Long-Term Ethanol Self-Administration by Cynomolgus Macaques Alters the Pharmacology and Expression of GABA<sub>A</sub> Receptors in Basolateral Amygdala

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Received May 28, 2004; accepted July 27, 2004

ABSTRACT

We have recently demonstrated that chronic ethanol ingestion alters the functional and pharmacological properties of GABA<sub>A</sub> receptors measured in acutely isolated rat lateral/basolateral amygdala neurons, a limbic forebrain region involved with fear-learning and innate anxiety. To understand relevance of these results in the context of primates, we have examined the effects of long-term ethanol self-administration on basolateral amygdala GABA<sub>A</sub> receptor pharmacology and expression in cynomolgus macaques (Macaca fascicularis). The impact of this 18-month-long exposure on GABA<sub>A</sub> receptor function was assessed in acutely isolated neurons from basolateral amygdala with whole-cell patch-clamp electrophysiology. Neurons from control animals expressed maximal current densities that were not significantly different from the maximal current densities of neurons from ethanol-treated animals. However, the GABA concentration-response relationships from ethanol-exposed neurons were significantly right-shifted compared with control neurons. These adaptations were associated with significant alterations in some characteristics of macroscopic current desensitization. To understand the mechanism governing these adaptations, we quantified GABA<sub>A</sub> α subunit mRNAs in basolateral amygdala from the same animals. mRNA levels of the α2 and α3 subunits were significantly decreased, whereas decreases in α1 expression only approached statistical significance. There were no changes in α4 mRNA levels. These findings indicate that ethanol-induced alterations in GABA<sub>A</sub> function may be regulated in part by selective changes in the expression of particular α subunits. We conclude that adaptations of basolateral amygdala GABA<sub>A</sub> receptors after long-term ethanol self-administration by the cynomolgus macaque are similar, but not identical, to those described in rodents after a brief forced ethanol exposure.

The amygdala is intimately associated with affective behaviors that contribute to the abuse of drugs, including ethanol. This limbic forebrain region is divided functionally into subdivisions that serve to organize inputs from various cortical and subcortical areas along with specific outputs to a wide array of central nervous system structures. The basolateral subdivision receives inputs from prefrontal cortex (Brinley-Reed et al., 1995), lateral entorhinal cortex (McDonald and Mascagni, 1997), and processes sensory information from the lateral amygdala (Pitkanen et al., 1997). It subsequently sends projections to the bed nucleus of the stria terminalis, ventral striatum, medial hypothalamus (McDonald and Culberson, 1986), the central amygdala (Pitkanen et al., 1997), and reciprocally to the prefrontal cortex (McDonald, 1987). This anatomical organization places the basolateral subdivision as a nexus in the integration of sensory/memory/cognitive information with areas that regulate psychological and physiological responses to emotionally relevant external cues. Not surprisingly, these responses are ultimately regulated by the balance between excitatory and inhibitory neurotransmitter systems.

Ligand-gated chloride channels such as the GABA<sub>A</sub> receptor are central regulatory elements in the basolateral amygdala. In rodents, it is well established that experimental manipulation of these receptors has profound behavioral consequences, from altering affective states (Sanders and Shekhar, 1995) to regulating drug discrimination (Hodge and Cox, 1998). GABA<sub>A</sub> receptors are multimeric complexes consisting of several related protein subunits. In general, these recep-

ABBREVIATIONS: RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, analysis of variance.
tors contain at least one $\alpha$ and one $\beta$ subunit, but a large population of receptors also contains a $\gamma$ subunit (Pritchett et al., 1989). Importantly, different combinations of subunits confer distinct pharmacological and channel properties on the receptor complex. For example, there is some suggestion that specific subunits may play an important role in conferring acute ethanol sensitivity on the GABA$_A$ receptor (Sundstrom-Poromaa et al., 2002). There is also an extensive literature indicating that chronic ethanol exposure leads to changes in GABA$_A$ receptor subunit composition and function (Mhatre et al., 1993; Devaud et al., 1997). In the context of the current manuscript, chronic exposure to an ethanol-containing liquid diet leads to alterations in subunit protein expression in a rat extended amygdala preparation (Papadeas et al., 2001). A similar exposure also enhances GABA-gated currents and decreases the apparent potency of GABA when measured using acutely isolated lateral/basolateral amygdala neurons (McCool et al., 2003), suggesting that alterations in subunit expression and receptor function may regulate pharmacological adaptations to chronic ethanol exposure. Because GABA$_A$ receptors have been strongly implicated in both alcohol self-administration (Hodge et al., 1995) and in amygdala-dependent anxiety behaviors, adaptations of basolateral amygdala GABA$_A$ receptors to chronic ethanol may be key for the behavioral consequences of long-term alcohol self-administration.

Ethanol exposure in rodents has been widely used to mimic various behavioral or neurobiological characteristics of human alcoholism, yielding considerable insight into the cellular and molecular events that underlie adaptations to chronic ethanol. However, the short life span of rodents makes the study of long-term ethanol exposure over months to years impractical. Furthermore, it has been difficult to train rodents to self-administer large daily levels of ethanol or to establish daily drinking patterns that are similar to those achieved by human alcoholics. Nonhuman primates provide unique research opportunities in this regard. Macaque monkeys have extensive capacities for complex cognitive behavior; and, their physiological, behavioral, and neuroanatomical similarities to humans also facilitate translation of findings in experimental primate models to human disease, including alcoholism. Importantly, cynomolgus macaques ($Macaca fascicularis$) will freely self-administer intoxicating quantities of ethanol with drinking patterns that mimic human alcoholics (Vivian et al., 2001). These nonhuman primates can therefore form a critical link between human clinical research and more fundamental studies in rodents.

### Materials and Methods

**Ethanol Exposure.** For functional studies, tissue was obtained from eight control animals (four males and four females) or six animals (two females and four males) that self-administered ethanol. For the molecular biology studies, additional tissue from the lateral aspect of the basolateral amygdala was obtained from a second cohort of ethanol-exposed animals that included two males and two females. All ethanol-exposed monkeys were trained to self-administer ethanol with a 3-month induction procedure, followed by 6 months of ethanol self-administration as described previously (Vivian et al., 2001). After this exposure, all “ethanol” monkeys were then given 12 months of abstinence from ethanol and then allowed to self-administer 4% ethanol (w/v) 22 h each day for 18 consecutive months with water and food ad libitum. These animals drank an average of 385 ± 57 g/kg ethanol over the 6 months before necropsy (range 169–680 g/kg, median 321 g/kg), with 18-month self-administration totals reaching 1086 ± 177 g/kg (range 587–2168 g/kg, median 1047 g/kg). These correspond to an average daily consumption of approximately 2 g/kg/day. This value is very similar to that reported for these same animals for the first 180 days of self-administration (Vivian et al., 2001), suggesting that self-administration by these monkeys is remarkably stable for months to years. Control animals were housed similarly to the ethanol-drinking monkeys and had free access to food and water.

**Slice Preparation.** Tissue from ethanol-exposed animals was obtained during necropsy immediately after their daily 22 h access to ethanol. All animals were sedated with ketamine (15 mg/kg i.m.) and brought to a deep surgical plane of anesthesia with intravenous pentobarbital administered to effect (20–40 mg/kg i.v.). After a partial craniotomy, animals were perfused through the left ventricle/ascending aorta with ice-cold, oxygenated Ringer’s solution (125 mM NaCl, 4.5 mM KCl, 1 mM MgCl$_2$, 26 mM NaHCO$_3$, 1.2 mM NaH$_2$PO$_4$, 10 mM D-glucose) for 2 min. The brain was removed, placed in an acrylic form, and sectioned according to topographical landmarks. Intact amygdala was rapidly dissected from temporal lobe. Coronal slices (400 μM) were made along the entire rostral/caudal extent of the basolateral nucleus in modified Ringer’s (194 mM sucrose and 30 mM NaCl replacing 125 mM NaCl in the standard solution) containing 10 μM ketamine at 4°C. Slices were stored in oxygenated standard Ringer’s solution containing 10 μM ketamine. The basolateral amygdala was immediately dissected from two to three coronal slices, flash frozen in liquid nitrogen, and stored at −80°C for the preparation of RNA. The remaining slices were stored for up to 12 h and used to acutely isolate individual basolateral amygdala neurons for electrophysiology experiments.

**Neuron Isolation and Whole-Cell Recordings.** Individual basolateral amygdala neurons were isolated from macaque brain slices with established methods (Floyd et al., 2003; McCool et al., 2003). Briefly, basolateral amygdala was dissected from the coronal slices and incubated at 35°C in Ringer’s solution containing 0.6–1.0 mg/ml Pronase protease (EMD Biosciences, San Diego, CA) for 20 min at 37°C with constant oxygenation. Tissue was then rinsed in isolation buffer (130 mM N-methyl-d-aspartate-mediated glutamatergic synaptic transmission in cynomolgus dentate gyrus are similar to those reported for rodent dentate in side-by-side comparisons (Ariwodola et al., 2003)). Similarly, chronic ethanol exposure in these monkeys was recently shown to increase dopamine clearance and enhance the efficacy of the dopamine D2 autoreceptor agonist quinpirole in the ventromedial caudate (Budygin et al., 2003). To date, however, there are no reports describing the impact of long-term ethanol self-administration on GABA receptor function in any primate species. In addition, neither the basic pharmacological properties of the receptors nor the functional/molecular consequences of chronic ethanol exposure for GABA$_A$ receptors have been reported for any nonhuman primate. We therefore examined the effects of long-term ethanol self-administration in cynomolgus macaques to better understand the relationship between chronic ethanol and amygdala neurobiology in the context of a primate model of human alcoholism.

After allowing neurons to adhere to the coverslip, we continuously perfused them with a HEPES-buffered external solution (140 mM...
was performed on 2 to 10 ng DNase I (QIAGEN, Valencia CA). The reverse transcription reaction was performed in three separate experiments for each sample. Determinations for each gene product were run in triplicate in a single plate to insure that they were directly comparable. Determi-nations from total cynomolgus thalamic RNA were run on the same plates as the samples to be assayed that were directly comparable. Determinations for each gene product were run in triplets in a single experiment. Expression values represent the mean ± S.E.M. for two to three separate experiments for each sample.

Results

Long-Term Ethanol Self-Administration Decreases GABA Potency. Most of the neurons acutely isolated from cynomolgus macaque basolateral amygdala morphologically resembled the "principle" pyramid-shaped neurons isolated from rat and mouse lateral/basolateral amygdala preparations (Fig. 1A; McCool et al., 2003). Currents elicited by GABA application to these neurons were robust and possessed activation and desensitization kinetics that were dependent upon GABA concentration (Fig. 1, B and C). To analyze the concentration-response data from different treatment groups, current amplitudes (picoampere, pA) at the apparent peak ("peak") of the response and 2.5 to 3 s after the apparent peak ("plateau") were normalized to cell capacitance (picofarad; pF) and expressed as a current density (pA/pF).

Table 1

<table>
<thead>
<tr>
<th>Gene Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
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<tbody>
<tr>
<td>β2-Microglobulin</td>
<td>CACCCCCCATGAAAAAGTGA</td>
<td>CTGGGCCCTTGACAAAGTCA</td>
</tr>
<tr>
<td>α1</td>
<td>GGATCTCTTATTAAGAAAAACACCTT</td>
<td>GGTCCACCCCTGGCCCAAAAT</td>
</tr>
<tr>
<td>α2</td>
<td>CAAATTTGCTGCTGTTTACAG</td>
<td>AAACAAAAAGCACAAACATT</td>
</tr>
<tr>
<td>α3</td>
<td>TGCAAATAATTTCCCGCATCA</td>
<td>TGCCCCGATGACCAAGATAGA</td>
</tr>
<tr>
<td>α4</td>
<td>GGTCTACTGGGAATJGGCTACCTA</td>
<td>TTATTCTTGCGGATAGCAG</td>
</tr>
</tbody>
</table>

Table 1. Primers and probes used for real-time RT-PCR of macaque basolateral amygdala GABA<sub>α</sub> receptors

Sequences for these primers and probes were obtained for cynomolgus macaque from the following GenBank entries: β2-microglobulin, AF485817; GABA<sub>α</sub> α1 subunit mRNA, AFY394495; GABA<sub>α</sub> α2 subunit, AFY394494; GABA<sub>α</sub> α3 subunit, AFY394495; and GABA<sub>α</sub> α4 subunit, AFY394496.
for each cell were derived from fits of data from individual cells with a standard logistic equation (Floyd et al., 2003). For the peak of the response, logEC50 values indicate that a substantial right-ward shift in GABA potency is associated with long-term ethanol self-administration. The mean logEC50 values were $-4.23 \pm 0.11$ for neurons ($n = 17$) from control animals and $-3.64 \pm 0.14$ for ethanol neurons ($n = 15$; Fig. 2B; $P < 0.01$; $t$ test). These values translate to an apparent GABA potency of $53 \, \mu M$ for neurons isolated from control monkeys and $164 \, \mu M$ for neurons from ethanol-exposed monkeys. Neither the maximal current density of the peak response at 1 to 3 mM GABA nor the Hill slope ($n_H$) were significantly different between control and ethanol neurons ($P > 0.05$; $t$ test). Analysis of the current densities 2.5 to 3 s after the apparent peak, during the plateau phase of the current response, revealed a similar shift in GABA potency resulting from long-term ethanol self-administration (Fig. 2, A and C). The logEC50 values for this nondesensitizing/slowly desensitizing component were $-4.63 \pm 0.13$ for control neurons and $-4.02 \pm 0.12$ for ethanol neurons ($P < 0.01$; $t$ test). These values are equivalent to an EC50 of $23 \, \mu M$ and $95 \, \mu M$ for control and ethanol cells, respectively. Neither the maximal plateau current density (1–3 mM) nor the apparent Hill slope were significantly different between neurons derived from these treatment groups ($P > 0.05$; $t$ test).

Comparing between animals instead of cells, peak and plateau GABA responses were also significantly less potent in ethanol-exposed monkeys compared with control individuals ($P < 0.001$; $t$ test on logEC50; data not shown). Control males and females had peak logEC50 values of $-4.30 \pm 0.111$ and $-4.32 \pm 0.08$, respectively, whereas drinking males and females had peak values of $-3.54 \pm 0.05$ and $-4.08 \pm 0.16$. Two-way ANOVA analysis using gender and treatment as dependent variables revealed a significant interaction between these variables for the peak response (Fig. 2D; $P = 14.2$; $P < 0.05$) for the interaction between gender and treatment; two-way ANOVA, suggesting that peak GABA potency was sensitive to ethanol in a gender-specific manner.

Long-Term Ethanol Self-Administration May Alter Current Desensitization Kinetics. Chronic ethanol exposure has been shown to dramatically influence the apparent desensitization kinetics of GABAergic, tetrodotoxin-resistant synaptic events (Cagetti et al., 2003). We examined whether long-term self-administration had similar effects by fitting
the desensitizing phase of maximal current responses (1–3 mM GABA) from each cell to a two-component exponential equation (Fig. 3A; Bianchi and Macdonald, 2002). Current responses with onset times >80 ms were presumed to represent slow solution exchange and were omitted from this analysis because substantial desensitization was likely to have occurred before the apparent peak of the response. The time constants, \( \tau_{\text{Fast}} \) and \( \tau_{\text{Slow}} \), were derived from fits in individual cells from the apparent peak of the current response to the plateau phase of the current (3–4 s after the peak). When analyzed across treatment groups, neither \( \tau_{\text{Slow}} \) (Fig. 3B) nor \( \tau_{\text{Fast}} \) were significantly affected by long-term ethanol self-administration. \( \tau_{\text{Fast}} \) was 366 ± 43 ms in control neurons (n = 18) and 345 ± 24 ms in ethanol neurons (n = 21; \( P > 0.05; t \) test). Similarly, \( \tau_{\text{Slow}} \) values were 1537 ± 115 ms in control neurons and 1647 ± 73 ms in neurons from ethanol-exposed animals (\( P > 0.05; t \) test). When the relative amplitudes of the two components were compared between treatment groups, there was a trend for the “fast” component to be more prominent in the ethanol-drinking monkeys. When expressed as a percentage of the total current amplitude, fast component contributions were 19 ± 2% of the total current amplitude in control neurons compared with 24 ± 3% in neurons from ethanol exposed animals (Fig. 3C; \( P < 0.1; t \) test). The normalized area under the curve during the first second of the current response was greater in control neurons compared with 24% in ethanol neurons (Fig. 3D; \( P < 0.05; t \) test). There were no apparent gender differences in this dataset and no significant correlations between the amount of alcohol consumed (last 6 months or entire 18 months self-administration) and the component contributions. Together, these results suggest that long-term ethanol self-administration has a modest influence on current desensitization, possibly by increasing the contribution of the fast component.

**Long-Term Ethanol Self-Administration Alters the Expression of Specific GABA\(_{\alpha}\) Subunit mRNAs.** Results in the previous sections indicate that long-term ethanol self-administration by cynomolgus macaques may substantially alter the pharmacological and functional properties of GABA\(_{\alpha}\) receptors expressed by basolateral amygdala neurons. To understand potential contributions by mRNA expression of the different \( \alpha \) subunits in this process, we examined macaque GABA\(_{\alpha}\) \( \alpha \) subunit mRNA expression using real-time RT-PCR. Importantly, total RNA from cynomolgus basolateral amygdala seemed to express only \( \alpha 1–4 \) subunits (Fig. 4A). To develop reagents for real-time RT-PCR, cDNAs corresponding to unique regions within each of these subunits were cloned and sequenced (GenBank accession nos. AY394493–AY394496). The expression level of each subunit within individual samples was measured using the 5exonuclease assay (Fig. 4B; Floyd et al., 2003) and normalized to levels of the ubiquitous mRNA for \( \beta_2 \)-microglobulin to control for experimenter error or differences in RNA quality between individual samples. We were able to add four additional basolateral amygdala samples from ethanol-drinking monkeys to these studies (2 male and 2 female) from fresh frozen tissue isolated from an earlier cohort subjected to identical self-administration procedures. Importantly, the levels of \( \beta_2 \)-microglobulin, expressed as a mass or “nanograms of RNA” equivalent in a given amount of our thalamic RNA standard (Floyd et al., 2003), were 3.7 ± 0.2 in control monkeys (n = 6) and 3.5 ± 0.2 in ethanol-exposed monkeys (n = 9; \( P > 0.05; t \) test; 8–10 replications per sample). Thus, long-term ethanol

![Fig. 3](https://via.placeholder.com/150)

**Fig. 3.** Long-term ethanol self-administration effects on GABA current apparent desensitization kinetics. A, this sample trace from a control neuron shows the two-component exponential fit (dashed line) to the desensitizing phase of the current response during the continued presence of 3 mM GABA (open bar above trace). The time constants \( \tau_{\text{Fast}} \) and \( \tau_{\text{Slow}} \) were 1580 and 340 ms for this trace, respectively. The relative contributions of “slow” and fast components in this cell were 87 and 23 pA/pF (normalized to cell size), respectively. B, ethanol exposure did not affect the apparent time constants or component amplitudes when fits were averaged across treatment groups. The time constant for the slow component (left) and fast component (right) is shown. C, relative contribution of the fast component is modestly decreased in monkeys self-administering ethanol (\( \pm ; P < 0.10 \), control versus ethanol neurons; \( t \) test). Here the fast component is expressed as a percentage of the total current (normalized to cell size; pA/pF) contributed by both fast and slow components (\( \Delta A_{\text{Fast}} \)). D, area under the curve (pA · s/pF) during the first second of the current response was significantly smaller in neurons from ethanol-exposed animals compared with control neurons (\( \pm ; P < 0.05; t \) test).
RNA levels for each of the subunits expressed in basolateral amygdala (α1–4) were quantified in control and ethanol-exposed individuals (Table 2). Across all individuals, only the expression levels of the α2 and α3 subunits were significantly decreased by long-term ethanol self-administration. Normalized levels of α2 expression were decreased by ~25% (Fig. 5B), from 100 ± 6% in control animals to 75 ± 8% in ethanol-exposed macaques (P < 0.05; t test). Similarly, α3 mRNA levels decreased from 100 ± 12 to 48 ± 9%, α > 50% change (Fig. 5C; P < 0.01; t test). α1 mRNA levels also seemed to decrease by ~30% from 100 ± 12 to 69 ± 13% (Fig. 5A). However, the changes in expression of this subunit only approached statistical significance, possibly due to substantial variance in the expression of this subunit in our samples. Levels of α4 mRNA decreased by only ~13% from 100 ± 5% in controls to 87 ± 9% in ethanol-exposed individuals (Fig. 5D; P > 0.05; t test).

When data were analyzed with two-way ANOVA across gender and treatment, there was not any significant effect of gender or any significant interaction between gender and ethanol exposure for any of the subunits examined. We also attempted to correlate relative levels of ethanol consumption with levels of mRNA expression. For the ethanol drinking monkeys, the mean total consumption during the 6 months before necropsy was 385 ± 64 g/kg with a range from 169 g/kg in the “lightest” drinker (a female) to 680 g/kg in the heaviest drinker (a male). The mean relative expression levels by a given animal (2–3 replicates) was significantly correlated with the total amount of ethanol consumed for both the α2 subunit (P < 0.05; Pearson R² = 0.31) and the α3 subunit (P < 0.01; Pearson R² = 0.43).

Discussion

Despite numerous structural and behavioral studies on GABA receptors in primates, our data are the first functional measures of GABA receptors in these models. A major finding was that long-term ethanol self-administration by the cynomolgus macaque significantly shifts GABA potency, but not the efficacy, for basolateral amygdala GABA receptors. This shift occurred for both the peak and plateau components (measured 2.5–3 s after the peak) of the GABA-gated current. The plateau current also possessed a higher apparent affinity (measured 2.5–3 s after the peak) of the GABA-gated current.

When data were analyzed with two-way ANOVA across gender and treatment, there was not any significant effect of gender or any significant interaction between gender and ethanol exposure for any of the subunits examined. We also attempted to correlate relative levels of ethanol consumption with levels of mRNA expression. For the ethanol drinking monkeys, the mean total consumption during the 6 months before necropsy was 385 ± 64 g/kg with a range from 169 g/kg in the “lightest” drinker (a female) to 680 g/kg in the heaviest drinker (a male). The mean relative expression levels by a given animal (2–3 replicates) was significantly correlated with the total amount of ethanol consumed for both the α2 subunit (P < 0.05; Pearson R² = 0.31) and the α3 subunit (P < 0.01; Pearson R² = 0.43).

**TABLE 2**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Chronic Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>α2</td>
<td>5.6 ± 0.3</td>
<td>4.2 ± 0.5**</td>
</tr>
<tr>
<td>α3</td>
<td>6.9 ± 0.5</td>
<td>3.8 ± 0.7**</td>
</tr>
<tr>
<td>α4</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

* n = 8 for control and 9 for ethanol animals.
* P < 0.05 and ** P < 0.01; two-tailed t test.
tion may lack the tors that have high agonist affinity and low/no desensitization. Forty days of forced-ethanol the length of exposure to ethanol can substantially influence cies are problematic and have not been reported. However, paradigms. Direct comparisons between these particular spe-
cific responses to chronic ethanol exposure; or the differences between these studies reflect the distinct ethanol-exposure rods and nonhuman primates represent true species-spe-
cific adaptations to chronic ethanol exposure, which are not identical. At present, we cannot distinguish two characteristic adaptations to chronic ethanol exposure, different sensitivi-
ties to allosteric modulators (Lim and Birnir, 2001), suggesting that distinct receptor populations with unique subunit combinations may be responsible for these distinct phases of the current response. Indeed, plateau phase GABA_A receptors that have high agonist affinity and low/no desensitization may lack the γ2 subunit (Dominguez-Perrot et al., 1996) and/or contain the δ subunit (Hevers et al., 2000). The impact of long-term ethanol exposure on these particular subunits remains to be investigated in our monkey model.

Treatment-dependent shifts in GABA potency are not unique to this monkey model. A similar shift in GABA potency was also found when rat lateral/basolateral amygdala neurons were examined during a 2-week exposure to an ethanol-containing liquid diet (McCool et al., 2003). However, agonist efficacy was also significantly enhanced in these rats after this exposure paradigm (Papadeas et al., 2001; McCool et al., 2003). Although these species therefore share some of the characteristic adaptations to chronic ethanol exposure, they are not identical. At present, we cannot distinguish two alternative interpretations: the subtle differences between rodents and nonhuman primates represent true species-specific responses to chronic ethanol exposure; or the differences between these studies reflect the distinct ethanol-exposure paradigms. Direct comparisons between these particular species are problematic and have not been reported. However, the length of exposure to ethanol can substantially influence GABA_A receptor adaptations. Forty days of forced-ethanol liquid diet, but not 14 days, causes significant changes in α4 peptide levels in rat hippocampus (Matthews et al., 1998). Similarly, 12 weeks of liquid-diet ethanol exposure, but not 1 to 4 weeks, reduces rat hippocampal α1 subunit peptide and mRNA levels (Charlton et al., 1997). Despite these indications, it is equally possible that GABA_A adaptations to ethanol self-administration are distinct from those found in forced exposure paradigms. The primate model used in these studies could provide the best opportunity to address such issues.

In addition to functional alterations in basolateral amygdala GABA_A receptors, long-term ethanol self-administration by cynomolgus macaques has robust effects on the mRNA expression levels of some GABA_A α subunits in this brain region. Our data are the first measurement of GABAA mRNA changes in response to ethanol exposure in monkeys. Both α2 and α3 levels were statistically significant in ethanol-exposed animals. A similar trend was noted for the α1 subunit although this did not reach statistical significance in our particular cohort of animals. Importantly, α4 mRNA levels were only slightly depressed in ethanol-exposed monkeys. These latter findings are similar to the lack of change in α4 mRNA levels reported in the frontal cortex of human alcoholics (Mitsuyama et al., 1998). Long-term ethanol self-administration by these monkeys therefore seems to selectively reduce expression of α2, α3, and perhaps α1 mRNAs without substantially influencing α4

**Fig. 5.** Long-term ethanol self-administration by cynomolgus macaques decreases the expression of GABA_A α subunits. The relative expression level of α subunit mRNAs in basolateral amygdala total RNA was normalized to β2-microglobulin expression in the same sample. “Percent control” values were obtained by normalizing these relative expression levels for each sample to the mean expression value of a given subunit obtained from control samples measured in a given experiment. A, expression of GABA_A α1 mRNA was depressed by ~30% in animals that self-administered ethanol, but these levels only approached statistical significance in these particular samples (vs. P < 0.1; t test). B and C, GABA_A α2 and α3 subunit mRNAs were significantly decreased in basolateral amygdala RNA isolated from ethanol-drinking monkeys (vs. P < 0.05; **, P < 0.01; t test). Expression levels were decreased by ~52% and ~52% for α2 and α3, respectively. D, long-term ethanol self-administration did not substantially affect the mRNA expression of the α4 subunit (13% decrease, P > 0.05).

![Graph A](image1.png) ![Graph B](image2.png) ![Graph C](image3.png) ![Graph D](image4.png)
expression. Unfortunately, mRNA expression response of GABA<sub>α</sub> subunits to chronic ethanol in rat lateral/basolateral amygdala has not yet been reported. However, there are numerous reports of mRNA measures in several rodent brain areas; and, region-to-region variability in mRNA responses to chronic ethanol is a frequent finding (Grobin et al., 2000). In general, most rodent studies have reported significant decreases in α1 mRNA levels and minor or no effects on α2 and α3 mRNA (Grobin et al., 2000). Our mRNA findings in primates therefore seem distinct from adaptations described in many rodent forebrain regions. We can better appreciate the impact of alterations in α2 and α3 mRNA levels by comparing expression values without normalizing to β<sub>2</sub>-microglobulin. α2 and α3 subunit mRNAs were expressed 4 to 5 times higher in the basolateral amygdala compared with our thalamic RNA standard, whereas α1 subunit mRNA levels were about 50% of the thalamic standard and α4 expression was approximately equivalent. Given that the relative levels of α subunit mRNAs in rhesus monkey lateral geniculate are α1 ≫ α2 = α3 = α4 (Huntsman et al., 1996), we propose that the relative levels of expression in control cynomolgus macaque basolateral amygdala are approximately α1 = α2 = α3 > α4, similar to the rank order of expression found in rat lateral/basolateral amygdala (Wisden et al., 1992). Large decreases in α2 and α3 subunit mRNAs would therefore have a major impact on GABA<sub>α</sub> subunit expression in general. These adaptations in mRNA levels may be the result of subunit-specific changes in mRNA transcription or mRNA stability.

The functional consequences of changes in relative mRNA expression are difficult to predict until we know more about potential alterations in polypeptide expression. Because GABA efficacy was not decreased in ethanol-drinking monkeys, the suppression of mRNA levels for the α2 and α3 subunits may not directly reflect their functional subunit contributions to whole-cell currents. Indeed, the decrease in GABA potency in these same animals might suggest a larger contribution by “low-affinity” α subunits like α3 and α4 (Smith et al., 2001) in the ethanol-exposed neurons. This is somewhat at odds with the profound decrease in α3 mRNA. We cannot rule out the possibility that mRNA measures would include non-neuronal cell types that have different sensitivities to chronic ethanol. However, it seems more reasonable to suggest that changes in mRNA expression for some subunits may not directly relate to, or even be opposite to, changes in their functional contributions. Chronic ethanol-induced alterations in receptor trafficking seem to be subunit-dependent in rodents (Kumar et al., 2003). Similarly, chronic ethanol exposure in rodents can influence the association between specific GABA<sub>α</sub> subunits and PKC<sub>γ</sub> (Kumar et al., 2002). Together with our findings, this raises the possibility that receptor phosphorylation or subunit-specific adaptations in receptor trafficking may play a significant role in the functional adaptations to long-term ethanol self-administration in nonhuman primates.

An important consideration in the present work is that our data have included neurons from both males and females. Several studies have failed to find significant differences between the sexes with regard to either absolute levels of GABA<sub>α</sub> receptors or their sensitivity to allosteric modulators. Indeed, there is no influence of gender on total muscimol binding in the rat amygdala (Davis and McCarthy, 2000), GABA<sub>α</sub> current density in monkey amygdala (this study), or benzodiazepine binding affinity in rat amygdala (Farabollini et al., 1996). However, gender-specific effects on α4 subunit protein expression were noted during withdrawal from chronic progesterone treatment (Gulinello et al., 2003). Furthermore, females are more sensitive than males to the anticonvulsant effects of the neurosteroid 3α,21-dihydroxy-5 α-pregnann-20-one during withdrawal from chronic ethanol (Devaud et al., 1998). Given the small number of “drinking” females in our studies, it is difficult for us to provide conclusive evidence of any gender-specific adaptation to ethanol exposure using the various functional and molecular parameters measured here. Regardless, decreases in GABA potency resulting from ethanol exposure were clearly more pronounced in males compared with females, this despite comparable GABA sensitivities in control neurons. However, females in our studies ingested a total of only 215 ± 46 g/kg for the functional study (n = 2) and 293 ± 71 for all females in the molecular study (n = 4) during the last 6 months before necropsy. This is in contrast with males who self-administered 396 ± 99 (functional studies; n = 4) or 459 ± 82 g/kg (molecular studies; n = 5) during this same period. Given that logEC<sub>50</sub> was significantly correlated with the total grams per kilogram during this period, it is possible that the apparent gender-specific effects of ethanol self-administration on GABA potency may be directly related to consumption.

In summary, acutely isolated basolateral amygdala neurons from cynomolgus macaque express GABA-gated currents that adapt to long-term ethanol self-administration. These adaptations include decreased GABA potency, but no change in efficacy, and alterations in apparent current desensitization kinetics. Although the levels of some α subunit mRNAs are profoundly decreased by this ethanol exposure, changes in mRNA expression are not robustly gender-specific and are not necessarily representative of any change in absolute levels of GABA receptor functional levels. Because acute ethanol facilitates function at some GABAergic synapses (Weiner et al., 1997) and this facilitation does not adapt during chronic ethanol in either rodents (Kang et al., 1998) or in our nonhuman primate model (J. L. Wiener, personal communication), decreases in GABA potency may be most relevant during withdrawal from chronic exposure where impaired/decreased GABAergic function would further upset the balance between excitation and inhibition. In the context of the amygdala, this imbalance could regulate the expression of fear/anxiety, emotions that influence (Kushner et al., 2000) the longitudinal severity of alcohol abuse.

References


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