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Prolonged Ethanol Inhalation Decreases γ-Aminobutyric Acid$_A$ Receptor α Subunit mRNAs in the Rat Cerebral Cortex

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SUMMARY

Ethanol administration to rats by ethanol vapor inhalation (14 days) results in a 40–50% reduction in the level of γ-aminobutyric acid$_A$ (GABA$_A$) receptor α_1 subunit mRNAs [4.4 and 4.8 kilobases (kb)] in the cerebral cortex. The level of α_2 subunit mRNA (8.0 kb) was also reduced by 29%, whereas there was no effect of prolonged ethanol exposure on the level of α_3 subunit mRNA (3.1 kb). Ethanol exposure did not alter the steady state levels of cerebral cortical glutamic acid decarboxylase or β-actin mRNAs. Moreover, no alterations in the levels of total RNA, poly(A)$^+$ RNA, or rRNA were observed, suggesting that the ethanol-induced reductions in GABA$_A$ receptor α_1 and α_2 subunit mRNAs were not the result of a generalized effect of ethanol administration on transcription or mRNA turnover. These ethanol-induced reductions in GABA$_A$ receptor α subunit mRNAs may underlie alterations in GABA$_A$ receptor function or number observed following prolonged ethanol exposure in rats.

Prolonged administration of ethanol to animals, including humans, results in the development of tolerance and dependence, the former manifested by a reduction in the behavioral effects of ethanol and the latter by a withdrawal syndrome following abrupt discontinuation (1, 2). The molecular and cellular mechanisms underlying the actions of ethanol, including the development of tolerance and dependence, are not well understood. Ethanol and other sedative-hypnotic drugs, such as the barbiturates and benzodiazepines, share a number of pharmacological properties (3), including the development of cross-tolerance and cross-dependence following prolonged administration (4). It is generally acknowledged that many of the pharmacological actions of benzodiazepines and barbiturates, which directly bind to central GABA$_A$ receptors, are mediated via an augmentation of GABAergic neurotransmission (5, 6). Recently, we and others have provided biochemical evidence that ethanol and related short chain alcohols augment GABA$_A$ receptor-mediated Cl^- ion uptake in cerebral cortical synaptoneurosomes (7), cerebellar microsacs (8), and embryonic neurons in culture (9), further suggesting that ethanol, like barbiturates and benzodiazepines, may enhance GABA$_A$ receptor-mediated Cl^- ion conductance.

We have recently shown that chronic ethanol administration to rats results in a reduction in GABA receptor-mediated $^{36}Cl^-$ uptake in cerebral cortical synaptoneurosomes, and we have postulated that this reduction in GABA$_A$ receptor-gated chloride ion channel function may contribute to the ethanol withdrawal syndrome (10). The mechanism(s) responsible for the ethanol-induced decrease in GABA-receptor-mediated $^{36}Cl^-$ uptake could include, among other possibilities, an alteration in the expression of GABA$_A$ receptor(s). GABA$_A$ benzodiazepine receptors are heteroligomeric protein complexes consisting of several homologous membrane-spanning glycoprotein subunits (α, β, γ, and δ) (11–13). Molecular cloning studies have isolated and identified multiple α, β, and γ subunit-encoding cDNAs from bovine, rat, and human brain cDNA libraries (11–13). Regional and developmental studies of the expression of subunit mRNAs using Northern analysis and *in situ* hybridization suggest the presence of multiple brain GABA$_A$ isoreceptors [see Schofield (13) for a recent review]. The expression of the various α subunit isoforms has been shown to result in GABA$_A$ receptors with different gating properties, which may account for the functional heterogeneity of GABA$_A$ receptors (12, 14). Measurement of GABA$_A$ receptor expression using radioligand binding techniques is limited by the fact that most GABA$_A$ receptor ligands do not distinguish between various GABA$_A$ receptor subunits or isoreceptors (15, 16). Alterations in the expression of a subpopulation of GABA$_A$ isoreceptors might not be detected using radioligands that are nonselective.

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ABBREVIATIONS: GABA, γ-aminobutyric acid; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; GAD, glutamic acid decarboxylase; TBPS, *t*-butylbicyclophosphorothionate; kb, kilobases; bp, base pairs; BEC, blood ethanol concentrations.

Recently, we have studied the regional and developmental expression of $GABA_A$ receptor α subunit mRNAs in rat, chick, and human brain. Using a series of α_1 subunit cRNA probes, several $GABA_A$ α subunit transcripts have been identified in mammalian brain (17). In the present study, subunit-specific oligonucleotide probes (derived from rat α_1, α_2, and α_3 subunit cDNA sequences) have been used to identify the transcripts expressed in rat brain that correspond to the α_1, α_2, and α_3 subunit mRNAs. In the present study, we used these probes to investigate whether prolonged ethanol inhalation alters the expression of these $GABA_A$ receptor subunits. We show that prolonged ethanol administration to rats results in a specific reduction in $GABA_A$ receptor α_1 and α_2 subunit mRNAs in the cerebral cortex.[2] In contrast, the levels of the $GABA_A$ receptor α_3 subunit are not altered. These data suggest that long term ethanol administration may decrease the synthesis of $GABA_A$ α_1 and α_2 subunits, which may in turn alter the expression of a specific population(s) of $GABA_A$ receptor(s).

Materials and Methods

Ethanol administration. Adult male Sprague Dawley rats (200–300 g) were maintained on a 12/12-hr light/dark cycle, with free access to food and water. In each experiment, 12 rats were exposed to ethanol vapor for 14 days in air-tight clear Plexiglass inhalation chambers, as described by Karanian *et al.* (19). Ethanol was vaporized throughout the chamber, and the concentration of ethanol was maintained at 25 ± 1 mg/liter for the 14-day period. Control rats (n = 6/experiment) were maintained under similar conditions but not exposed to ethanol. At the end of the ethanol exposure, rats were removed from the inhalation chambers and euthanized by decapitation within 15 min. BEC were measured in trunk blood obtained at the time of sacrifice. BEC were greater than 150 mg/100 ml in all animals used for these studies (BEC = 207 ± 21 mg/100 ml; n = 21). This BEC has been shown to be associated with dependence upon ethanol, as determined by the appearance of withdrawal symptoms when ethanol administration is discontinued (10, 19). Individual cerebral cortices were rapidly dissected on ice, frozen on dry ice, and stored at −80°.

Preparation of RNA and Northern analysis. Total RNA was extracted using the method of Chirgwin *et al.* (20). Briefly, tissues were homogenized in 4 M guanidine thiocyanate, using a Polytron (Brinkman Instruments), and RNA was purified by ultracentrifugation over a 5.7 M CsCl cushion, followed by extraction with phenol/chloroform/isoamyl alcohol (50:49:1) and repeated precipitation with ethanol. Total RNA (5 μg) was denatured in a formamide/formaldehyde solution at 65° and separated by electrophoresis overnight in a 1% agarose gel containing 6% formaldehyde. β-Actin sense RNA (0.6 kb) and $GABA_A$ α_1 subunit sense RNA (0.8 kb) were also electrophoresed, as internal standards. The RNA was transferred overnight by capillary action under pressure to nitrocellulose membranes (BRL 1058HY), which were baked at 80° under vacuum for 2 hr.

Four different [^{32}P]CTP-labeled antisense cRNA probes, synthesized by SP6 or T7 RNA polymerase using linearized cDNAs as templates, were used in the present study. The cDNAs used were the human $GABA_A$ receptor α subunit (21), glutamic acid decarboxylase (22), β-actin (23), and rRNA (24). The $GABA_A$ receptor α_1 subunit cRNA probe used in the present experiments corresponds to 726 bp of 5′ coding sequence and identifies two α_1 transcripts, of 4.4 and 4.8 kb, on Northern analysis of total cerebral cortex RNA (21). Two additional transcripts are detected by Northern analysis using purified poly(A)$^+$ RNA (see below). We have previously shown that this probe fails to hybridize to rat β subunit transcripts under the hybridization conditions used in these experiments (17). As has been previously reported for rat brain, the transcripts for GAD and β-actin were 3.7 and 1.9 kb, respectively (22, 23). The rRNA cRNA probe labeled the 6-kb genomic sequence coding for the 28 S and 16 S rRNA subunits (24).

The hybridizations utilizing cRNA probes were conducted following prehybridization of the membranes for 1 hr at 55° in 50% formamide, 50 mM Na_2HPO_4, 5× SSC, 0.1% SDS, 1 mM EDTA, 0.05% Ficoll, 0.05% polyvinylpyrolidone, 200 μg/ml salmon sperm DNA. The appropriate [^{32}P]CTP-labeled antisense RNA probe (5 × 10^5 cpm/ml) was added to the same solution, and the hybridization was carried out for at least 16 hr at 55°. Following hybridization, the filters were washed three times in 0.1% SDS, 0.1% SSC, air dried, and exposed to X-ray film (Kodak), using intensifying screens.

To identify the individual α subunit transcripts in rat brain, subunit-specific oligonucleotide probes for the α_1 [nucleotides 1048–1095 of the reported sequence (25)], α_2 (nucleotides 24–72),[3] and α_3 [nucleotides 1246–1293 (26)] subunits were obtained. The probes were synthesized utilizing a cyclone DNA synthesizer (MilliGen Biosearch) and end-labeled with [α-^{32}P]dATP using terminal deoxytransferase (Bethesda Research Laboratories), according to the procedure recommended by the supplier. These oligonucleotide probes were used to confirm the identity of the α subunit transcripts detected with the human α subunit cRNA probes.

To quantify the poly(A)$^+$ RNA fraction of each total RNA sample, total RNA was applied to nitrocellulose membranes under vacuum, using a minifold dot blot apparatus (Schleicher and Schuell), heated at 80°, and subsequently hybridized with a deoxythymidine oligonucleotide probe labeled with [α-^{32}P]dTTP. The probe was synthesized at 37°, using a deoxythymidine homopolymer (19–24-mer; Pharmacia) as a template for terminal deoxytransferase, in 50 μl of reaction mix containing 100 mM potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM dithiothreitol, 0.5 μM deoxythymidine homopolymer, and 120 μCi of [^{32}P]dTTP (800 Ci/mmol; Amersham). Terminal deoxytransferase (50 units; BRL) was added after 1 min of preincubation at 37° and again after 20 min, for a total reaction time of 40 min. The oligonucleotide probes were purified by ethanol precipitation, yielding an average specific activity of 4.0 ± 0.65 × 10^{11} dpm/μg. Using various amounts of rRNA or tRNA in the presence and absence of poly(A)$^+$ RNA, we have demonstrated that this probe specifically hybridizes to polyadenylated mRNAs (see Fig. 3).[3] No cross-hybridization with rRNA or tRNA was detected, even at very high concentrations (30 μg).

The oligonucleotide probes were hybridized with Northern and dot blots following prehybridization at 60° for 1 hr in 6× SSC, 0.5% SDS, 10× Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 0.05% pyrophosphate, 50 μg/ml salmon sperm DNA. Prehybridization solution was removed, and the hybridization was conducted overnight at 42° in a solution containing 6× SSC, 10× Denhardt's solution, 0.1% pyrophosphate, 50 μg/ml salmon sperm DNA, 50 μg/ml tRNA, and the appropriate oligonucleotide probe (20 × 10^6 dpm/ml). The filters are washed successively at room temperature with 4× SSC, 10 mM Na_2PO_4 in 2× SSC, and 10 mM Na_2PO_4 in 1× SSC. The final wash was carried out with 10 mM Na_2PO_4 in 1× SSC, at 48°.

Following hybridization, the filters were washed, air dried, and exposed to X-ray film (Kodak), using intensifying screens, as previously described (21). Densitometric measurements of the films were made using a light box (Northern Light Precision Illuminator), a video camera (model 890; Sierra Scientific), and image analysis software (Image 1.15), as previously described (17, 18). Absorbance measurements of the autoradiogram signals, reflecting the specific hybridization to the appropriate mRNAs, were obtained for each sample of rat cerebral cortical total RNA. Densitometric measurements were obtained following calibration employing Kodak calibration step tablet 809ST601 as standard. Statistical comparisons of the densitometric measurements were made using analysis of variance or Student's t test for paired samples.

[2] A preliminary communication of some of these data has been published elsewhere (18).

[3] P. Montpied, A. L. Morrow, S. M. Paul, unpublished data.

Results

GABA$_A$ receptor α subunit mRNA levels were measured first in samples of total RNA prepared from individual animals and then in the poly(A)$^+$ RNA fraction purified from pooled samples of total RNA from ethanol-treated and control animals. Hybridization of total RNA with the 726-bp ^{32}P-labeled antisense α subunit riboprobe (21) detects the two most abundant GABA$_A$ receptor (α_1 subunit) mRNAs expressed in rat cerebral cortex (4.8 and 4.4 kb), whereas hybridization of poly(A)$^+$ RNA reveals two additional mRNA species (8.0 and 3.1 kb). We have previously reported the presence of five α subunit transcripts (2.9, 3.1, 4.4, 4.8, and 8.0 kb) in adult and embryonic rat brain (17). Using oligonucleotide probes specific for the reported rat α_1, α_2, and α_3 subunit cDNAs sequences, we demonstrate that these transcripts correspond to the α_1 subunit (4.4 and 4.8 kb), α_2 subunit (2.9 and 8 kb), and α_3 subunit (3.1 kb) mRNAs (Fig. 1).

Prolonged ethanol inhalation decreased the levels of GABA$_A$ receptor α subunit mRNAs in the cerebral cortex of rats (Fig. 2). The α_1 subunit transcripts (4.8 and 4.4 kb) were reduced by approximately 49 and 39%, respectively, compared with controls ($p < 0.01$, Students t test) (Figs. 2 and 3). Control rats, maintained in the same chambers but not exposed to ethanol, had identical GABA$_A$ receptor α_1 subunit mRNA levels as normally housed control rats (4.8 kb, O.D. = 0.183 versus 0.176; 4.4 kb, 0.166 versus 0.162; $n = 6$/group).

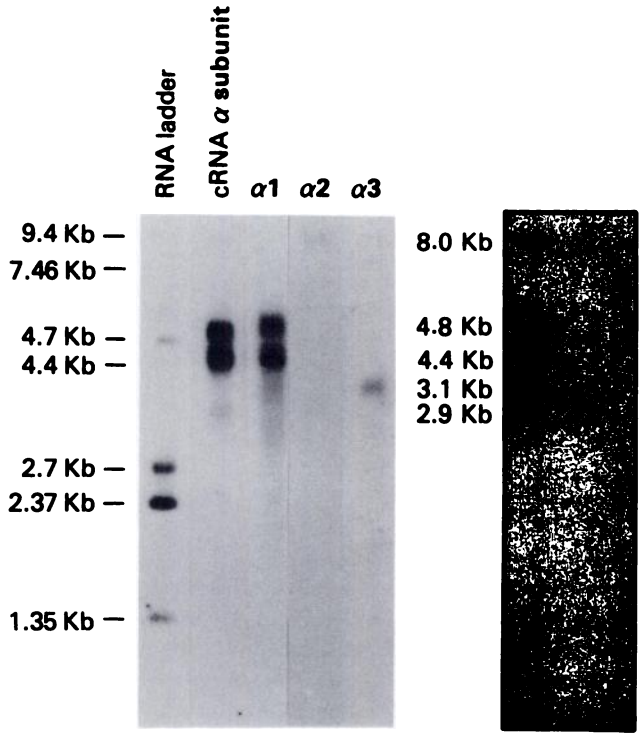

Fig. 1. Identification of the GABA$_A$ receptor α subunit transcripts labeled using a GABA$_A$ receptor α subunit cRNA probe. Poly(A)$^+$ RNA (2.0 μg/lane) from various brain regions was hybridized with human GABA$_A$ receptor α subunit riboprobe (726 bp), rat α_1 subunit oligonucleotide probe [nucleotides 1048–1095 of the reported sequence (25)], rat α_2 subunit oligonucleotide probe (nucleotides 24–72),[3] or rat α_3 subunit oligonucleotide probe [nucleotides 1246–1293 (26)]. Poly(A)$^+$ RNA purified from whole rat brain was used with the α_1 subunit cRNA probe, α_2 probes, cerebral cortex with the α_1 probe, and hippocampus with the α_3 probe. Data are from a representative experiment, which shows the size of the α_1 (4.8 and 4.4 kb), α_2 (8.0 and 2.9 kb), and α_3 (3.1 kb) subunit-encoding mRNAs.

To investigate the possibility that prolonged ethanol administration nonspecifically reduced the steady state level(s) of all mRNAs expressed in rat cerebral cortex, the concentration of poly(A)$^+$ RNA was quantified in the samples of total RNA prepared from each cerebral cortex. Using a ^{32}P-labeled deoxythymidine homopolymer probe (30–50 bp), we quantified the poly(A)$^+$ RNA content of each sample of total RNA, using a dot blot method (Fig. 4). Standard curves for hybridization of the [^{32}P]oligo(dT) with poly(A)$^+$ RNA or deoxyadenine homopolymer were analyzed in parallel, to validate the linearity and sensitivity of each assay for quantifying poly(A)$^+$ RNA. No significant difference in the poly(A)$^+$ RNA concentrations in total RNA samples prepared from the cerebral cortices of control or ethanol-treated animals were observed (Fig. 4).

In order to further control for possible nonspecific effects of ethanol on brain levels of mRNA, we quantified the level of β-actin mRNA by rehybridization to membranes that had been analyzed with the GABA$_A$ receptor probe. No alteration in the level of cerebral cortical β-actin mRNA was observed (Table 1; Fig. 3). Similarly, we investigated whether prolonged ethanol administration altered the level of GAD mRNA. No change in the level of GAD mRNA was observed in the cerebral cortices of ethanol-treated versus control rats (Table 1; Fig. 3).

Because poly(A)$^+$ RNA could not be purified from cerebral cortex of individual animals in sufficient amounts for quantitative analysis of GABA$_A$ receptor α subunit mRNAs, we purified poly(A)$^+$ RNA from pooled samples of total RNA (50 μg/rat) prepared from control and ethanol-treated animals. Fig. 5 illustrates a representative Northern blot of GABA$_A$ receptor α subunit mRNAs in rat cerebral cortex, using poly(A)$^+$ RNA from control and ethanol-treated animals. The levels of α_1 subunit mRNAs (4.4 and 4.8 kb) were reduced by 39 and 53%, respectively ($p < 0.001$, paired t test), in ethanol-treated rats compared with controls. In addition, there was a significant reduction in the α_2 subunit (8.0 kb) mRNA (29%, $p < 0.025$, paired t test), but no alteration in the level of the α_3 subunit (3.1 kb) transcript was detected (Fig. 6).

Discussion

In the present series of experiments, we observed a reduction (approximately 40–50%) in the levels of GABA$_A$ receptor α_1 subunit mRNAs in the cerebral cortex of rats exposed to prolonged (2 weeks) ethanol inhalation. No change in the absolute levels of total RNA, rRNA, or poly(A)$^+$ RNA or in levels of β-actin or GAD mRNAs were observed, suggesting that the ethanol-induced alteration in α subunit mRNAs was not a result of a generalized or nonspecific "drug effect" on transcription or mRNA turnover. Using an identical method of chronic ethanol administration to rats, we have previously reported a reduction in the function of GABA$_A$ receptor-gated Cl$^-$ channels in cerebral cortical synaptoneurosomes following chronic ethanol exposure (10). Chronic ethanol exposure has also been shown to reduce the behavioral responses of rats given injections of muscimol in the substantia nigra (27), suggesting that GABAergic function is reduced in these neurons as well. In contrast, muscimol-stimulated ^{36}Cl$^-$ uptake was not altered in cerebellar or cerebral cortical microsacs (28, 29) of mice fed an alcohol-enriched diet, although ethanol enhancement of muscimol-stimulated ^{36}Cl$^-$ uptake was abolished in

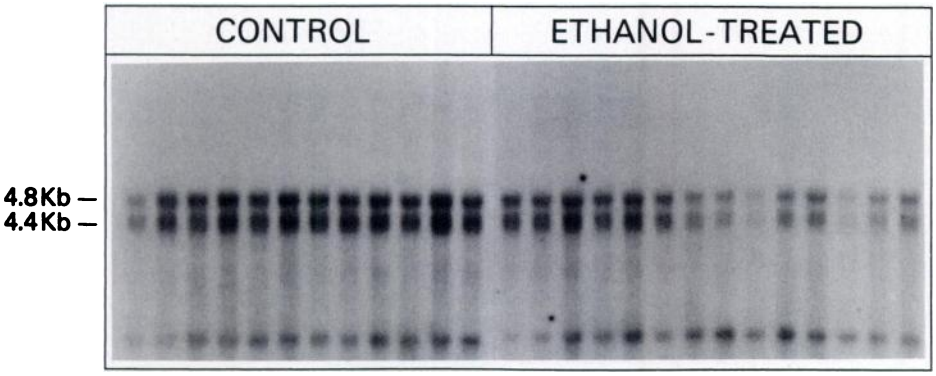

Fig. 2. Prolonged ethanol inhalation reduces the level of $GABA_A$ receptor α_1 subunit mRNA in the rat cerebral cortex. Rats were administered ethanol by inhalation for 14 days, producing BEC of 207 ± 21 mg/100 ml. Total RNA was prepared from cerebral cortex of individual rats, and Northern blot analysis was conducted on two or three blots for each experiment. Hybridization signals were adjusted to be within the linear range of the densitometric readings obtained. The 4.8- and 4.4-kb species of α subunit mRNA were routinely detected in the cerebral cortex of each animal. The blots were stripped of $GABA_A$ receptor α subunit cRNA probe and rehybridized with the β-actin riboprobe. Densitometric measurements were made without knowledge of the treatment group. Prolonged ethanol administration reduced the level of the 4.8-kb species of α subunit mRNA by 49% ($p < 0.01$) and reduced the level of the 4.4-kb species by 39% ($p < 0.01$). The level of β-actin mRNA was not altered by prolonged ethanol administration. Data shown are representative of three experiments, in which multiple Northern blots were performed with similar results. Each lane represents the hybridization signal using 5 μg of total RNA from a single animal.

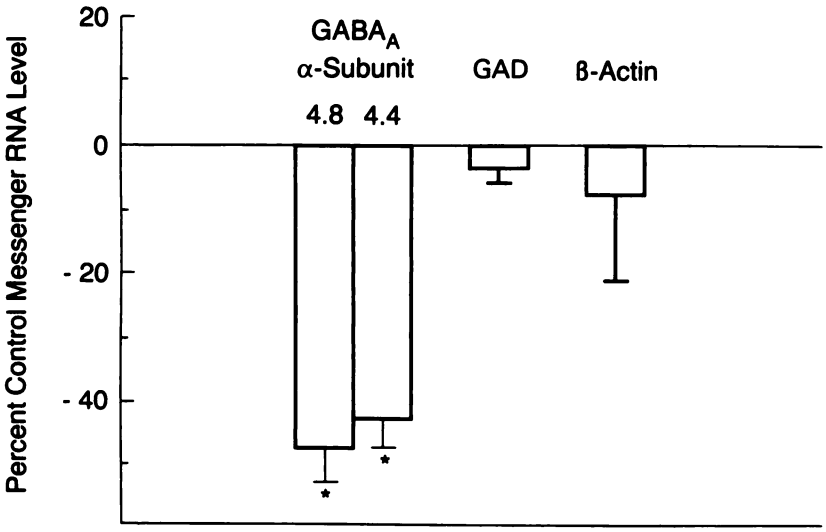

Fig. 3. Prolonged ethanol exposure does not alter the levels of GAD mRNA or β-actin mRNA. Northern blots were made as described in Fig. 2 and hybridized with GAD and β-actin cRNA probes, as well as the $GABA_A$ receptor α subunit probe. Hybridization signals were measured as described in the text, and the O.D. values were analyzed by Student's *t* test. Data shown represent the percentage of control O.D. values for the densitometric measurements from two experiments (control, $n = 12$; ethanol-treated, $n = 14$). *, $p < 0.01$.

cerebellum (28) and benzodiazepine potentiation of GABA was reduced in cerebral cortex (29).

The measurement of $GABA_A$ receptor mRNA levels is a more specific indicator of $GABA_A$ receptor subunit expression than radioligand binding techniques because radioligands do not readily distinguish highly homologous subunits of the receptor (15, 16). However, mRNA levels are an indirect measure of $GABA_A$ receptor subunit expression and should be interpreted with caution. The development of subunit-specific polyclonal or monoclonal antibodies for immunohistochemical or Western blot analysis could help delineate whether actual changes in the expression of specific subunit proteins occur in ethanol-treated animals.

The reduction in the levels of $GABA_A$ receptor α subunit mRNAs following prolonged ethanol exposure is consistent with studies showing a decrease in the density of low affinity binding sites for [^{3}H]GABA (30) or [^{3}H]muscimol (31) following chronic ethanol administration. Because GABA receptor-gated chloride uptake is mediated by low affinity binding sites (32), these data are also consistent with the reductions in GABA receptor function in the cerebral cortex discussed above. In contrast, however, no effect on high affinity ^{3}H-agonist binding sites has been observed (33, 34). Recently, deVries *et al.* (35) have reported a reduction in the ability of GABA to enhance [^{3}H]flunitrazepam binding in brain membranes prepared from ethanol-treated mice. Because the enhancement of benzodiazepine binding induced by GABA is mediated by "low affinity" $GABA_A$ binding sites (36), these data further suggest an ethanol-induced reduction in at least one population of $GABA_A$ receptors.

It is important to recognize that several laboratories have demonstrated that there is no alteration in the number of [^{3}H]flunitrazepam (37–39) or [^{35}S]TBPS binding sites (37, 40) in brain tissue from rodents chronically administered intoxicating

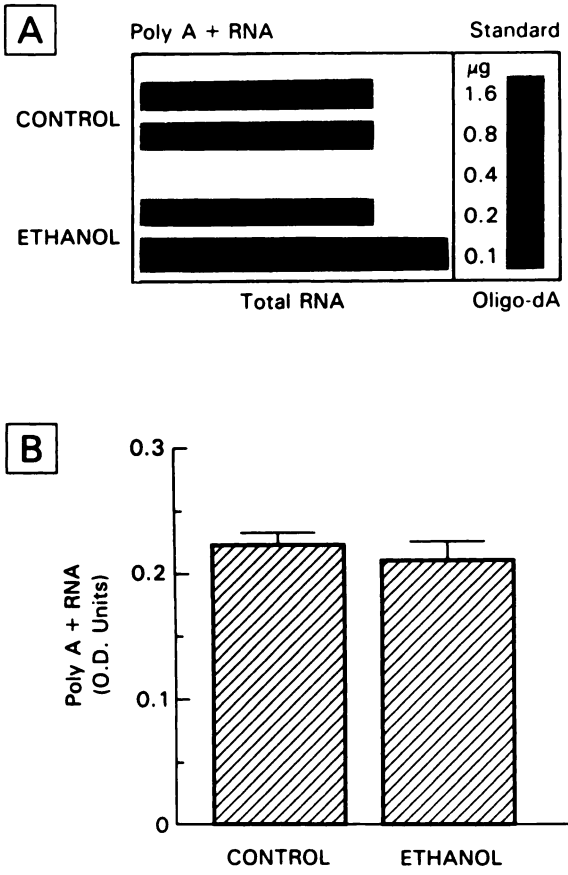

Fig. 4. Prolonged ethanol exposure has no effect on the steady state levels of poly(A)$^+$ RNA in rat cerebral cortex. Poly(A)$^+$ RNA was quantified in samples of total RNA (2.5 μg/rat) as described in the text. Briefly, total RNA was directly applied under vacuum to nitrocellulose membranes, using a dot blot minifold, and hybridized with a ^{32}P-labeled deoxythymidine oligonucleotide probe [35–50 bp; specific for poly(A)$^+$ RNA]. The specificity of the probe was tested by its linear hybridization with purified poly(A)$^+$ RNA and deoxyadenine oligonucleotide [oligo(dA)], as well as its failure to hybridize with ribosomal RNA. Total RNA samples were analyzed from the same rats used to measure GABA$_A$ receptor α subunit mRNA levels. No difference in the level of poly(A)$^+$ RNA was detected in ethanol-treated rats compared with controls (control, 216 ± 10 versus ethanol-treated, 209 ± 16 absorbance units). Data are representative of three experiments.

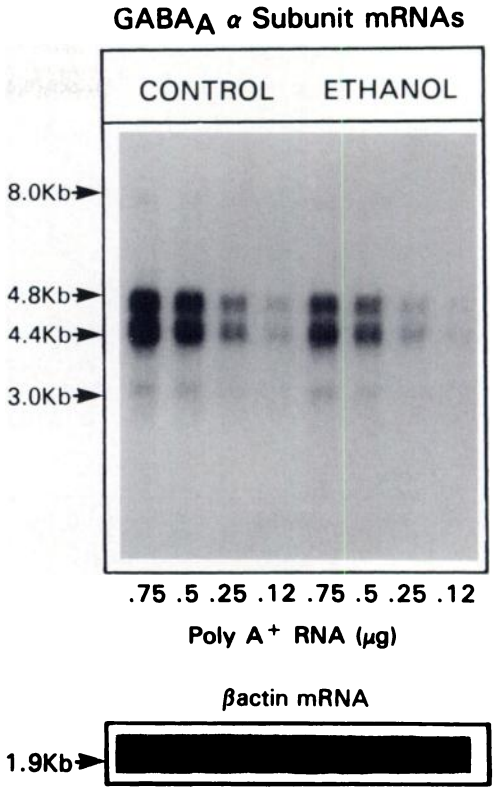

Fig. 5. Prolonged ethanol administration reduces GABA$_A$ receptor α_1 and α_2 subunit mRNAs in samples of cerebral cortical poly(A)$^+$ RNA. Total RNA (50 μg/rat) was pooled for each treatment group, and poly(A)$^+$ RNA was purified from the pooled samples using oligo(dT) column chromatography (48). For each group, increasing concentrations of poly(A)$^+$ RNA (0.75–0.125 μg) were separated by gel electrophoresis and hybridized with the GABA$_A$ receptor α subunit riboprobe. Four GABA$_A$ receptor α subunit transcripts were detected in cerebral cortex, corresponding to the α_1 (4.8 and 4.4 kb), α_2 (8.0 kb), and α_3 (3.1 kb) mRNAs. Prolonged ethanol administration reduced the level of the 4.8-kb (53%), the 4.4-kb (39%), and the 8.0-kb (29%) species but had no significant effect on the level of the 3.1-kb species. The exposure shown was optimal for quantitation of the α_1 subunit mRNAs. Longer exposures were used for quantitation of the α_2 and α_3 subunit mRNAs. Northern blots were then rehybridized with β-actin riboprobe. No alteration in the level of β-actin mRNA was detected. Triplicate Northern blots were performed on the pooled sample of poly(A)$^+$ RNA derived from 12–14 rats/group.

TABLE 1

Chronic ethanol administration reduces the level of GABA$_A$ receptor α_1 subunit mRNA levels in rat cerebral cortex

Total RNA from individual animals (n = 6–8/group) was hybridized with radioactive cRNA probes for each of the mRNAs shown. Densitometric readings of the autoradiograms were made by an observer in a blinded fashion. Data were analyzed by analysis of variance (F = 24, $p < 0.001$). The levels of the GABA$_A$ receptor α subunit transcripts were significantly reduced in ethanol-treated rats, compared with controls. Data are from a representative experiment, repeated twice with similar results.

mRNA	O.D. Units	
	Control	Ethanol-treated
4.8-kb GABA α subunit	1.44 ± 0.11	0.82 ± 0.05[a]
4.4-kb GABA α subunit	1.40 ± 0.10	0.89 ± 0.07[a]
GAD	1.03 ± 0.16	0.96 ± 0.07
β-Actin	1.33 ± 0.05	1.58 ± 0.18

[a] $p < 0.01$, Dunnett's t test.

doses of ethanol. Conflicting data on the effects of chronic ethanol administration on low affinity [^{3}H]GABA versus [^{3}H] flunitrazepam or [^{35}S]TBPS binding sites have been widely attributed to the use of different experimental protocols (species, doses of ethanol, routes of administration, etc.) or different radioligand binding conditions. Alternatively, it is possible that chronic ethanol administration has differential effects on the expression of specific populations of GABA$_A$ isoreceptors and that radioligands that measure all GABA receptors are too insensitive to detect changes in one or more subpopulations of GABA$_A$ isoreceptors. The demonstration that GABA$_A$ receptor α_1 and α_2 subunit mRNAs, but not α_3 subunit mRNAs, are reduced following prolonged ethanol administration suggests that prolonged ethanol administration may reduce the synthesis of GABA$_A$ isoreceptors containing these subunits. Increased synthesis of other GABA$_A$ receptor subunits may account for the lack of reduction in binding sites for ^{3}H-benzodiazepines or [^{35}S]TBPS. Recently, Mhatre *et al.* (41) have reported an increase in the density of specific binding sites for [^{3}H]Ro15-

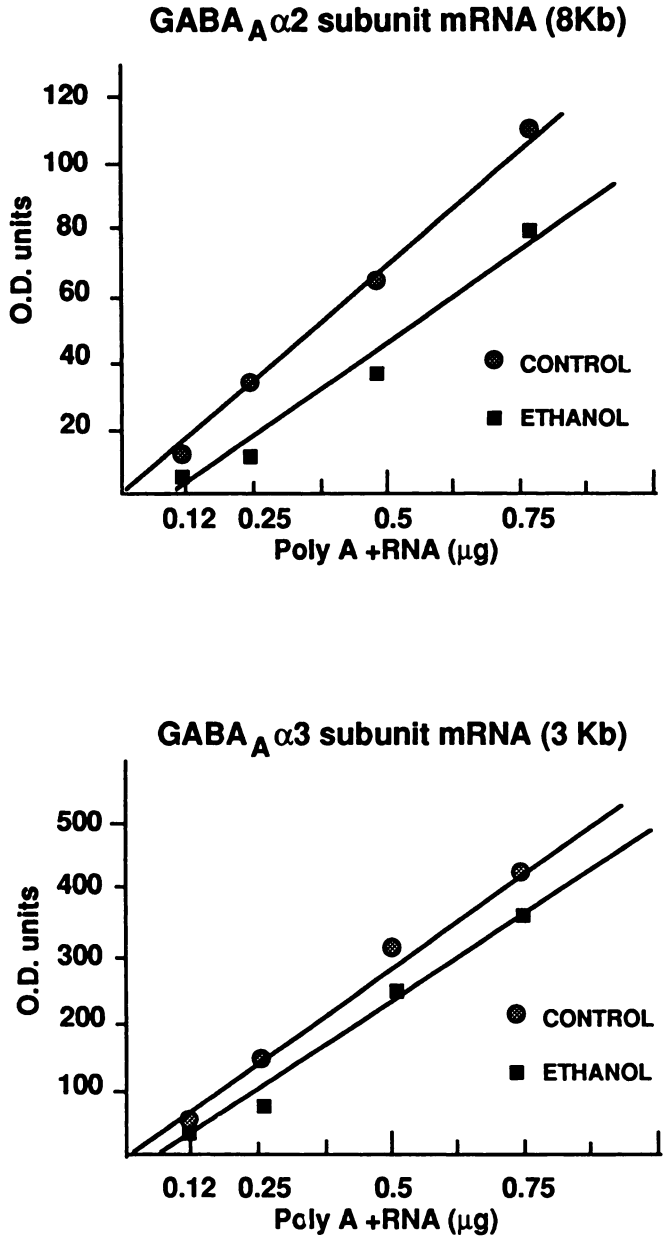

Fig. 6. Prolonged ethanol exposure reduces $GABA_A$ receptor α_2 subunit mRNA levels without altering α_3 subunit mRNA levels. Increasing concentrations of poly(A)$^+$ RNA were electrophoresed in parallel, and the blots were hybridized with the $GABA_A$ receptor α subunit riboprobe. All hybridization signals were linear with respect to RNA concentration. $GABA_A$ receptor α_2 subunit mRNA levels were reduced 29% following prolonged ethanol exposure. Data are representative of two blots using poly(A)$^+$ RNA derived from 12–14 rats/group.

4513 in the rat cerebellum following chronic ethanol administration, as well as increased sensitivity to its behavioral effects (42). Ro15-4513 selectively labels a unique α subunit (43) and, therefore, the expression of this subunit (α_6) may be increased following chronic ethanol administration. Further studies on the regulation of other $GABA_A$ receptor subunits by ethanol will be required to investigate this possibility.

The effects of ethanol on $GABA_A$ receptor α_1 and α_2 subunit mRNA levels were studied following a prolonged exposure period (2 weeks) in order to compare the receptor mRNA levels with previously published studies of $GABA_A$ receptor function (10) and radioligand binding, using methods of ethanol administration that produce comparable blood ethanol levels. The inhalation method of ethanol administration has been well established to produce tolerance to ethanol and withdrawal upon cessation of treatment (19, 44). As with all animal models of ethanol abuse, it is difficult to rule out the contribution of stress, nutritional, and selection factors on the effects reported here. Furthermore, the effects of ethanol on $GABA_A$ receptor α subunit mRNA levels may not require the 2-week exposure period. It is possible that more acute ethanol administration would have similar effects on $GABA_A$ receptor subunit expression. We are presently investigating this possibility.

The cellular mechanism(s) resulting in the reductions in α_1 subunit mRNAs in the cerebral cortices of rats exposed to prolonged ethanol inhalation could involve a decrease in α_1 subunit gene transcription or mRNA stability. The fact that the cerebral cortical concentrations of poly(A)$^+$ RNA, GAD mRNA, and β-actin mRNA were unaffected by prolonged ethanol exposure shows that the decrease in α subunit mRNAs was relatively selective and not due to a generalized change in transcription or mRNA processing. We have recently found rather marked reductions in the levels of $GABA_A$ receptor α subunit transcripts in primary chick neurons exposed to GABA for 48 hr *in vitro*.[4] This down-regulation of α subunit mRNAs induced by GABA is receptor mediated, because it is blocked by the GABA receptor antagonist SR-95531. The effects of prolonged ethanol administration on α subunit mRNAs could, therefore, be mediated via an ethanol-induced augmentation of GABA receptor-mediated Cl^- ion conductance, rather than a direct effect of ethanol itself. However, repeated administration to rats of barbiturates, which also enhance GABA receptor-mediated Cl^- conductance, has no effect on the level of $GABA_A$ receptor α_1 subunit mRNA levels in the cerebral cortex (18).

Regardless of the underlying mechanism(s), however, our data suggest that prolonged administration of a relatively nonspecific drug, like ethanol, to rats can result in a relatively selective reduction in $GABA_A$ receptor α_1 and α_2 subunit transcripts in the cerebral cortex. Given that ethanol has also recently been shown to interact with excitatory amino acid neurotransmitter receptors of the *N*-methyl-D-aspartate subtype (45–47), it is possible that the expression of other receptor-gated ion channel subunit proteins is altered following ethanol administration. Alterations in the expression of various receptors and ion channels could, therefore, underlie the well known changes in central nervous system excitability observed following prolonged ethanol administration.

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[4] P. Montpied E. I. Ginns, B. M. Martin, D. Roca, D. H. Farb, and S. N. Paul. GABA induces a receptor-mediated reduction in GABA, receptor α submit messenger RNAs in embryonic chick neurons in culture. *J. Biol. Chem.*, in press..

References

1. Taberner, P. V. The GABA system in functional tolerance and dependence following barbiturates, benzodiazepines or ethanol: correlation or causality? *Comp. Biochem. Physiol.* **93**:241–245 (1989).
2. Sellers, E. M., and H. Kalant. Alcohol intoxication and withdrawal. *N. Eng. J. Med.* **294**:757–760 (1976).
3. Boisse, N. N., and M. Okamoto. Ethanol as a sedative-hypnotic: comparison with barbiturate and nonbarbiturate sedative-hypnotics, in *Alcohol Tolerance*

and Dependence (H. Rigter and J. C. Crabbe, eds.). Elsevier, Amsterdam, 265–292 (1980).
4. Le, A. D., J. M. Khanna, H. Kalant, and F. Grossi. Tolerance to and cross-tolerance among ethanol, pentobarbital, and chlordiazepoxide. *Pharmacol. Biochem. Behav.* **24**:93–98 (1986).
5. Olsen, R. W. Drug interactions at the GABA receptor ionophore complex. *Annu. Rev. Pharmacol. Toxicol.* **22**:245 (1982).
6. Skolnick, P., and S. M. Paul. Benzodiazepine receptors in the central nervous system, in *International Review of Neurobiology* (J. R. Smythies and R. J. Bradley, eds.). Academic Press, New York, 103–140 (1982).
7. Suzdak, P. D., R. D. Schwartz, P. Skolnick, and S. M. Paul. Ethanol stimulates γ-aminobutyric acid receptor-mediated chloride transport in rat brain synaptoneurosomes. *Proc. Natl. Acad. Sci. USA* **83**:4071 (1986).
8. Allan, A. M., and R. A. Harris. *Gamma*-aminobutyric acid and alcohol actions: neurochemical studies of long sleep and short sleep mice. *Life Sci.* **39**:2005–2015 (1986).
9. Ticku, M. K., P. Lorimore, and P. Lehoullier. Ethanol enhances GABA-induced $^{36}Cl^-$ flux in primary spinal cord cultured neurons. *Brain Res. Bull.* **17**:123–126 (1986).
10. Morrow, A. L., P. D. Suzdak, J. W. Karanian, and S. M. Paul. Chronic ethanol administration alters γ-aminobutyric acid, pentobarbital and ethanol-mediated $^{36}Cl^-$ uptake in cerebral cortical synaptoneurosomes. *J. Pharmacol. Exp. Ther.* **246**:158–164 (1988).
11. Schofield, P. R., M. G. Darlison, N. Fujita, D. R. Burt, F. A. Stephenson, H. Rodriguez, L. M. Rhee, J. Ramachandran, V. Reale, T. A. Glencorse, P. H. Seeburg, and E. A. Barnard. Sequence and functional expression of the GABA-A receptor shows a ligand-gated super-family. *Nature (Lond.)* **328**:221–227 (1987).
12. Levitan, E. S., P. R. Schofield, D. R. Burt, L. M. Rhee, W. Wisden, M. Kohler, N. Fujita, H. F. Rodriguez, F. A. Stephenson, M. G. Darlison, E. A. Barnard, and P. H. Seeburg. Structural and functional basis for GABA receptor heterogeneity. *Nature (Lond.)* **335**:76–79 (1988).
13. Schofield, P. R. The $GABA_A$ receptor: molecular biology reveals a complex picture. *Trends Pharmacol. Sci.* **10**:476–478 (1989).
14. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338**:582–585 (1989).
15. Pritchett, D. B., H. Luddens, and P. H. Seeburg. Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
16. Bureau, M., and R. W. Olsen. γ-Aminobutyric acid/benzodiazepine receptor protein carries binding sites for both ligands on two major protein subunits. *Biochem. Biophys. Res. Commun.* **153**:1006–1011 (1988).
17. Montpied, P., E. I. Ginns, B. M. Martin, D. Stetler, A.-M. O'Carroll, S. J. Lolait, L. C. Mahan, and S. M. Paul. Multiple $GABA_A$ receptor α subunit mRNAs revealed by developmental and regional expression in rat, chicken and human brain. *FEBS Lett.* **258**:94–98 (1989).
18. Morrow, A. L., P. Montpied, A. Lingford-Hughes, and S. M. Paul. Chronic ethanol and pentobarbital administration in the rat: effects on $GABA_A$ receptor function and expression in brain. *Alcohol* **7**:237–244 (1990).
19. Karanian, J. W., J. Yergey, R. Lister, N. D'Souza, M. Linnoila, and N. Salem. Characterization of an automated apparatus for precise control of inhalation chamber ethanol vapor and blood ethanol concentrations. *Alcohol. Clin. Exp. Res.* **10**:443–447 (1986).
20. Chirgwin, J. M., A. E. Przbyla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
21. Montpied, P., B. M. Martin, S. L. Cottingham, B. K. Stubblefield, E. I. Ginns, and S. M. Paul. Regional distribution of the $GABA_A$/benzodiazepine receptor (α-subunit) mRNA in rat brain. *J. Neurochem.* **51**:1651–1654 (1988).
22. Kim, S. K., J. W. Thomas, N. J. K. Tillakaratne, P. Montpied, P. D. Suzdak, C. Banner, A. J. Tobin, and S. M. Paul. Glutamic decarboxylase mRNA in rat brain: regional distribution and effects of intrastriatal kainic acid. *Mol. Brain Res.* **3**:77–82 (1987).
23. Cleveland, D. W., M. A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. *Cell* **20**:95–105 (1980).
24. Chikaraishi, D. M., L. Buchanan, K. J. Danna, and C. A. Harrington. Genomic organization of rat rDNA. *Nucleic Acids Res.* **11**:6437–6452 (1983).
25. Khrestchatisky, M., A. J. MacLennan, W. Xu, M. B. Jackson, N. Brecha, C. Sternini, R. W. Olsen, and A. J. Tobin. A novel α subunit in rat brain $GABA_A$ receptors. *Neuron* **3**:745–753 (1989).
26. Malherbe, P., E. Sigel, R. Baur, E. Persohn, J. G. Richards, and H. Möhler. Functional expression and sites of gene transcription of a novel α subunit of the $GABA_A$ receptor in rat brain. *FEBS Lett.* **260**:261–265 (1990).
27. Gonzalez, L. P., and J. F. Czachura. Reduced behavioral responses to intranigral muscimol following chronic ethanol. *Physiol. Behav.* **46**:473–477 (1989).
28. Allan, A. M., and R. A. Harris. Acute and chronic ethanol treatments alter GABA receptor-operated chloride channels. *Pharmacol. Biochem. Behav.* **27**:665–670 (1987).
29. Buck, K. J., and R. A. Harris. Benzodiazepine agonist and inverse agonist actions on $GABA_A$ receptor-operated chloride channels. II. Chronic effects of ethanol. *J. Pharmacol. Exp. Ther.* **253**:713–719 (1990).
30. Ticku, M. K., and T. Burch. Alterations in γ-aminobutyric acid receptor sensitivity following acute and chronic ethanol treatments. *J. Neurochem.* **34**:417–423 (1980).
31. Unwin, J. W., and P. V. Taberner. Sex and strain differences in GABA receptor binding after chronic ethanol drinking in mice. *Neuropharmacology* **19**:1257–1259 (1980).
32. Luu, M. D., A. L. Morrow, and R. D. Schwartz. Characterization of $GABA_A$ receptor-mediated ^{36}chloride uptake in rat brain synaptoneurosomes. *Life Sci.* **41**:1277–1287 (1987).
33. Volicer, L. GABA levels and receptor binding after acute and chronic ethanol administration. *Brain Res. Bull.* **5**:809–813 (1980).
34. Volicer, L., and T. M. Biagoni. Effect of ethanol administration and withdrawal on GABA receptor binding in rat cerebral cortex. *Substance Alcohol Actions-Misuse* **3**:31–39 (1982).
35. deVries, D. J., G. A. R. Johnston, L. C. Ward, P. A. Wilce, and B. C. Shanley. Effects of chronic ethanol inhalation on the enhancement of benzodiazepine binding to mouse brain membranes by GABA. *Neurochem. Int.* **10**:231–235 (1987).
36. Burch, T. P., R. Thyagarajan, and M. K. Ticku. Group-selective reagent modification of the benzodiazepine-γ-aminobutyric acid receptor-ionophore complex reveals that low affinity γ-aminobutyric acid receptors stimulate benzodiazepine binding. *Mol. Pharmacol.* **23**:52–59 (1983).
37. Rastogi, S. K., R. Thyagarajan, J. Clothier, and M. K. Ticku. Effect of chronic treatment of ethanol on benzodiazepine and picrotoxin sites on the GABA receptor complex in regions of the brain of the rat. *Neuropharmacology* **25**:1179–1184 (1986).
38. Volicer, L., and T. M. Biagioni. Effect of ethanol administration and withdrawal on benzodiazepine receptor binding in the rat brain. *Neuropharmacology* **21**:283–286 (1982).
39. Karobath, M., J. Rogers, and F. E. Bloom. Benzodiazepine receptors remain unchanged after chronic ethanol administration. *Neuropharmacology* **19**:125–128 (1980).
40. Thyagarajan, R., and M. K. Ticku. The effect of *in vitro* and *in vivo* ethanol administration on [^{35}S]*t*-butylbicyclophosphorothionate binding in C57 mice. *Brain Res. Bull.* **15**:343–345 (1985).
41. Mhatre, M., A. K. Mehta, and M. K. Ticku. Chronic ethanol administration increases the binding of the benzodiazepine inverse agonist and alcohol antagonist [^{3}H]Ro15-4513 in rat brain. *Eur. J. Pharmacol.* **153**:141–145 (1988).
42. Mehta, A. K., and M. K. Ticku. Chronic ethanol treatment alters the behavioral effects of Ro 15-4513, a partially negative ligand for benzodiazepine binding sites. *Brain Res.* **489**:93–100 (1989).
43. Luddens, H., D. B. Pritchett, M. Kohler, I. Killisch, K. Keinanen, H. Monyer, R. Sprengel, and P. H. Seeburg. Cerebellar $GABA_A$ receptor selective for a behavioral alcohol antagonist. *Nature (Lond.)* **346**:648–651 (1990).
44. Goldstein, D. Relationship of alcohol dose to intensity of withdrawal signs in mice. *J. Pharmacol. Exp. Ther.* **180**:203–215 (1972).
45. Lovinger, D. M., G. White, and F. F. Weight. Ethanol inhibits NMDA-activated ion currents in hippocampal neurons. *Science (Washington D. C.)* **243**:1721–1724 (1989).
46. Hoffman, P. L., C. S. Rabe, F. Moses, and B. Tabakoff. *N*-Methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *J. Neurochem.* **52**:1937–1940 (1989).
47. Lima-Landman, M. T., and E. X. Albuquerque. Ethanol potentiates and blocks NMDA-activated single-channel currents in hippocampal pyramidal cells. *FEBS Lett.* **247**:61–67 (1989).
48. Aviv, H., and P. Leder. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* **69**:1408–1412 (1972).

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