the down-regulation of α subunit transcripts is not accompanied by a change in the binding sites for different ligands that bind to the GABA$_A$ receptor (13, 21, 37). Several laboratories have failed to find changes in the number of [^{3}H]flunitrazepam (12, 20, 35, 36) or [^{35}S]*t*-butylbicyclophosphoro[^{35}S]thionate binding sites (13, 21) after chronic ethanol administration. It is possible that the radioligands used to study the changes may not be sensitive enough to detect a change in a particular subpopulation of GABA$_A$ receptors, due to the complexity and heterogeneity of receptor recognition sites in the CNS. Pharmacological and biochemical studies have suggested that benzodiazepine agonists, such as flunitrazepam, bind with similar affinity to multiple subtypes of benzodiazepine receptors (38, 39). Thus, a change in the expression of any particular subtype of the receptor may not be reflected in these particular ligand-binding experiments. Another reason could be a difference in the coupling mechanisms of these sites. The ratio of coupled receptor to uncoupled receptor may change after the functional disturbance induced by chronic treatment. Harris and Allan (40) reported that the genetic difference

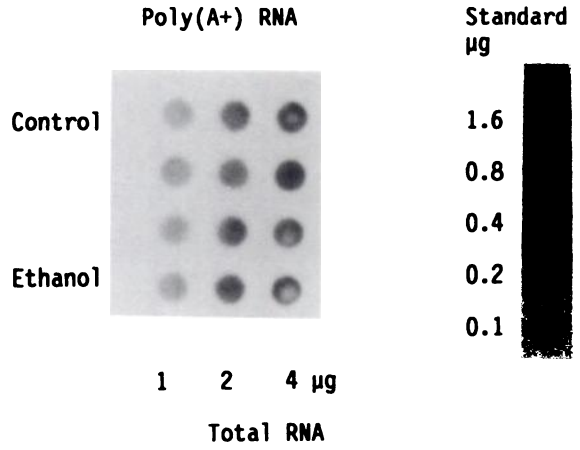

Fig. 6. Chronic ethanol treatment had no effect on the steady state levels of poly(A)$^+$ RNA in rat cerebral cortex. Poly(A)$^+$ RNA was quantitated in samples of total RNA (1, 2, or 4 µg/rat), as described in the text. Total RNA was directly applied, under vacuum, to Nytran membranes, using a dot blot apparatus, and was hybridized with [^{32}P]deoxythymidine oligonucleotide probe [35–50 base pairs; specific for poly(A)$^+$ RNA]. The specificity of the probe was tested by its linear hybridization with purified poly(A)$^+$ RNA and oligo(dA). Total RNA samples from 12 control and 12 ethanol-treated rats were analyzed. No difference in the level of poly(A)$^+$ RNA was detected in the cerebral cortex of ethanol-treated rats, compared with controls (control, 11 ± 3, 20 ± 1, and 35 ± 2; ethanol group, 12 ± 0.5, 22 ± 0.8, and 37 ± 1 absorbance units for 1, 2, and 4 µg of total RNA, respectively; n = 12). The above figure is representative of six experiments. *Rows 1* and *2* contain samples from two control rats, whereas *Rows 3* and *4* contain samples from two ethanol-treated rats.

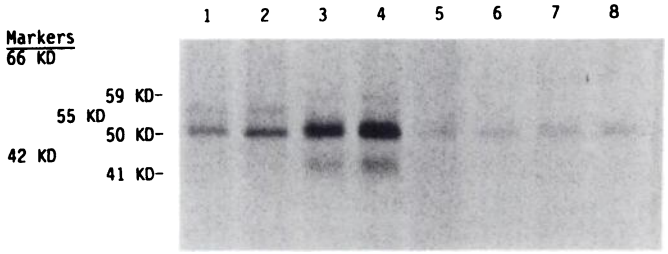

Fig. 7. Comparison of proteins photolabeled by [^{3}H]Ro15–4513 in membrane preparations from the cerebellum (*lanes 1* and *2*) and the cerebral cortex (*lanes 3* and *4*) of chronically ethanol-treated rats or control rats. The membranes were prepared (20) and specifically photolabeled with [^{3}H]Ro15–4513 (10 nM) or [^{3}H]flunitrazepam (10 nM), and equal amounts of protein were loaded for SDS-polyacrylamide gel electrophoresis. The gel was stained and scanned (to check the protein concentrations in each lane). After fluorography, the gel was dried and subjected to autoradiography. The apparent molecular masses of the protein were determined by calibrating the gel with standard proteins of known molecular weight. *Lanes 1* and *2*, cerebellar proteins photolabeled by [^{3}H]RO15-4513 from control and ethanol-treated rats, respectively; *lanes 3* and *4*, cortical proteins photolabeled by [^{3}H]R015-4513 from control and ethanol-treated rats. *Lanes 5* (control) and *6* (ethanol-treated), cerebellar proteins photolabeled by [^{3}H]flunitrazepam; *lanes 7* (control) and *8* (ethanol-treated), cortical proteins photolabeled with [^{3}H]flunitrazepam. The experiment was performed eight times using cerebral cortex and three times using cerebellum, with similar results. The percentage increases in the photolabeling of various proteins after chronic ethanol treatment are summarized in Table 3.

between long sleep (alcohol-sensitive) and short sleep (alcohol-insensitive) mice showed that both strains had the same number of flunitrazepam receptor sites but differences existed in terms of coupled and uncoupled receptors, thus suggesting that not all subtypes are coupled to the chloride channel. Also, a significant decrease in the capacity of GABA to increase [^{3}H] flunitrazepam binding in brain membranes prepared from

TABLE 3

Percentage increase in [^{3}H]Ro15-4513 photolabeling of various protein components in rat cerebral cortex and cerebellum after chronic ethanol treatment

The cortical and cerebellar membranes were prepared and photolabeled with [^{3}H] Ro15-4513 as described for Fig. 7. The differences in photolabeling in ethanol-treated rats were statistically significant ($p < 0.005$, Student's t test), relative to control values. The photolabeling by [^{3}H]flunitrazepam of the P_{50} band was not significantly different in control and ethanol-treated groups. The absorbance of P_{50} was very low, and we could not analyze the image statistically.

	[^{3}H]Ro15-4513 binding		
	P_{41}	P_{50}	P_{55}
	% increase over control group		
Cerebral cortex (n = 8)	59 ± 2	43 ± 3	–[a]
Cerebellum (n = 3)	–	50 ± 2	113 ± 3

[a] –, not detectable.

chronic ethanol-treated mice was found by de Vries *et al.* (41). Furthermore, allosteric modulation of muscimol-activated chloride conductance by flunitrazepam (benzodiazepine agonist) and DMCM (benzodiazepine inverse agonist) was also reduced in ethanol-treated oocytes (34), which supports the aforementioned hypothesis.

The withdrawal studies indicate that α subunit mRNAs remained decreased at 24 hr and returned to normal values by 36 hr. These data suggest that these changes observed during 24-hr withdrawal may contribute to the ethanol withdrawal symptoms. In fact, genetic experiments with withdrawal seizure-prone and withdrawal seizure-resistant mice provide evidence for such a relationship (42).

It is intriguing that both chronic benzodiazepine and ethanol treatments produce similar changes in gene expression, because chronic benzodiazepine treatment has been reported to produce down-regulation of α_1 subunit mRNA of $GABA_A$ receptors (43, 44). Also, Montpied *et al.* (32) found marked reductions in the levels of $GABA_A$ receptor α subunit mRNAs in primary chick neurons exposed to GABA for 48 hr *in vitro*. These data suggest that down-regulation of α transcripts after chronic ethanol, benzodiazepine, or GABA treatment could be an adaptive response to augmentation of chloride conductance due to these drugs. However, it is not clear why pentobarbital, which has a similar effect on GABA receptor-gated chloride ion conductance, fails to alter $GABA_A$ receptor α_1 subunit mRNA levels in the cerebral cortex, when given chronically (34). The determination of mRNA levels for other subunits of the $GABA_A$ receptor could probably provide an explanation for this.

Recently, we reported an increase in binding sites for [^{3}H] Ro15–4513 in rat cerebral cortex and cerebellum after chronic ethanol administration (13). Ro15–4513 is selective in antagonizing the sedative and intoxicating effects of ethanol (11) and is a partial inverse agonist for the benzodiazepine receptor. Recent studies have discovered a novel α subunit variant (α_6) present in cerebellar granular cells. Luddens *et al.* (31) have shown that recombinant receptors composed of α_6, β_2, and γ_2 subunits bind with high affinity to the GABA agonist [^{3}H] muscimol and [^{3}H]Ro15–4513 but not the other benzodiazepines or β-carbolines. The marked increase in the α_6 subunit mRNA level in the cerebellum observed in the present study is consistent with an increase in the photolabeling by [^{3}H]Ro15–4513 of the P_{55} band in the cerebellum (Fig. 7; Table 3) after chronic ethanol treatment and withdrawal. We did not find detectable expression of the α_6 subunit in the cerebral cortex of ethanol-treated rats. However, photoaffinity labeling studies

indicate a significant increase in P_{41} and P_{50} protein components, which bind to [^{3}H]Ro15–4513 irreversibly in the cortex of ethanol-treated rats. The α_6 subunit is reported to encode only the P_{56} component in the cerebellum (31), but both P_{50} and P_{55} components were increased in the cerebellum after ethanol treatment (Fig. 7). The photoaffinity labeling study clearly indicated the presence of diazepam-insensitive sites in the P_{50} region as well.[1] It is very likely that the increase in α_6 mRNA might be related to the increased labeling of P_{55} and partially responsible for the increased binding in cerebellum. It is also possible that the increased binding observed in P_{41} and P_{50} in the cerebral cortex and in the P_{50} component in the cerebellum may be due to increased levels of some other untested subunit polypeptide. Behavioral studies also support these data, in as much as chronic ethanol treatment was found to increase selectively receptor sensitivity to Ro15–4513 (45). In the study of Buck and Harris (46), acute exposure to ethanol was found to enhance the ability of the benzodiazepine inverse agonist DMCM to reduce muscimol-activated $^{36}Cl^-$ uptake by membranes isolated from mouse cerebral cortex. This increased sensitivity to an inverse agonist is apparently due to a change in the subunit expression, which can lead to functionally different receptors. This might explain both the development of tolerance and the phenomenon of kindling and seizures produced during withdrawal. After longer ethanol treatment, such a change might lead to a decrease in sensitivity in GABAergic transmission, causing symptoms of withdrawal and dependence. Thus, the available data indicate the possible existence of another subunit(s), and knowledge of such subunit(s) may well lead to a greater understanding of the mechanisms responsible for chronic ethanol action and tolerance. It is worth noting that, although the α_6 subunit is not expressed in the cerebral cortex, diazepam-insensitive [^{3}H]Ro15–4513 binding sites are present in this region (47).[1]

Thus, in brief, the change in the function of $GABA_A$ receptors may be associated with an alteration in α subunit mRNA levels. The increased binding of [^{3}H]Ro15–4513 in the cerebellum is partly due to increased levels of α_6 mRNA. The changes observed in steady state mRNA levels of a subpopulation of $GABA_A$ receptors could be the consequence of altered regulation of the rate of transcription or processing of the transcripts or changes in mRNA stability. Taken together, these data indicate that chronic ethanol treatment and its withdrawal alter $GABA_A$ receptor gene expression, which could be related to cellular adaptation to the functional disturbance caused by ethanol.

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