GABA$_A$ Receptor α_1, α_4, and β_3 Subunit mRNA and Protein Expression in the Frontal Cortex of Human Alcoholics

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Animal studies have shown that chronic ethanol consumption produces physical dependence upon ethanol and alters γ-aminobutyric acid-A (GABA$_A$) receptor subunit gene expression in brain. Although extensive investigation has been conducted in animal models, relatively little work has been performed directly on human alcoholic brain tissue to determine if there are alterations in GABA$_A$ receptor gene expression. In this study, GABA$_A$ receptor α_1, α_4, and β_3 subunit mRNA and peptide expression in postmortem frontal cortex from human alcoholics ($n = 15$) and age- and sex-matched controls ($n = 13$) were measured by quantitative, competitive reverse transcription polymerase chain reaction and Western blot analysis. GABA$_A$ receptor β_3 subunit mRNA expression was 35% greater ($p < 0.05$) in alcoholics, compared with nonalcoholic controls. We found no significant difference in α_1 and α_4 subunit mRNA levels between groups. However, there was a trend toward greater (21%) α_1 subunit mRNA expression. There was no difference in α_1, α_4, or $\beta_{2/3}$ subunit peptide levels in frontal cortex between controls and alcoholics. Neither the age of the subjects nor the postmortem interval correlated with mRNA or peptide levels. Blood ethanol content also did not correlate with mRNA or peptide expression in alcoholic samples. These data suggest that GABA$_A$ receptor adaptations, resulting from prolonged alcohol consumption in human alcoholics, may differ from animal models of alcohol dependence. These differences may be related to the longevity of alcohol exposure in human alcoholics, as well as variability in the dependence/withdrawal state of the human subjects. Therefore, further studies in human postmortem brain tissue are warranted.

Key Words: GABA$_A$ Receptors, Ethanol Dependence, Alcoholics, Postmortem Brain.

γ-Aminobutyric acid (GABA) is the most prevalent inhibitory neurotransmitter in the brain. GABA acts primarily at GABA$_A$ receptors, which are pentameric structures comprised of homologous protein subunits from six major classes: α, β, γ, δ, ϵ, and ρ, each of which has several isoforms (see Sieghart[1] for review). These receptors contain recognition sites for benzodiazepines, barbiturates,

and neuroactive steroids that allosterically modulate chloride channel gating of the receptors (see Morrow[2] for review). Ethanol enhances GABA$_A$ receptor-mediated chloride conductance, but not all GABA$_A$ receptors in brain are sensitive to ethanol (see Crews et al.[3] for review). The biophysical and pharmacological properties of GABA$_A$ receptors have been studied in neurons, where these receptors modulate CNS excitability by gating Cl$^-$ conductance. GABA$_A$ receptors are also present on glia,[4–6] but the physiological function of GABA$_A$ receptors in glial cells is largely unknown.

Chronic ethanol administration alters GABA$_A$ receptor-mediated chloride channel function in rats and mice (see Morrow[2] for review). These studies have demonstrated cellular tolerance to ethanol,[7–9] cross-tolerance to benzodiazepines[10] and barbiturates,[7] and sensitization to neurosteroids[11,12] and inverse agonists.[10,13] Human alcoholics exhibit behavioral changes that parallel rodent models of alcohol dependence, including ethanol tolerance and benzodiazepine and barbiturate cross-tolerance.[14,15] The neurochemical correlates of alcohol dependence in postmortem human brain suggests that alterations in GABA$_A$ receptors may be involved. [^{3}H]Muscimol binding density is greater in alcoholic cerebral cortex[16] and superior frontal gyrus of noncirrhotic alcoholics[17] versus controls. Contradictory effects on benzodiazepine binding have been reported in different studies of human alcoholics.[17–19] Likewise, conflicting results have also been obtained on chronic ethanol-induced changes in the density and affinity of GABA$_A$/benzodiazepine receptors in the rat brain (see Morrow[2] for review).

Whereas the possibility of alterations in density or affinity of GABA$_A$/benzodiazepine receptors is still in question, it is also possible that a change in GABA$_A$ receptor subunit expression accounts for the alterations that result from prolonged ethanol consumption. A recent study suggests that GABA$_A$ receptor α_1 subunit mRNA expression is elevated in human alcoholic postmortem frontal cortex, compared with nonalcoholic controls.[18]

Previous work has shown that chronic ethanol administration to rats results in selective alterations in GABA$_A$ receptor subunit mRNA and peptide expression. GABA$_A$ receptor α_1 subunit mRNA and peptides are significantly decreased,[19–26] whereas α_4 and α_6 subunit mRNA and

Table 1. Control and Alcoholic Subject Information

Age	Sex	Race	PMI	Cause of Death	Other Diagnoses	EtOH Level (g/dL)
Controls (13)						
25	M	W	20	Chest trauma-falling tree		0.00
39	M	B	8	Gun shot wound-abdomen	Occasional EtOH use	0.05
39	M	W	10	Myocardial infarction	High blood pressure, taking tenormin and dyazide	0.00
47	M	W	19	Myocardial infarction		0.00
27	M	W	20	Electrocution		0.00
35	F	W	12	Cardiac	Oral Contraceptives	0.00
29	M	W	40	Gun shot wound-abdomen	Osteomyelitis-foot	0.00
61	M	W	19	Trauma-aircraft		0.00
45	M	W	17	Trauma-aircraft		0.00
80	M	B	18	Aortic aneurysm		0.00
69	M	W	19	Gun shot wound-neck	High blood pressure, taking dyazide	0.00
49	M	W	15	Cardiac		0.00
36	M	W	15	Cardiac		0.00
44.7 ± 4.6			*17.9 ± 2.1*			
Alcoholic (15)						
31	M	W	6	Gun shot wound-neck	Liver fatty infiltration	0.18
42	M	W	7	Gun shot wound-chest	High blood pressure, taking inderal, vasotech, dyazide	0.10
45	M	W	12	Myocardial infarction	Hx peptic ulcer disease	0.00
52	M	W	29	GI bleeding	Possible cirrhosis	0.00
34	M	W	28	Ethanol overdose	Possible cirrhosis	0.30
33	F	B	9	Cardiac	Severe liver fatty infiltration	0.06
43	M	B	11	Gun shot wound-chest		0.25
52	M	B	27	Cardiac		0.28
50	M	B	18	Cirrhosis		0.08
74	M	B	12	Carbon monoxide poisoning		0.29
62	M	B	14	Gun shot wound-head	Liver fatty infiltration, depressed mood, adjustment disorder	0.21
69	M	W	38	Gun shot wound-chest	Hx gastric ulcer	0.12
29	F	B	10	Stab wound-chest		0.17
57	M	W	15	Cardiac		0.05
39	M	B	10	Gun shot wound-head	Regular marijuana use	0.16
47.5 ± 3.6			*16.4 ± 2.5*			*0.15 ± 0.026*

PMI—Post mortem interval.
Italics—Mean values.

peptides are increased[23,25,26] in ethanol-dependent rats after prolonged ethanol administration. There are also increases in β subunit mRNA and peptide expression, depending on the state of ethanol dependence versus withdrawal and the dose of ethanol.[25-27] During the peak of ethanol withdrawal, we found α_1 and α_4 subunit mRNA expression had returned to control levels, whereas β_3 subunit mRNA expression increased by 82%, compared with controls.[12] Interestingly, α_1, α_4, and $\beta_{2/3}$ subunit peptide expression in ethanol dependent rats was nearly identical to the peptide expression in ethanol withdrawn rats, with both groups displaying a significant decrease in α_1, a significant increase in α_4, and a significant increase in $\beta_{2/3}$ subunit peptide expression.[25] Thus, alterations in peptide expression are a more stable adaptation to long-term ethanol consumption in the rat. These alterations in $GABA_A$ receptor subunit gene expression could potentially contribute to the development of physical dependence, tolerance, and withdrawal symptoms associated with prolonged alcohol consumption.

The present study was undertaken to quantitatively measure the potential effects of long-term alcoholism on specific $GABA_A$ receptor subunits in postmortem human frontal cerebral cortex. $GABA_A$ receptor α_1, α_4, and β_3 subunit mRNA expression was measured in the frontal cortex of

alcoholics and controls using quantitative, competitive reverse transcriptase-polymerase chain reaction (RT-PCR). Western blot analysis with subunit selective antibodies was used to determine α_1, α_4, and $\beta_{2/3}$ subunit peptide expression. These subunits were studied because they are abundant in human frontal cortex and sensitive to prolonged ethanol exposure in rat models of alcohol dependence.

MATERIALS AND METHODS

Tissue Collection and Preparation

Postmortem brain samples were obtained at autopsy as authorized by the Office of the Chief Medical Examiner, State of North Carolina. Family members provided informed consent under guidelines approved by the Institutional Review Board of the University of North Carolina. Samples were obtained from 28 subjects who died suddenly from accidents ($n = 6$), assault ($n = 10$), cardiac causes ($n = 10$), gastrointestinal bleeding ($n = 1$), and cirrhosis ($n = 1$). Information regarding subjects was gathered through personal and phone interviews conducted by a research psychiatrist and a social worker. Based on all available evidence and relying most heavily on quantitative data from relatives, DSM-III-R psychiatric diagnoses[28] were assigned at a consensus conference by several clinicians. Ethanol-dependent subjects were chosen from a pool of 82 potential subjects. Controls demonstrated the absence of DSM-III-R substance abuse or other diagnoses and were selected on the basis of age, postmortem interval, sex, and race to match alcoholic subjects. Sufficient material was not available from each subject to measure all mRNA and peptide

Table 2. Subunit-Selective PCR Primers for GABA$_A$ Receptor Subunit mRNAs and Corresponding Human mRNA Segments

Subunit	Primer (5' to 3')	Position
α_1 sense	AGC-TAT-ACC-CCT-AAC-TTA-GCC-AGG	
Corresponding human mRNA	AGC-TAC-ACC-CCT-AAT-TTG-GCC-AGG	1334–1357
α_1 antisense	AGA-AAG-CGA-TTC-TCA-GTG-CAG-AGG	
Corresponding human mRNA	ATA-AAT-AAA-TTC-CCA-GTG-CAG-AGG	1615–1638
α_4 sense	AAA-TGC-AGC-TGA-GAC-TAT-CTC-TGC	
Corresponding human mRNA	AAA-TGC-AGC-TGA-AAC-CAT-ATC-TGC	1331–1354
α_4 antisense*	CCA-TAG-TGT-CCT-TAG-ATA-AAT-AAA	
Corresponding human mRNA	CCA-TAG-TGT-CCT-TAG-ATA-AAT-AAA	1633–1656
β_3 sense	GAA-ATG-AAT-GAG-GTT-GCA-GGC-AGC	
Corresponding human mRNA	GAA-ATG-AAT-GAG-GTC-TCA-GGC-GGC	1182–1205
β_3 antisense	CAG-GCA-GGG-TAA-TAT-TTC-ACT-CAG	
Corresponding human mRNA	CAG-GCA-GAG-TAA-TAT-TTC-ACT-CAG	1513–1536

* Identical to human GABA$_A$ receptor subunit mRNA.

levels; therefore, the number of subjects in each comparison is shown in the "Results" section.

Brain samples were dissected from the frontal pole, containing Broadmann's areas 9 and 10, quickly frozen on dry ice, and stored at $-70°C$. Subjects were free of chronic neuropathological changes on microscopic examination of hippocampal sections. Urine or serum samples from subjects were assayed quantitatively for the presence of stimulants, anxiolytics, marijuana, antidepressants, antipsychotics, and ethanol. These data, as well as clinical details regarding the subjects, are shown in Table 1. One control subject was positive for ethanol, but was included in the control group as evidence indicated that he was not a chronic user of ethanol.

Quantitative Competitive RT-PCR

Total RNA was extracted as previously described.[26] Briefly, tissue was homogenized with a Polytron homogenizer in guanidine isothiocyanate solution, followed by overnight centrifugation in cesium chloride solution. Protein contaminants were removed from the RNA by two phenol and chloroform/isoamyl alcohol extractions, each followed by an overnight precipitation in ethanol. Optical density measurements were taken to ensure purity and ascertain yield of RNA.

Internal standards were used that had been generated according to the method of Grayson et al.[29] The cRNA templates were derived from rat α_1, α_4, and β_3 subunits[26] and were 91%, 76%, and 93% homologous to the corresponding segments of α_1, α_4, and β_3 human RNA, respectively. All internal standards contained a BglII restriction site so that internal standard cRNAs could be distinguished from native RNA by digestion with BglII. The α_1 internal standard generated a 251 base pair (bp) polymerase chain reaction (PCR) product that was digested into two 125 kB fragments. The β_3 internal standard generated a 355 bp PCR product that was digested into two 178 bp fragments. Because the human α_4 subunit mRNA differed from the rat cRNA standard, the antisense primer (1633 to 1656 bp) was designed to amplify human α_4 mRNA (Table 2), as well as a truncated segment of the rat α_4 cRNA internal standard (323 bases). This shifted the relative position of the BglII restriction site in the cRNA standard, resulting in cRNA amplification products of unequal length after digestion, as evidenced by the two bands of internal standard amplification products. Hence, the α_4 internal standard generated a 325 bp PCR product that was digested into 200 and 123 bp fragments.

Quantitative, competitive RT-PCR was conducted using various known concentrations of cRNA that were added to a constant amount of total

RNA, subjected to identical reverse transcription conditions, and amplified using a pair of subunit specific primers that recognized both the native and internal standard transcripts (Table 2). The amplification products were digested with the restriction enzyme BglII to separate the cRNA standards from the target mRNA. Quantitation of the target mRNA was achieved by measuring [32P] incorporation in the amplified target and internal standard products, calculating the ratio of cRNA/target mRNA for each cRNA concentration, and estimating the concentration of equivalent [32P] incorporation by linear regression analysis of the log-transformed data.

Reverse transcription was conducted in 50 mM Tris-HCl, 3 mM MgCl$_2$, and 75 mM KCl buffer with 10 mM dithiothreitol, 1 mM dNTPs, 1 mM random hexamers, and 200 units M-Mlv RT (Boehringer Mannheim, Indianapolis, IN) at 37°C for 75 min. The resultant cDNA was heat-denatured at 95°C for 5 min, then amplified in a buffer containing 50 mM Tris-HCl, 20 mM ammonium sulfate, 1 μM each of 5' (sense) and 3' (antisense) subunit specific primers (Lineberger Cancer Research Center Oligonucleotide Laboratory, University of North Carolina, Chapel Hill, NC), 200 μM dNTPs, 1 unit Hot Tub polymerase (Amersham, Arlington Heights, IL), and 2 or 4 mM MgCl$_2$. In addition, 1 μCi [32P]dCTP was added to each tube. The α_1 amplification was conducted using 4 mM MgCl$_2$, and the α_4 and β_3 amplifications were conducted using 2 mM MgCl$_2$. The amplification reaction was also optimized for each subunit by altering the annealing temperature and number of cycles. For the α_1 and α_4 subunits, each cycle consisted of 94°C for 40 sec, followed by 58°C (the annealing temperature) for 50 sec, and ended with 72°C for 50 sec. The annealing temperature utilized for β_3 amplification was 55°C. The number of amplification cycles utilized for the α_1, α_4, and β_3 subunits were 35, 35, and 28 cycles, respectively. The amplification was conducted utilizing a Barnstead/Thermolyne Amplitron II (Dubuque, IA). Control tubes with no template were run adjacent to sample tubes to measure background radiation and to ensure the absence of contaminants in the PCR. Controls for contamination by genomic DNA were conducted by amplification RNA samples that were not reverse-transcribed. Genomic DNA contamination was not observed.

PCR products were digested overnight with BglII, and run on a 1.8% agarose gel (Sea Kem agarose, FMC Corp, Rockland, ME) in 0.5 × Tris/borate/EDTA buffer. Each cRNA concentration was run in triplicate, and the bands for cRNA and target mRNA were measured by phosphorous detection using a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Data was analyzed by ANOVA or independent t tests.

(A)

α1

(B)

α4

(C)

β3
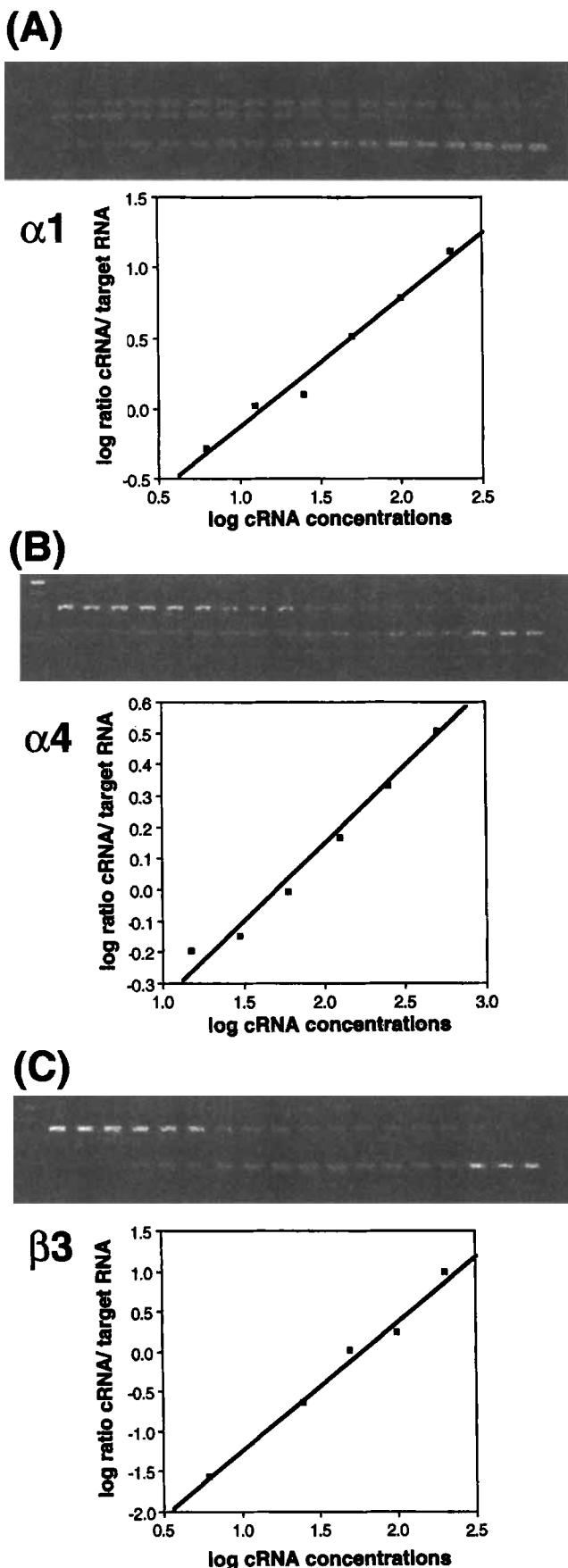
The native human α_1 mRNA target PCR product contains an endogenous BglII recognition site. Therefore, digestion of the target PCR product with BglII enzyme produced two α_1 subunit fragments (251 bp and 54 bp in length, respectively). An additional PCR product ~300 bp in length was also observed. BglII digestion, utilizing 5 to 10 times the normal amount of BglII enzyme, failed to cleave this product into the two expected fragments of 251 and 54 bp, signifying that this PCR product clearly lacked the BglII recognition site present in the reported GABA$_A$ receptor α_1 subunit mRNA sequence (Genbank Accession no. X14766). Therefore, this product was not included in the quantitation of α_1 subunit mRNA levels.

Western Blot Analysis

Tissue was homogenized in 30 volumes of 0.32 M sucrose in phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4), and centrifuged at 1000 × g. The supernatant solution was centrifuged at 12,000 × g, and the resultant pellet was resuspended in 8 volumes of PBS (3 mg/ml), aliquoted, and stored at −80°C until use.

Protein samples were diluted 1:1 with Tris-glycine sodium dodecyl sulfate buffer and denatured at 85°C for 3 min with 0.1 volume 1 M dithiothreitol. Proteins (24 μg/well) were separated on 8 to 16% Tris-glycine gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Novex Xcell II minicell apparatus (San Diego, CA). Proteins were transferred to Immobilon-P polyvinylidine fluoride membranes (Millipore, Bedford, MA), and blocked for 6 hr with a solution of PBS, 1% milk powder, and 0.05% Tween 20. The membranes were incubated overnight with primary antibody specific for α_1,[30,31] α_4,[32] or $\beta_{2/3}$ (bd17, BMB, Indianapolis, IN) subunit peptides. After two washes with PBS and 0.05% Tween 20, secondary antibodies consisting of horseradish peroxidase conjugated anti-rabbit (α_1[Pharmingin] and α_4), anti-guinea pig (α_1), or anti-mouse ($\beta_{2/3}$) were applied for 60 min. Two α_1 subunit antibodies were used for these studies, because there are minor differences in the rat and human peptide sequences used to generate the antiserum. Both antibodies recognized a single peptide band at ~51 kDa. Protein bands were visualized by enhanced chemiluminesence substrate (Pierce, Rockford, IL) and exposed to x-ray film under nonsaturating conditions. Densitometric measurements were made using NIH Image 1.47, and data was analyzed by ANOVA or independent t tests. Equivalent protein loading was verified by staining with coomassie blue. Data are presented as the percent difference relative to control values from the same gel.

RESULTS

Representative data utilized to determine GABA$_A$ receptor α_1, α_4, and β_3 subunit mRNA levels by quantitative competitive RT-PCR are shown in Fig. 1. Absolute quantification of α_1 subunit mRNA expression utilizing RT-PCR showed no significant difference between alcoholics

Fig. 1. Quantitative competitive RT-PCR determination of GABA$_A$ receptor α_1 (A), α_4 (B), and β_3 (C) subunit mRNA levels in human frontal cortex. Various concentrations of cRNA were utilized to compete for amplification with the target mRNA (α_1: 6.25–200 pg; α_4: 15–500 pg; β_3: 6.25–300 pg). [^{32}P] Incorporation was determined by phosphor detection using a Storm phosphorimager. The amount of target mRNA was determined by linear regression of log [cRNA] versus log [cRNA/target RNA]. The point of equivalence (cRNA = target RNA) denotes the absolute concentration of target mRNA in the samples (pg/μg of total RNA). (A) In the digitized gel photographs of ethidium bromide fluorescence, the α_1 subunit target PCR product is the middle band (251 bp), and the internal standard fragments appear as one band (125 bps). (B) The upper band represents the amplified target α_4 subunit PCR product (325 bp), and digestion of the internal standard results in 200 and 123 bp fragments. (C) The β_3 target PCR products (upper band) are 355 bp, and the internal standard digest produces two 178 bp fragments.

Fig. 2. GABA$_A$ receptor α_1, α_4, and β_3 subunit mRNA levels in frontal cortex of alcoholics versus nonalcoholic controls. There was no detectable difference in α_1 or α_4 subunit mRNA expression between groups. However, human alcoholics exhibited 35% greater GABA$_A$ receptor β_3 subunit mRNA levels, compared with age-matched, nonalcoholic controls (*p < 0.05).
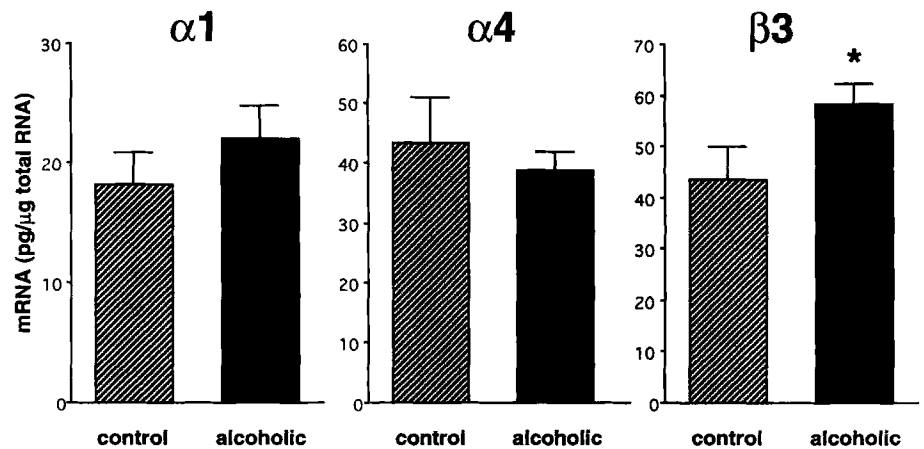
and nonalcoholic controls. However, as shown in Fig. 2 and Table 3, α_1 subunit mRNA expression in the alcoholic group showed a trend toward greater expression (21%) over matched controls. Similarly, α_4 subunit mRNA showed no significant difference between alcoholics and matched controls. However, α_4 subunit mRNA expression exhibited a trend toward lower expression (11%) in alcoholics versus controls (Fig. 2 and Table 3). Absolute quantification of β_3 subunit mRNA expression exhibited a 35% greater (p < 0.05) expression of mRNA in alcoholics versus control subjects, as shown in Fig. 2 and Table 3.

GABA$_A$ receptor α_1, α_4, and $\beta_{2/3}$ subunit peptide expression in alcoholic and age-matched controls was measured by Western blot analysis with subunit specific antibodies.[25] Figure 3 displays representative blots for each subunit studied. The α_1 antibodies recognized a 51 kDa band, the α_4 antibody recognized a band at 67 kDa, and the $\beta_{2/3}$ antibody recognized a doublet at 56 and 58 kDa. As shown in Fig. 4, there was no difference in α_1, α_4, or $\beta_{2/3}$ subunit peptide expression in alcoholic frontal cortex, compared with controls.

Previous studies have suggested that complicating factors, such as cirrhosis of the liver, can alter mRNA expression[18] and the affinity and density of GABA$_A$/benzodiazepine receptors[17,33] in humans. Six of the 15 alcoholic patients in the present study showed cirrhosis or displayed fatty infiltration of the liver. Therefore, mRNA and peptide data for the alcoholic samples was separated between subjects who had cirrhosis or liver fatty infiltration and cirrhosis-free alcoholics to determine if a difference could be observed between control versus cirrhotic or noncirrhotic

alcoholics. No difference in α_1 or α_4 subunit mRNA expression between controls and either cirrhotic or noncirrhotic alcoholics was observed. However, β_3 subunit mRNA expression in noncirrhotic alcoholics (n = 5) was 53% greater (ANOVA, Tukey Kramer post-hoc comparison: p < 0.05) compared with controls (n = 10), whereas β_3
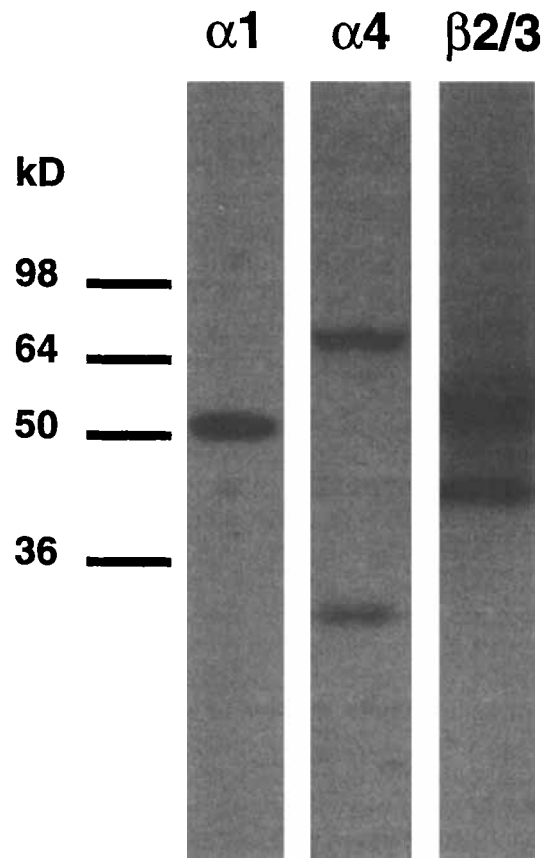

Fig. 3. Western-blot analysis of GABA$_A$ receptor α_1, α_4, and $\beta_{2/3}$ subunit peptides in human frontal cortex. Protein bands were visualized by enhanced chemiluminesence substrate and exposed to x-ray film. Densitometric readings were made using NIH Image 1.47. The α_1 antibody recognized a 51 kDa band, the α_4 antibody recognized a band at 67 kDa, and the $\beta_{2/3}$ antibody recognized a doublet at 56 and 58 kDa. Representative Western blots are shown.

Table 3. Quantification of mRNA Levels for GABA$_A$ Receptor Subunits in Human Frontal Cortex

Subunit	Control	(n)	Alcoholic	(n)
α_1	18.2 ± 2.6	(11)	22.0 ± 2.8	(9)
α_4	43.3 ± 7.7	(10)	38.8 ± 3.2	(12)
β_3	43.5 ± 6.5	(8)	58.5 ± 3.7*	(11)

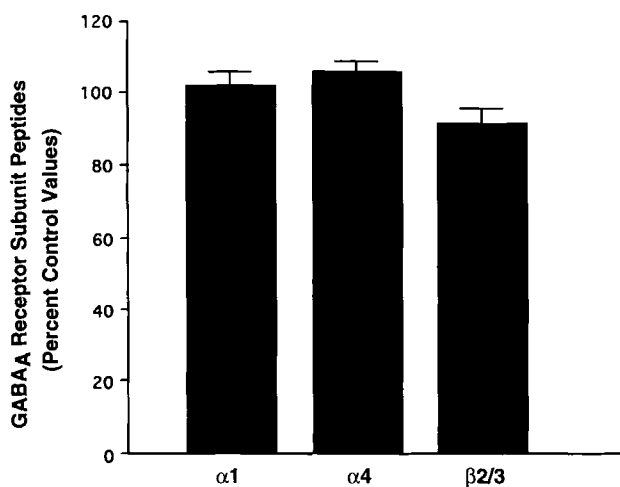Data are presented as pg/μg total RNA.
*p < 0.05.

Fig. 4. GABA$_A$ receptor α_1, α_4, and $\beta_{2/3}$ peptide expression in frontal cortex of alcoholics versus nonalcoholic controls. There was no significant difference in α_1, α_4, and $\beta_{2/3}$ subunit peptide levels between groups (n = 10 alcoholics, 9 controls for α_1 subunits; n = 9 alcoholics, 9 controls for α_4 subunits; n = 10 alcoholics, 9 controls for $\beta_{2/3}$ subunits).

mRNA expression in cirrhotics (n = 6) showed a nonsignificant trend toward greater expression of 20%. There was no difference in GABA$_A$ receptor α_1, α_4, or $\beta_{2/3}$ subunit peptide expression between cirrhotic or noncirrhotic alcoholics, compared with controls.

DISCUSSION

The present study was undertaken to determine whether chronic alcohol consumption results in measurable alterations in GABA$_A$ receptor subunit gene expression in postmortem tissue from human alcoholics. Positron emission tomography studies show a decrease in benzodiazepine binding in the frontal lobes of human alcoholics relative to normal controls.[34] Preliminary single position emission computerized tomography (SPECT) studies suggest a decrease in benzodiazepine binding in the inferior medial frontal cortex, temporal, and parietal cortices.[35,36] Previous work undertaken in this laboratory[12,25,26] has supported the hypothesis that chronic alcohol administration leads to alterations in GABA$_A$ receptor subunit expression in rat brain. To determine if the same adaptations were present in human alcoholics, GABA$_A$ receptor α_1, α_4, and β_3 subunits were measured by quantitative competitive RT-PCR and Western blotting analysis in postmortem frontal cortex of alcoholics, compared with age-matched controls.

The present findings showed no difference in GABA$_A$ receptor α_1 or α_4 subunit expression between human alcoholics and matched control subjects. Although greater β_3 subunit mRNA levels were observed in alcoholic versus nonalcoholic subjects, no difference in $\beta_{2/3}$ peptide expression was observed. This result could indicate that a change in β_3 peptide expression was masked by simultaneous measurement of both β_2 and β_3 subunit peptides. Further studies with selective β_3 subunit antibodies will be required to address this possibility.

The present results differ from a recent study showing greater levels of GABA$_A$ receptor α_1 subunit mRNAs in noncirrhotic alcoholics, compared with control subjects.[18] In the present study, we observed a trend toward greater α_1 subunit mRNA levels in alcoholics, but this trend was not observed among the noncirrhotic alcoholics, possibly due to the small sample size. Further, no difference in GABA$_A$ receptor α_1 subunit peptide levels were observed between alcoholics and controls, irrespective of liver pathology.

In the present study, the use of primers initially designed for use with rat RNA appeared to competently amplify human cDNA. There is a significant amount of homology between human and rat α_1 (89%), α_4 (86%), and β_3 (92%) subunit cDNAs, respectively. Furthermore, there is minimal difference in the base pairs between the primers and the human mRNA sequence (Table 2).

The control and alcoholic groups were well matched for both postmortem interval and age, and neither factor showed a significant correlation with overall levels of mRNA or peptide expression. There was a significant correlation between postmortem interval and α_4 peptide expression in the alcoholic group. However, because sample sizes were small and the slope of the regression was positive, it is likely that the correlation was anomalous. Similarly, regression analyses between age and the levels of mRNA or peptide expression yielded nonsignificant correlations. These results suggest that neither age nor postmortem interval systematically confounded the observed results, and concur with a recent study in human alcoholics[18] that failed to observe a significant correlation between postmortem interval or age with levels of mRNA expression. Additional studies measuring GABA$_A$/benzodiazepine receptor binding sites in human alcoholic brain samples have shown no significant correlation between postmortem interval or age with B_{max} values, suggesting that GABA$_A$ receptor protein subunits are stable for a period of time after death.[17,33,37] Age or postmortem interval do not correlate with alterations in either neuronal counts or neuronal area in control and alcoholic brain tissue as well.[38]

In addition to the subjects being matched for age and postmortem interval, the subjects in this study were selected based on the incurrence of sudden death. Tissue preparations made from such subjects have been shown to be virtually identical in functionality for up to 24 hr, when compared with fresh tissue obtained through surgery or biopsy.[39] The mean postmortem interval for control and alcoholic groups in this study was 17.9 ± 2.1 hr and 16.4 ± 2.5 hr, respectively, both well within the 24-hr period.

The results of this study in human alcoholics clearly differs from previous studies in rodent models of alcohol dependence. In rodent studies, chronic ethanol exposure alters GABA$_A$ receptor α_1, α_4, and β_3 subunit expression, and there is good agreement between the alterations in mRNA and peptide expression in dependent animals.[23,25-27] These effects were not observed in human

alcoholics in the present study. There are a number of important differences between human alcoholism and animal models of ethanol dependence that may contribute to these differences. The major difference between human and rat models is the chronicity of ethanol dependence in human alcoholics. Postmortem analysis of alcoholic subjects may reveal changes that represent the effects of neuronal loss and shrinkage,[40] as well as alterations in neurotransmission. There may be selective loss of specific cell populations that are most sensitive to ethanol exposure, complicating the interpretation of measurements of gene expression relative to total protein or total RNA. During ethanol withdrawal in rats, mRNA levels change rapidly,[12] but peptide levels remain at levels observed in the dependent state.[25] These data suggest that the state of ethanol dependence/withdrawal at the time of death may also contribute to variability in human postmortem studies.

Another potentially important difference between human postmortem studies and rat studies of ethanol dependence involves the regional analysis of ethanol's effects. Rat studies were conducted in homogenates of the entire cerebral cortex, whereas the studies in postmortem human tissue were conducted from frontal cortical tissue samples. Previous studies have show regional differences within cerebral cortex in human alcoholic postmortem tissue.[18] This factor might contribute to the results of the present study, if there were subtle differences in frontal cortical dissections across subjects. Therefore, studies of GABA_A receptor regulation in human alcoholics may require greater sensitivity that could be achieved by in situ hybridization and immunocytochemistry of brain sections.

The timing, method, and amount of alcohol consumption also differs in human and animal studies. Differences in the amount of alcohol imbibed may influence mRNA levels,[26,27] and this factor may contribute to greater variability in human studies. Although it is not known how differing alcohol consumption by humans could alter mRNA and peptide levels in postmortem tissue, such uncontrolled environmental factors, as well as the genetic diversity encountered with human subjects, could explain the differences observed between humans and animal models of alcohol dependence. Because human studies involve far more variables that cannot be controlled experimentally, larger sample sizes may be required to detect differences in GABA_A receptor gene expression between alcoholics and nonalcoholics.

GABA_A receptor expression is present in glial cells,[4-6] although the physiological role of these receptors is unknown. Human alcoholics exhibit neuronal shrinkage, death, and glial proliferation in the frontal cortex[38,40,41] that may alter measurements of GABA_A receptor expression. These factors may also contribute to the differences between human alcoholics and rat models of alcohol dependence.

In summary, GABA_A receptor α_1, α_4, and $\beta_{2/3}$ subunit peptide expression did not differ in postmortem frontal cortex of human alcoholics versus nonalcoholic controls. Greater levels of β_3 subunit mRNA were found in alcoholics, compared with control subjects. No difference in GABA_A receptor α_1 or α_4 subunit mRNAs were observed. These data suggest that the effects of chronic ethanol exposure in human alcoholics differs from rat models of alcohol dependence. GABA_A receptor adaptations in human alcohol dependence may require further investigation with larger sample sizes, more complete information on the subjects' state of alcohol dependence/withdrawal, more precise regimal analysis, and differentiation between neuronal and glial receptor populations.

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