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Chronic Ethanol Consumption Differentially Alters the Expression of γ-Aminobutyric Acid$_A$ Receptor Subunit mRNAs in Rat Cerebral Cortex: Competitive, Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis

LESLIE L. DEVAUD, F. DONELSON SMITH, DENNIS R. GRAYSON, and A. LESLIE MORROW

Bowles Center for Alcohol Studies (L.L.D., F.D.S., A.L.M.) and Department of Psychiatry (L.L.D., A.L.M.), University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7178, and Neuroscience Research Center, Department of Psychiatry, Medical College of Pennsylvania and Hahnemann University, Pittsburgh, Pennsylvania 15211-4772 (D.R.G.)

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SUMMARY

The molecular mechanisms that underlie ethanol dependence appear to involve alterations in GABA$_A$ receptor function and gene expression. In rat cerebral cortex, chronic exposure to ethanol alters many functional properties of GABA$_A$ receptors, including reduction of GABA$_A$ receptor-mediated chloride uptake. These functional alterations occur without a concomitant alteration in total receptor density or affinity. Previous investigations have shown that chronic ethanol exposure elicits alterations in mRNA and polypeptide levels for several abundant GABA$_A$ receptor subunits. For example, α1 and α2 subunit mRNA and polypeptide levels have been shown to decrease with chronic ethanol exposure. The present study was undertaken to further investigate the effects of chronic ethanol consumption on GABA$_A$ receptor subunit mRNA levels in rat cerebral cortex by using a competitive, quantitative reverse transcriptase-polymerase chain reaction assay that incorporates subunit-specific internal standards and allows for the absolute quantification of mRNA levels. We find that chronic ethanol consumption elicits a significant increase in α4 subunit mRNA levels that is equal, in absolute amount, to a decrease in α1 subunit mRNA levels. There is a small (30%) increase in γ2S but not γ2L subunit mRNA levels after chronic ethanol consumption. In addition, γ1 subunit mRNA levels are increased by 70%, whereas α5, β1, β2, β3, γ3, and δ subunit mRNA levels do not change. We also reproduced results obtained previously by Northern blot analysis showing a 40% reduction in α1 mRNA levels with no change in β2 subunit mRNA levels after chronic ethanol consumption. These results are consistent with the hypothesis that chronic ethanol consumption alters the function of GABA$_A$ receptors by eliciting changes in receptor subunit assembly. These changes may underlie the development of ethanol dependence.

Chronic ethanol consumption results in the development of behavioral tolerance, physical dependence, and rebound central nervous system hyperexcitabiltiy. Extensive evidence suggests that these changes are mediated, in part, by adaptations in GABAergic neurotransmission. However, the exact mechanisms that account for these effects are not clear. Although chronic ethanol exposure alters the functional properties of GABA$_A$ receptors in many brain regions, primary cultured neurons, and recombinant systems (1–4), these effects are not explained by correlating alterations in GABA$_A$ receptor number or affinity.

In the cerebellum, there is a good correlation among the effects of chronic ethanol exposure on GABA$_A$ receptor α6 subunit levels (5, 6), recognition sites for the inverse agonist [^{3}H]Ro 15–4513 (7), and the efficacy of inverse agonists in GABA$_A$ receptor-mediated chloride uptake (3). However, in the cerebral cortex, chronic ethanol consumption decreases GABA-, muscimol-, pentobarbital-, or benzodiazepine-induced effects on chloride flux (1–4) with no consistent change in binding to high affinity muscimol recognition sites, benzodiazepine recognition sites, or [^{35}S]*t*-butylbicyclophosphorothionate recognition sites (4, 7–9). Therefore, alterations in GABA$_A$ receptor density or affinity do not appear to be a mechanism underlying the functional alterations observed in GABA$_A$ receptors after chronic ethanol consumption. Moreover, these functional effects, as well as increases in the density of [^{3}H]Ro 15–4513 recognition sites and increases in the efficacy of inverse agonists in chloride flux assays after chronic ethanol exposure (3, 7), cannot be explained by alterations in GABA$_A$ receptor subunit mRNA and polypeptide levels measured to date.

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ABBREVIATIONS: GABA, γ-aminobutyric acid; RT, reverse transcriptase; PCR, polymerase chain reaction; PK, protein kinase; dNTP, deoxynucleoside-5′-triphosphate.

The apparent inconsistency between the effects of chronic ethanol exposure on $GABA_A$ receptor-mediated responses and binding properties in rat cerebral cortex could be explained by several possibilities. $GABA_A$ receptors are heteroligomeric complexes composed of multiple subunits, each with several isoforms (10, 11). Alterations in the function of $GABA_A$ receptors can be achieved by changes in subunit composition. Transient expression studies using various recombinant $GABA_A$ receptors have shown that expression of different subunit combinations results in receptors with different functional and pharmacological properties (12–14). Therefore, alterations in the subunit composition of $GABA_A$ receptors may be one mechanism that mediates some of the observed effects of chronic ethanol exposure. This possibility has been supported by evidence that $GABA_A$ receptor subunit steady state mRNA levels are differentially altered by chronic ethanol exposure (5, 6, 15, 16).

Loss of the ability of ethanol to potentiate GABA- or muscimol-stimulated chloride uptake after chronic ethanol exposure could represent cellular tolerance to ethanol, but the molecular mechanisms underlying this effect have also not been shown. Ethanol sensitivity is postulated to require the expression of γ2L subunits phosphorylated by PKC in oocyte expression systems (17) and to require the expression of zolpidem binding sites in rat brain, which appear to contain both γ2S and γ2L subunits (18). Radioligand binding studies in transient expression assays and immunoprecipitation studies have shown that zolpidem selectively recognizes α1 subunit-containing $GABA_A$ receptors (14, 19). Because ethanol sensitivity is lost after chronic ethanol administration, alterations in [^{3}H]zolpidem binding, γ2 subunit expression, or its phosphorylation state might be associated with ethanol tolerance to GABA responses. However, chronic ethanol administration does not reduce [^{3}H]zolpidem binding in rat cerebral cortex and cerebellum (20, 21), although site-selective alterations in [^{3}H]zolpidem binding levels have been observed in several key brain regions where ethanol enhances $GABA_A$ receptor-mediated chloride conductance (21, 22).

The observed alterations in $GABA_A$ receptor subunit mRNA and polypeptide levels induced by chronic ethanol administration cannot be reconciled with the effects on [^{3}H]Ro 15–4513 and [^{3}H]zolpidem binding. The present study was undertaken to investigate the effects of chronic ethanol consumption on mRNA levels for additional $GABA_A$ receptor subunits that may be associated with these sites. The competitive, quantitative RT-PCR assay controls for nonequivalent amplification of experimental samples and allows absolute quantification of subunit mRNA levels. This approach was used to explore the effects of chronic ethanol administration on $GABA_A$ receptor α1, α4, α5, β1, β2, β3,γ1, γ2S, γ2L, γ3, and δ subunit expression in the male rat cerebral cortex.

Experimental Procedures

Materials. Hot Tub polymerase was purchased from Amersham (Arlington Heights, IL); M-MLV RT, dNTPs, and random hexamers were purchased from Boehringer Mannheim (Indianapolis, IN); and *Bgl*II was purchased from Stratagene (La Jolla, CA). Sea Kem agarose was purchased from FMC Corp. (Rockland, ME), and PCR primer pairs were synthesized at the University of North Carolina by the Lineberger Cancer Research Center Oligo Laboratory.

Chronic ethanol administration. Male Sprague-Dawley rats weighing 150–180 g (Charles River, Raleigh, NC) were individually housed and administered a nutritionally complete liquid diet (Dextrose Diet, ICN Biochemicals, Costa Mesa, CA). Groups of rats were given a liquid diet for 3 days to allow acclimation to the diet. Ethanol administration was begun with the addition of 5% ethanol (v/v) to the diet for 1 week followed by 7.5% ethanol (v/v) for the second week. Control animals were pair-fed the identical diet with dextrose substituted for ethanol. Water was available *ad libitum* to both groups. Dietary consumption of each animal was monitored daily. The typical daily ethanol consumption was 10–12 g/kg. The mean body weights for the control and pair-fed animals were similar at the termination of the experiment. Tolerance to and dependence on

TABLE 1

Subunit-selective PCR primers for $GABA_A$ receptor subunit mRNAs

Primer pairs for each subunit were designed from original published sequences.

Subunit	Product size	Primer (5′ TO 3′)	Position
α1 sense	304	AGCTATACCCCTAACTTAGCCAGG	1178–1482
α1 antisense		AGAAAGCGATTCTCAGTGCAGAGG	
α4 sense	394	AAATGCAGCTGAGACTATCTCTGC	1315–1709
α4 antisense		AGACAGTCTGTATTTCCATCACGG	
α5 sense	338	CAAGAAGGCCTTGGAAGCAGCTAA	1188–1526
α5 antisense		TCTTACTTTGGAGAGGTAGCCCCT	
β1 sense	341	CCTGGAAATCAGGAATGAGACCAG	1190–1531
β1 antisense		GGAGTCTAAACCGAACCATGAGAC	
β2 sense	317	TGAGATGGCCACATCAGAAGCAGT	1201–1518
β2 antisense		TCATGGGAGGCTGGAGTTTAGTTC	
β3 sense	355	GAAATGAATGAGGTTGCAGGCAGC	1199–1554
β3 antisense		CAGGCAGGGTAATATTTCACTCAG	
γ1 sense	360	CAGAGACAGGAAGCTGAAAAGCAA	1098–1458
γ1 antisense		CGAAGTGATTATATTGGACTAAGC	
γ2S sense	336	AAGAAAAACCCTGCCCCTACCATT	1156–1492
γ2S antisense		TTCGTGAGATTCAGCGAATAAGAC	
γ2L sense	390	CTTCTTCGGATGTTTTCCTTCAAG	1168–1534
γ2L antisense		CATAGGGTATTAGATCGTTGGACT	
γ3 sense	398	CACCACGGTGCTAACCATGACCAC	972–1370
γ3 antisense		TCCTCATAGCAGCAGAAGAAGCTC	
δ sense	333	TGAGGAACGCCATTGTCCTCTTCT	1097–1430
δ antisense		ACCACCGCACGTGGTACATGTAAA	

ethanol have been demonstrated with this protocol (5). Animals were killed on day 15 while still ethanol dependent. Brain areas were rapidly dissected over ice and stored at −80° until use. Trunk blood was collected at the time of death. Blood ethanol concentrations were 150–250 mg/100 ml.

Competitive RT-PCR using internal standards. Total RNA was extracted from cortex through homogenization and ultracentrifugation with the guanidine thiocyanate/CsCl extraction procedure. Extraneous protein was removed by consecutive extractions with equal volumes of Tris-buffered phenol (pH 6.0) and chloroform/isoamyl alcohol (50:49:1) followed by repeated precipitation with 100% ethanol. Removal of contaminating DNA was confirmed by both Northern blot analysis and PCR analysis of total RNA samples (excluding RT).

Generation of internal standards was conducted according to the method of Grayson *et al.* (23). This approach generates a cRNA template that is identical to the target mRNA except for the inclusion of a *Bgl*II restriction site midway through the template. Therefore, RT conditions are identical for both the cRNA (added at a known quantity) and the target mRNA. PCR is performed with a single pair of subunit selective primers, and the internal standard products are separated from the target mRNA PCR products by digestion with the restriction enzyme *Bgl*II. Quantification is achieved by the inclusion of various concentrations of internal standard cRNAs that will compete with the target message for amplification by subunit-selective primers. Linear regression analysis of the log-transformed ratios (cRNA/target RNA) versus the amount of cRNA added generates the point of equivalent amplification (when the ratio is 1), which determines the absolute concentration of mRNA.

Aliquots of cerebral cortical total RNA (1 μg/tube) and a series of internal standard cRNAs were reverse transcribed in 50 mM Tris·HCl, 3 mM $MgCl_2$, and 75 mM KCl with 10 mM dithiothreitol, 1 mM dNTPs, 1 mM random hexamers, and 200 units M-Mlv RT at 37° for 60 min. The resulting cDNA was heat denatured at 95° for 5 min, and tubes were kept on ice until ready for PCR. The PCR reaction was conducted in buffer containing 50 mM Tris·HCl, 20 mM ammonium sulfate, and 1.5–3.0 mM $MgCl_2$, with 1 μM each of 5′ (sense) and 3′ (antisense) primers, 200 μM dNTPs, and 1 unit Hot Tub polymerase, with the addition of 1 μCi [^{32}P]deoxycytidine triphosphate per tube. Magnesium concentration was optimized for each primer pair and determined to be 1.5 mM for the series of α subunit mRNAs, 2.0 mM for β and δ subunit mRNAs, and 3.0 mM for γ subunit mRNAs. The PCR was performed with 30 cycles, with each cycle consisting of 94° for 45 sec, 60° for 45 sec, and 72° for 1 min, followed by a final elongation step (72° for 5 min) in a Perkin Elmer 480 Thermal Cycler. PCR products were digested overnight with 10 units *Bgl*II and separated on a 1.8% agarose gel in 0.5× Tris/Borate/EDTA buffer. Bands for both the native RNA product and the cRNA product were analyzed by phosphorous detection using a Phoshorimager SI (Molecular Dynamics, Sunnyvale, CA). Adjacent lanes with control PCR samples (no template) were always included to determine background radiation. Controls for contamination by genomic DNA were also conducted, with PCR amplification of total RNA samples (rather than cDNA). No evidence of genomic DNA was present. No extraneous bands were observed with any of the $GABA_A$ subunit-selective primers employed.

Data are presented as the ratio of counts incorporated into the amplified cRNA internal standard products to counts incorporated into the corresponding target mRNA amplification product. The native concentration of target RNA is determined by the point of equivalent incorporation using linear regression analysis of log-transformed data.

Subunit-selective PCR primers for $GABA_A$ receptor subunit mRNAs. Subunit-selective PCR primers for $GABA_A$ receptor subunit mRNAs are given in Table 1.

Statistical analysis. Statistical analysis was performed using the paired Student's *t* test with 8–12 individual pairs per subunit derived from two or three independent chronic ethanol exposure experiments with each determination run in triplicate. A value of $p < 0.05$ was considered statistically significant.

Results

The effects of chronic ethanol administration were investigated with competitive, quantitative RT-PCR analysis to determine absolute levels of $GABA_A$ receptor subunit mRNA in rat cerebral cortex. Fig. 1 shows representative gels for

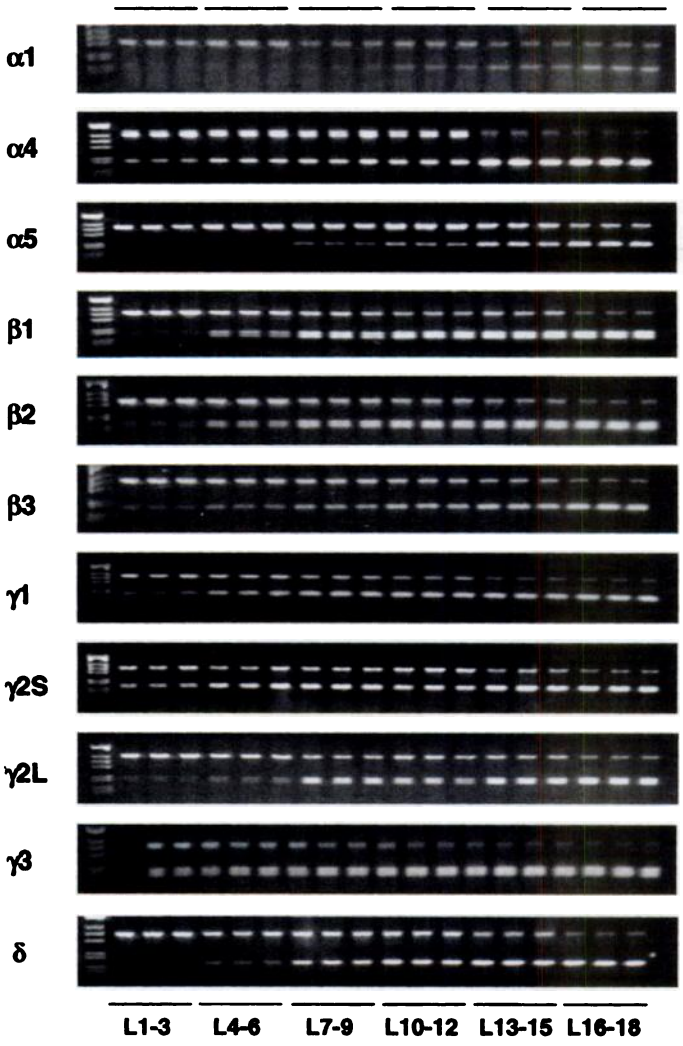

Fig. 1. Representative gels for $GABA_A$ receptor subunit mRNA levels assayed in this study. A series of six increasing concentrations of internal standard cRNA were added to each tube containing 1 μg of total RNA. The PCR products from each tube are shown in triplicate (*solid lines*) for each subunit. For example, α1 internal standard cRNA was added at concentrations of 5 pg (*lanes [L]1–3*), 10 pg (*L4–6*), 20 pg (*L7–9*), 40 pg (*L10–12*), 60 pg (*L13–15*), and 100 pg (*L16–18*). *Top bands*, PCR products of target mRNA. *Bottom bands*, *Bgl*II-digested internal standard PCR products. Note that increasing concentrations of internal standards compete with target mRNA for amplification. The concentration range for each subunit was α1, 5–200 pg; α4, 5–250 pg; α5, 1–50 pg; β1, 1–100 pg; β2, 5–200 pg; β3, 5–200 pg; γ1, 1–40 pg; γ2S, 5–50 pg; γ2L, 1–40 pg; γ3, 1–40 pg; and δ, 1–50 pg. The point of equivalence was determined by linear regression analysis of the ratio of counts incorporated into the internal standard PCR product divided by counts incorporated into the target PCR product across the series of concentrations of internal standards. The point of equivalence (when the ratio is 1) is the absolute concentration of mRNA/μg of total RNA (see Table 2). This figure was generated using digitized images captured from photographs of ethidium bromide-stained agarose gels.

$GABA_A$ receptor subunit mRNA levels measured in this study. Initial experiments with the competitive, quantitative RT-PCR technique verified results obtained previously with Northern blot analysis. The present results also provided absolute quantification of initial mRNA levels for individual $GABA_A$ receptor subunits.

Although a combination of $\alpha 1$, $\beta 2$, and $\gamma 2S$ and/or $\gamma 2L$ subunits appear to be the predominant population of $GABA_A$ receptors in rat brain (10, 24), it is possible that other subunit combinations present in the cortex may be altered by chronic ethanol administration. Alterations in subunit assembly of populations of $GABA_A$ receptors could influence the binding of ligands such as [^{3}H]Ro 15–4513 and [^{3}H]zolpidem. As shown in Fig. 2, although $\alpha 1$ subunit mRNA levels significantly decreased by 43% after chronic ethanol consumption, $\alpha 4$ subunit mRNA levels significantly increased by 89% in cortex from the same pairs of animals (Fig. 2 and Table 2).

Previous studies have reported conflicting results on the effect of chronic ethanol exposure on β subunit mRNA levels using different ethanol administration paradigms (25, 26). In the present study, chronic ethanol consumption had no effect on $\beta 1$, $\beta 2$, or $\beta 3$ subunit mRNA concentrations (Table 2). Likewise, mRNA levels for the $\alpha 5$ and δ subunits did not change with chronic ethanol consumption. Previous investigations with Northern blot analysis have shown that $\alpha 2$ mRNA levels decrease, whereas there is no change in $\alpha 3$ mRNA levels with chronic ethanol consumption (15).

The basal mRNA level for the $\gamma 2S$ subunit splice variant was higher than that for the $\gamma 2L$ variant (Table 2). Because the expression of $\gamma 2S$ or $\gamma 2L$ splice variants may be important for ethanol sensitivity of $GABA_A$ receptors (17), we next determined whether chronic ethanol administration would alter mRNA levels for either of these subunits. Although $\gamma 2L$ subunit mRNA levels were not altered, $\gamma 2S$ subunit mRNA levels significantly increased by 32% after chronic ethanol consumption (Fig. 3). Investigation of chronic ethanol-induced alterations of mRNA levels for additional γ subunits showed that $\gamma 1$ levels increased by 70%, whereas the levels of mRNA for the $\gamma 3$ subunit did not change (Fig. 4).

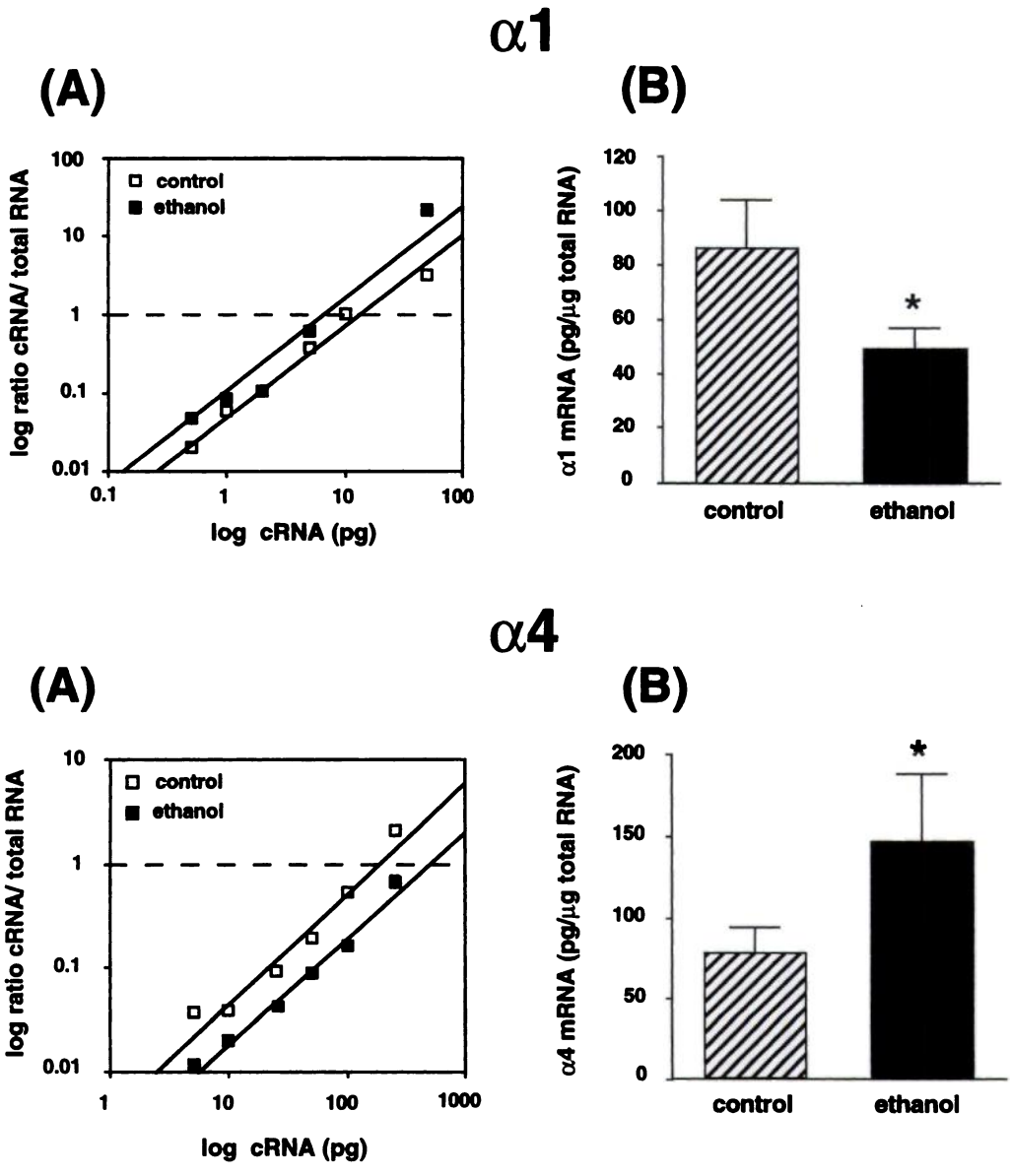

Fig. 2. A, Representative determination of $\alpha 1$ and $\alpha 4$ subunit mRNA levels in cortex between a pair-fed (*control*) and ethanol-fed rat. *Dashed line*, point of equivalence, which is shifted to the left (decreased) for $\alpha 1$ and shifted to the right (increased) for $\alpha 4$ after chronic ethanol consumption. B, Summary of the effects of chronic ethanol consumption on absolute concentrations of $\alpha 1$ and $\alpha 4$ subunit mRNA levels. $\alpha 1$ subunit mRNA levels were significantly decreased (*, $p < 0.01$), whereas $\alpha 4$ subunit mRNA levels significantly increased (*, $p < 0.05$) with chronic ethanol consumption. Results represent 10–12 pairs across three independent experiments.

TABLE 2

Quantification of mRNA levels for $GABA_A$ receptor subunits in rat cerebral cortex

Subunit	Pair-fed	Ethanol-fed
	pg/μg total RNA	
α1	86.5 ± 17.8	49.1 ± 7.9[a]
α4	77.5 ± 15.9	146.2 ± 41.3[b]
α5	29.4 ± 3.4	25.8 ± 3.5
β1	30.9 ± 4.8	35.5 ± 5.1
β2	47.9 ± 8.0	60.7 ± 12.9
β3	71.0 ± 21.8	84.2 ± 21.7
γ1	3.5 ± 0.6	6.0 ± 1.3[b]
γ2S	23.5 ± 3.1	31.1 ± 4.2[b]
γ2L	13.8 ± 2.1	14.9 ± 2.6
γ3	4.6 ± 1.2	3.9 ± 0.6
δ	14.9 ± 2.0	19.3 ± 2.9

Data are presented as the mean ± standard error for 8–12 individual pairs across three independent experiments.

[a] $p < 0.01$

[b] $p < 0.05$ by paired *t* test.

Discussion

The present study was undertaken to determine whether alterations in gene expression for subunits of $GABA_A$ receptors may be a mechanism underlying the changes in the functional responsiveness of $GABA_A$ receptors observed after chronic ethanol consumption. These functional alterations cannot be explained by alterations in receptor density or affinity. Furthermore, recombinant expression studies have shown that $GABA_A$ receptor responses are influenced by subunit composition. We found that chronic ethanol exposure reduces α1 subunit mRNA concentrations and increases α4 subunit mRNA concentrations by approximately equal amounts in rat cerebral cortex.

The partial inverse agonist Ro 15–4513 is a selective antagonist of ethanol intoxication as well as ethanol potentiation of $GABA_A$ receptor-mediated chloride uptake (27). Ticku *et al.* (7) observed a large increase in [^{3}H]Ro 15–4513 binding density in rat cortex after chronic ethanol exposure. Recombinant expression studies have shown that [^{3}H]Ro 15–4513 has high affinity for α4 and α6 subunit-containing $GABA_A$ receptors, which is in contrast to typical benzodiazepines, which have low affinity for α4 and α6 subunit-containing $GABA_A$ receptors (28, 29). However, the α6 subunit is not detectable in rat cortex (28). Therefore, [^{3}H]Ro 15–4513 must recognize $GABA_A$ receptors containing other α subunits, with the α4 subunit being a likely candidate. The present results, showing a large increase in α4 subunit mRNA levels in cortex after chronic ethanol consumption, add additional support to this suggestion. Chronic ethanol-induced substitution of α4 subunits for α1 subunits in a population of $GABA_A$ receptors could account for the increased [^{3}H]Ro 15–4513 binding noted by Ticku *et al.* (7). Using the competitive, quantitative RT-PCR technique, we verified that chronic ethanol consumption decreases $GABA_A$ receptor α1 mRNA levels, as was previously observed with the use of Northern blot analysis (5, 15).

The competitive, quantitative RT-PCR technique allowed separate determination of the effects of chronic ethanol consumption on mRNA levels for the two γ2 subunit splice variants. This subunit is of particular interest because the γ2L splice variant may be required for ethanol potentiation of GABA responses in some, but not all, recombinant expression systems (17, 30). In the present study, we found no changes in mRNA levels of the γ2L subunit in ethanol-dependent rats. Rather, a significant increase in γ2S subunit mRNA levels was noted after chronic ethanol consumption. Recent studies have shown that the γ2S and γ2L splice variants are both expressed in the rat cortex (22, 31), and there is evidence that there is considerable colocalization within the same receptor complex (32). This evidence, taken together with the present findings, suggests that there may be a role for the γ2S splice variant in responding to the effects of chronic ethanol consumption. In addition, a significant increase in the concentration of the γ1 but not γ3 subunit mRNA was noted after chronic ethanol consumption. It is not known at the present time whether these subunits influence ethanol sensitivity of $GABA_A$ receptors in brain.

No effect of chronic ethanol administration on β subunit mRNA levels was observed. These results do not agree with the report of Mhatre *et al.* (25) showing substantial increases in these subunits after chronic ethanol administration. These conflicting results may be explained by differences in the doses of ethanol administered. Although rats in both studies exhibited physical dependence on ethanol, the blood ethanol concentrations in the present study were approximately 200 mg/100 ml, whereas the blood ethanol concentrations in the former study were 400 mg/100 ml or higher. Therefore, it unclear whether alterations in β subunit expression are related to the development of ethanol dependence. The lack of effect on β subunit mRNA levels is consistent with previous studies using Northern blot analysis (26).

There is generally good agreement when comparisons are made between the absolute levels of mRNA for $GABA_A$ receptor subunits obtained in the present study (Table 2) and the relative levels of these subunit mRNAs observed with *in situ* hybridization (10, 24). For example, $GABA_A$ receptor α1 mRNA is more than 3-fold more abundant in the cortex than α5 mRNA as determined with each approach. Message levels for the γ2S splice variant are present at larger concentrations than for the γ2L variant. In addition, basal levels of mRNA for the α4 subunit are comparable to α1 levels.

The present investigation into the effects of chronic ethanol consumption on $GABA_A$ receptor gene expression in rat cortex has demonstrated subunit-specific and bidirectional effects. This result supports accumulating evidence that chronic ethanol consumption exerts selective effects at $GABA_A$ receptors. Although the relationship between alterations in receptor subunit expression and function is unknown, there is increasing evidence that chronic exposure to a number of GABAergic agents selectively alters subunit mRNA levels in particular brain regions. For example, chronic GABA exposure reduces α1 subunit expression in cultured chick neurons (33) and cortical neurons (34). Chronic lorazepam or diazepam exposure reduces mRNA levels for the α1 and γ2 subunits in cerebral cortex but not cerebellum or hippocampus (35, 36). Conversely, the inverse agonist FG 7142 increases levels of mRNA for α1 and γ2 in the cortex (36). Therefore, chronic ethanol consumption, like a number of other GABAergic compounds, elicits alterations in subunit mRNA levels.

The increased concentration of α4, γ1, and/or γ2S subunit mRNAs observed in the present study, along with the reductions in α1 and α2 subunit expression, may explain the alterations in $GABA_A$ receptor function associated with ethanol dependence. GABA- or muscimol-stimulated chloride

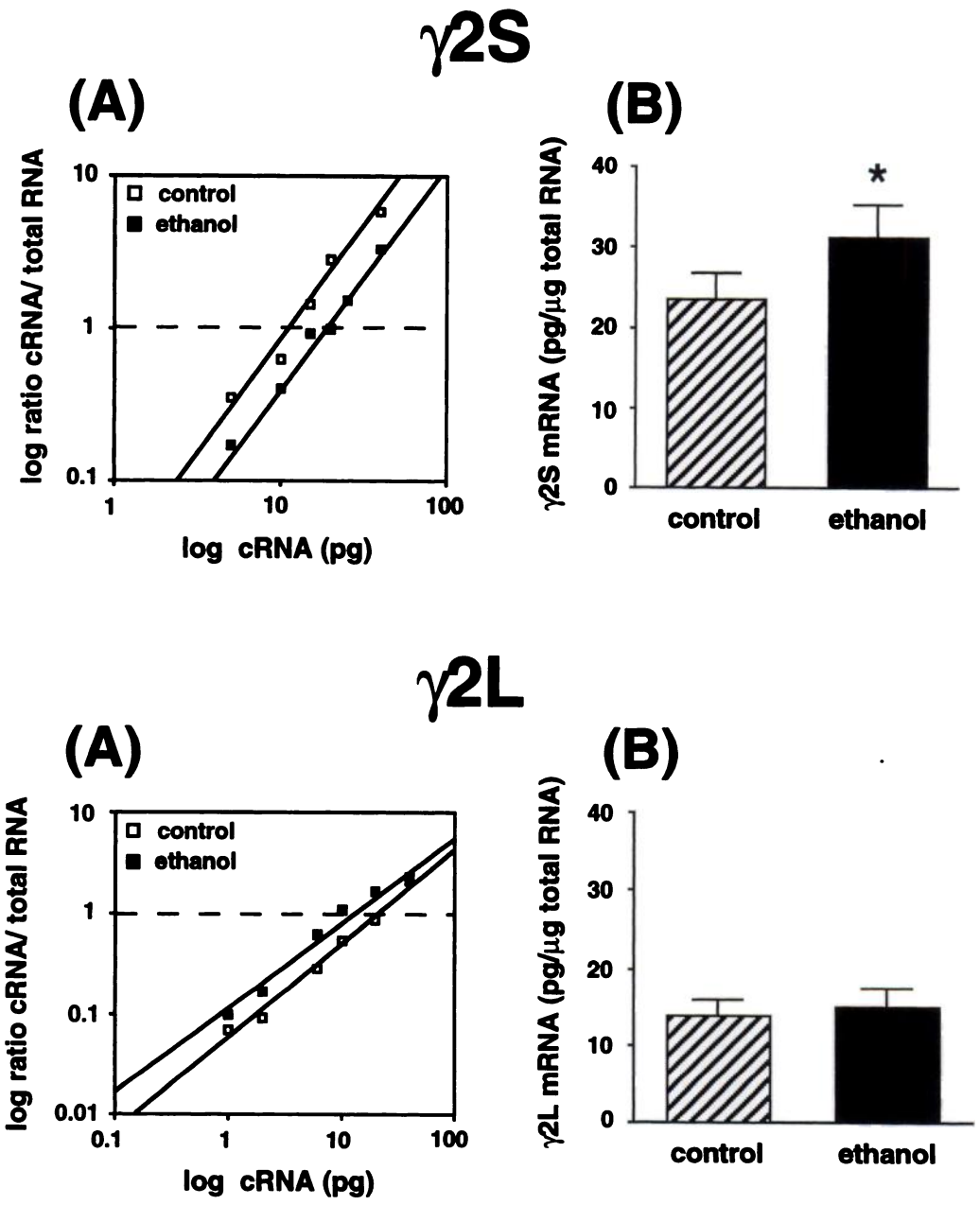

Fig. 3. A, Representative determination of γ2S and γ2L subunit mRNA levels in cortex between a pair-fed (*control*) and ethanol-fed rat. B, Summary of the effects of chronic ethanol consumption on absolute concentrations of γ2S and γ2L subunit mRNA levels. γ2S subunit mRNA levels significantly increased (*, $p < 0.05$), whereas there was no change in γ2L subunit mRNA levels with chronic ethanol consumption. Results represent 8–11 pairs across three independent experiments.

uptake, as well as benzodiazepine and ethanol potentiation of chloride uptake, are reduced after chronic ethanol exposure (1, 2, 4). Because $GABA_A$ receptor function has been shown to be influenced by the expression of different subunit combinations in recombinant expression assays (12–14), alterations in subunit composition resulting from alterations in subunit expression could elicit functional changes in $GABA_A$ receptors. Our results suggest that loss of responsiveness to the effects of ethanol after chronic ethanol consumption is not due to decreased levels of γ2L subunit mRNA.

Although the physiological relevance of alterations in $GABA_A$ receptor subunit mRNA levels after chronic ethanol consumption has not yet been elucidated, it is probable that continuing investigations will discern important roles for a number of subunit isoforms in modulating $GABA_A$ receptor function. It has been shown that the expression of various $GABA_A$ receptor subunits influences the pharmacology and the binding characteristics of GABAergic ligands, including [^{3}H]Ro 15–4513 and [^{3}H]zolpidem (12–14, 37). However, alterations in subunit mRNA levels may not directly translate into changes in polypeptide levels or $GABA_A$ receptor stoichiometry. To address this point, recent evidence provided by Ticku *et al.* suggests that $GABA_A$ receptor subunit mRNA levels show good correlation with changes in polypeptide levels (16, 25, 38). Immunoprecipitation studies with subunit-selective antibodies will be required to directly demonstrate whether chronic ethanol consumption alters $GABA_A$ receptor subunit composition.

$GABA_A$ receptor function is also influenced by post-translational receptor modifications, including phosphorylation (see Ref. 39 for review), as are many other ligand-gated ion channels (40). For example, a number of protein kinases, including PKA and PKC, influence $GABA_A$ receptor-mediated chloride uptake. Most $GABA_A$ receptor subunits contain

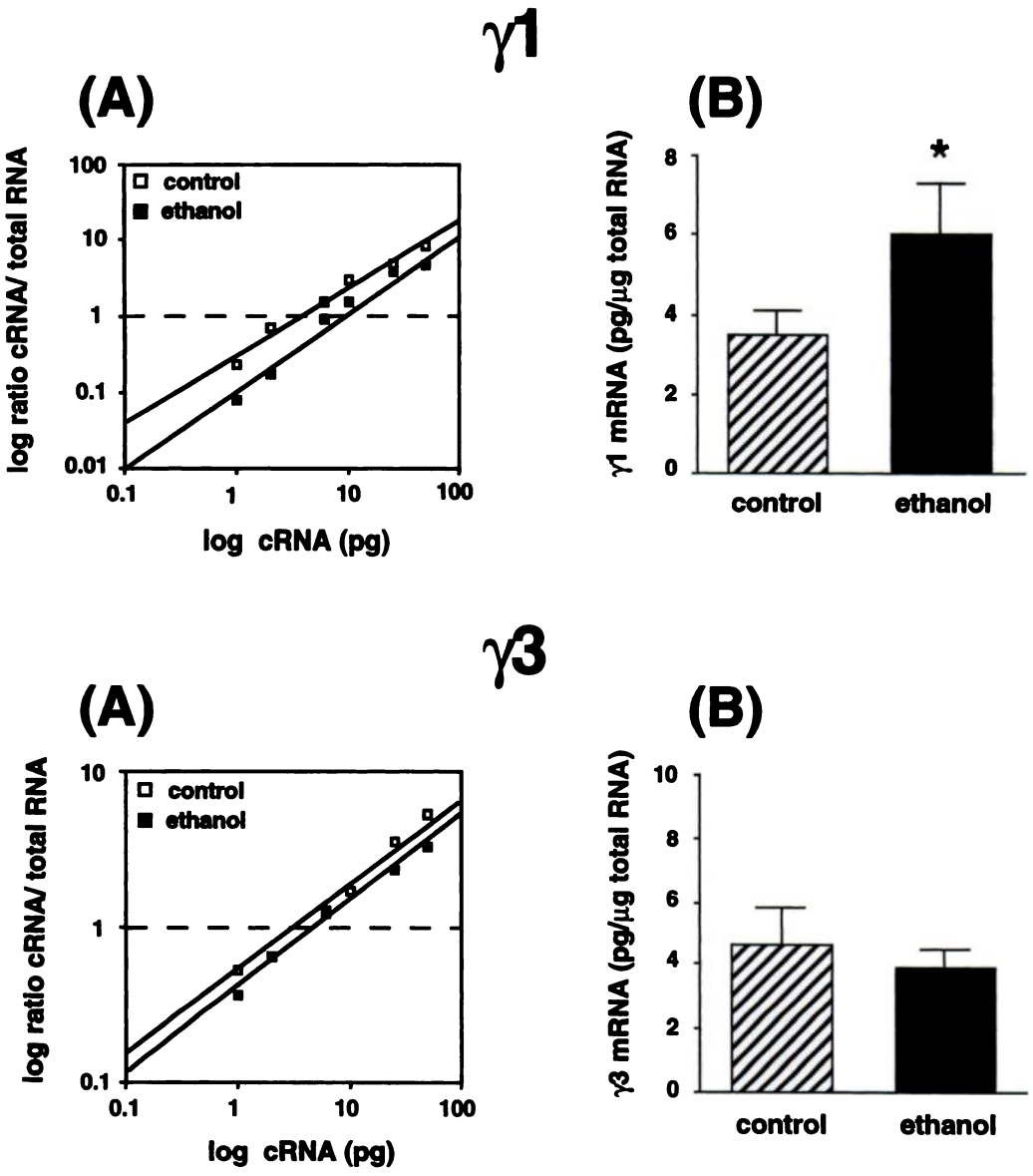

Fig. 4. A, Representative determination of γ1 and γ3 subunit mRNA levels in cortex between a pair-fed (*control*) and ethanol-fed rat. B, Summary of the effects of chronic ethanol consumption on absolute concentrations of γ1 and γ3 subunit mRNA levels). γ1 subunit mRNA levels significantly increased (*, $p < 0.05$), whereas there was no change in γ3 subunit mRNA levels with chronic ethanol consumption. Results represent 8–10 pairs across three independent experiments.

phosphorylation sites, some of which may exist endogenously in a phosphorylated state (41). The present study does not rule out alterations in post-translational processing as a mechanism for decreased $GABA_A$ receptor function after chronic ethanol exposure. Therefore, alterations in phosphorylation states of particular subunits, as well as subunit assembly, may be important mechanisms underlying the development of ethanol dependence.

The present findings support the hypothesis that chronic ethanol consumption alters $GABA_A$ receptor subunit composition, possibly through alterations in subunit assembly. This mechanism may explain the functional alterations of $GABA_A$ receptors associated with the development of ethanol dependence. However, there is no evidence for concomitant changes in polypeptide levels for most of these subunits. Therefore, additional studies are necessary to determine whether chronic ethanol consumption alters the subunit composition of $GABA_A$ receptor macromolecules.

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Send reprint requests to: Leslie L. Devaud, Ph.D., Bowles Center for Alcohol Studies, CB#7178, UNC School of Medicine, Chapel Hill, NC 27599-7178.