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Long-term Ethanol Self-Administration by the Non-Human Primate, *Macaca fascicularis*, Decreases the Benzodiazepine Sensitivity of Amygdala GABA_A Receptors

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Abstract

Background—Rodent models of chronic alcohol exposure are typically constrained to relatively short periods of forced ethanol due to the lifespan of these animals. Non-human primate models, particularly those employing long-term self-administration, are conceptually more similar to human alcoholics.

Methods—We performed whole-cell patch clamp recordings on acutely dissociated amygdala neurons isolated from cynomolgus macaque coronal temporal lobe slices. Slices were prepared from control monkeys or monkeys allowed to self-administer oral ethanol for eighteen months. Flunitrazepam and acute ethanol modulation of currents gated by exogenous GABA application was assessed in these isolated neurons. Complementary experiments were performed on amygdala total RNA using quantitative real-time reverse transcription/polymerase chain reaction to understand potential ethanol-dependent adaptations to subunit composition.

Results—GABA-gated currents from ethanol-exposed macaque amygdala neurons exhibited reduced modulation by flunitrazepam compared to control neurons. However, this was specific for benzodiazepines since the modest inhibition of GABA-gated currents by acute ethanol was not affected by the chronic ethanol consumption. We also measured mRNA expression levels for the β , γ , and δ subunits in total amygdala RNA isolated from control and ethanol-drinking animals. β_1 and γ_2 expression was significantly reduced in samples from ethanol-exposed amygdala.

Conclusions—Our findings demonstrate that chronic ethanol self-administration reduces the benzodiazepine sensitivity of amygdala GABA_A receptors. This reduced sensitivity may be the result of decreased expression of an amygdala γ subunit. These findings suggest that, while rodent and non-human primate models of chronic ethanol exposure share many characteristics, the specific molecular adaptations associated with the amygdala GABAergic system may not be identical.

Keywords

whole-cell patch-clamp electrophysiology; real-time RT-PCR; flunitrazepam; isolated neurons

Introduction

Animal models of chronic ethanol exposure yield insight into the cellular and molecular mechanisms governing ethanol abuse. While offering the greatest experimental flexibility, rodent models of chronic ethanol exposure generally depend on non-contingent ethanol administration like oral gavage, liquid diets, injections, or vapor inhalation. This is because out-bred strains of rodents do not typically self-administer quantities that parallel daily ethanol intakes achieved by human alcoholics. Furthermore, alcoholics typically ingest large quantities of ethanol over many years, a time-span impossible to achieve with rodent models of chronic ethanol exposure. In this regard, a recently described non-human primate model of chronic ethanol exposure mitigates many of the rodent-model short-comings by allowing the animals to self administer large quantities of ethanol continuously over 12–18 months (Vivian et al., 2001). This model could therefore potentially provide an important “translational bridge” (Grant and Bennett, 2003) between human and rodent studies of chronic ethanol exposure. However, characterization of ethanol-dependent neural adaptations to chronic ethanol in the non-human primate model has just begun.

The lateral and basolateral subdivisions (BLA) represent the primary input nuclei of the amygdala. Together, they integrate processed sensory information, memory, and executive processes before projecting to regions like the nucleus accumbens (Friedman et al., 2002) and prefrontal cortex (Porrino et al., 1981). Not only do these projections help govern the acquisition of traditional fear conditioning (Muller et al., 1997), the lateral and basolateral amygdala also appear to help regulate drug seeking behaviors (Di Ciano and Everitt, 2004; Lee et al., 2005; Rizos et al., 2005; See, 2005). While a multitude of neurotransmitters appear to be involved with these amygdala-dependent behaviors, modification of the balance between excitatory glutamatergic and inhibitory GABAergic transmission in the BLA is likely to be the ultimate mechanism by which these complex outcomes are established (see (Quirk and Gehlert, 2003)).

As in other brain regions, amygdala GABA_A receptors appear to be multi-subunit complexes composed of distinct family members consisting at least of α and β subunits, and typically associated with γ or δ subunits as well (Benke et al., 1996; Fritschy and Mohler, 1995). Unique subunit combinations confer unique pharmacological sensitivity to the native receptor. For example, the combination of specific α subunits with a γ subunit appears to be required for formation of functional benzodiazepine binding sites (Pritchett et al., 1989). Acute sensitivity to ethanol may also be influenced by the presence of the δ -subunit (Wallner et al., 2003; Wei et al., 2004) although this has not been consistently observed (Borghese et al., 2006; Yamashita et al., 2006).

The GABAergic system is dynamic and has been found frequently to adapt to chronic ethanol exposure. More robust rodent models of chronic ethanol exposure have found pronounced behavioral tolerance or sensitization to various GABA_A-acting modulators (Becker et al., 1998; Cagetti et al., 2003; Finn et al., 2000; Masur and Boerngen, 1980). In some brain regions, the behavioral adaptations are associated with decreased sensitivity of GABAergic synaptic transmission to allosteric modulators (Cagetti et al., 2003), altered potency of GABAergic agonists (McCool et al., 2003), and altered probability of neurotransmitter release (Roberto et al., 2004). These functional adaptations are frequently mirrored by pronounced alterations of GABA_B subunit mRNA and protein expression (Cagetti et al., 2003; Devaud et al., 1997; Papadeas et al., 2001).

Although primate models of chronic ethanol have not been as extensively characterized, we recently reported that long-term ethanol self-administration caused a decrease in apparent agonist potency at amygdala GABA_A receptors that was associated with decreased α_2 and α_3

subunit mRNA expression (Floyd et al., 2004). The expression of several α , β , and γ subunits was also altered by chronic ethanol in several prefrontal cortical areas in this same model (Hemby et al., 2006). Together, these molecular biological findings suggest that the GABAergic system of non-human primates may be as dynamic as that found in rodents, although it is not clear how many parallels may exist. To address this, the current study was undertaken to specifically examine the effects of long-term ethanol self-administration on allosteric modulation and β/γ subunit expression of amygdala GABA_A receptors in cynomolgus macaque.

Materials and Methods

Animals

The animals used for this study are the same as those described previously that showed decreased agonist potency at amygdala GABA_A receptors with concurrent decreased α_2 and α_3 subunit mRNA expression (Floyd et al., 2004). For the studies reported here, tissue from a total of 10 ethanol-exposed monkeys (6 males, 4 females) was prepared (see below) for either electrophysiology (n=4 males and 2 females) or molecular biology (n = 5 males and 4 females). With the exception of one additional male, the animals used in the electrophysiology experiments were also used for the molecular assessments. The lifetime administration of ethanol (g/kg) over a total of 24 months of access to 4% (w/v) ethanol, an average daily intake over the last 6 months prior to euthanasia, blood-ethanol data, and monkeys contributing to the molecular biology and electrophysiology are indicated in Table 1. While this manuscript was under review, another study describing drinking data from many of these same individuals was published (Hemby et al., 2006). These data are provided here to facilitate comparisons with the current work and to illustrate novel individuals used in the current study. Regardless, the lifetime consumption for the animals used in the electrophysiology experiments was 1297 ± 320 g/kg and was not significantly different from the lifetime consumption by all animals in this manuscript (1479 ± 219 g/kg; $P > 0.05$ with Student's t-test). For BEC determinations, blood samples (20 μ l) were taken from the saphenous vein using awake venipuncture every fifth day from every monkey just prior to the lights turning off in the room and approximately 7 hr following the onset of the drinking session (see (Vivian et al., 2001) for details). Blood samples were sealed in air-tight vials containing 0.5 ml of distilled water and 0.02 ml of isopropanol (10%; internal standard), and stored at -4°C until assayed using gas chromatography (Hewlett-Packard 5890 Series II, Avondale, PA, equipped with a headspace autosampler, flame ionization detector, and a Hewlett Packard 3392A integrator).

The control macaques used in the current study are the same as those described previously (Ivester et al., 2007). Briefly, adult macaques were purchased from a commercial vendor (Primate Products) and, following two months of quarantine, were individually housed. Control animals were placed on the same diet as the ethanol-drinking animals (Vivian et al., 2002) and had very similar daily routines to the ethanol-drinking animals. Control individuals remained in the laboratory for six months prior to euthanasia.

Brain Slice Preparation and Neuron Isolation

Coronal slices from a single temporal lobe containing the lateral and basolateral amygdala were obtained within 20 minutes of sacrifice as previously described (Floyd et al., 2004). Slices were incubated in artificial cerebrospinal fluid (aCSF, in mM: 124 NaCl, 2.5 KCl, 1.5 MgCl_2 , 26 NaHCO_3 , 1.2 NaH_2PO_4 , 10 Glucose, bubble constantly with 95% O_2 /5% CO_2) for <1hr (molecular biology) or up to 10hr (electrophysiology) after preparation.

Individual neurons were obtained from slices by incubation aCSF containing 0.6-1mg/ml Pronase protease (CalBiochem) at 37°C for 20min followed by mechanical trituration with

fire-polished Pasteur pipettes as described previously (Floyd et al., 2004; McCool and Botting, 2000). Individual acutely isolated neurons were plated onto Alcian-blue coated coverslips and allowed to attach to the substrate for 5 minutes before beginning the electrophysiology experiments. For preparation of total RNA, individual subdivisions were dissected, flash frozen on dry ice, and stored at -80°C until use.

Whole-cell Patch-Clamp Electrophysiology

Following isolation, neurons were continuously perfused with HEPES-buffered saline containing (HBS, in mM): 140 NaCl, 10 HEPES, 2.5 KCl, 2.5 CaCl_2 , 2.0 MgCl_2 , 10 glucose; pH 7.4 with HCl; osmolality adjusted to 330 mmol/kg with sucrose. 0.5 μM tetrodotoxin was added immediately prior to use. The intracellular pipette solution contained (in mM): 100 CsCl, 10 HEPES, 10 EGTA, 1 CaCl_2 , 4 Mg-ATP; pH adjusted to 7.2 with CsOH; osmolality adjusted to 300-310mmol/kg with sucrose. Whole-cell series resistance and capacitance were determined from fits to capacitive currents during square-wave depolarizations using pClamp 9.2 (Molecular Devices); neither measure was different between treatment groups ($P>0.05$, t-test). These parameters were corrected manually prior to each recording and were monitored throughout the experiment.

Cells were voltage-clamped at -30 mV holding potential using an Axoclamp 200B (Molecular Devices, Sunnyvale CA). Whole-cell currents were elicited by a 4 second local application of GABA-containing (3 μM) HBS solution using a series of square glass pipettes (0.7 mm I.D.) placed within 100 μm of the cell. Rapid (≤ 100 ms) switching between HBS only (control) and GABA-containing HBS solutions was achieved by connecting the drug-application pipettes to a computer-controlled stepper motor (Perfusion Fast Step System; Warner Instruments, Hamden CT). For drug comparing drug-effects in control and ethanol-exposed neurons, flunitrazepam or ethanol was added to the GABA-containing HBS solutions; and, these data are expressed as a percentage calculated from the GABA-alone responses measured in the same cells. The apparent peak of the current response was used for all experiments; these currents likely represent functional contributions by all GABA_A receptors expressed in the somatic compartment and the peri-somatic dendritic processes. Where current responses themselves were compared between treatments or groups, current density was used to reduce cell-to-cell variance. Current density was calculated by dividing the peak of the current response (pA) by the apparent whole-cell capacitance (see above) determined at the beginning of the experiment. There was no significant effect of ethanol drinking on whole cell capacitance (control = 19.6 ± 5.4 pF, ethanol = 14.6 ± 2.8 pF, $P>0.05$ with t-test) or on access resistance (control = 10.7 ± 0.9 M Ω , ethanol = 9.5 ± 0.4 M Ω , $P>0.05$ with t-test). Signals were digitized at 10 kHz and filtered at 2 kHz during data acquisition.

RNA Isolation

Total RNA was isolated from 50-100 mg pieces of cynomolgus lateral amygdala and trace DNA contamination was removed using the RNAqueous 4-PCR kit (Ambion; Austin, TX) following the manufacturer's recommendations. The RNA was quantified using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington DE). Mean OD 260/280 ratio was 2.01 ± 0.04 for the samples used in this study. First strand cDNA was synthesized from 20 ng total RNA in 10 μl reactions using SuperScript II (Invitrogen; Carlsbad, CA) with random primers as previously described (Floyd et al., 2004).

Real-time RT-PCR

Real-time PCR was performed using an Opticon II System (BioRad Laboratories, Hercules CA) with SYBR Green reagents (Applied Biosystems; Foster City, CA) according to established protocols using 2 ng cDNA and 1 μM primers per 25 μl reaction volume in triplicate. Primers were constructed using PrimerExpress software (Applied Biosystems;

Foster City, CA) and purchased through Integrated DNA Technologies (Coralville, IA). Primers for β_2 -microglobulin were reported previously (Floyd et al., 2004). Primers for GABA_A β_{1-3} , γ_{1-3} , and δ have been reported previously (Hemby et al., 2006). Primers for β -actin were identified from *Macaca fascicularis* cDNA sequences (GenBank# MFU20576): forward – GCGCGGCTACAGCTTCA (nt# 200–216); reverse – TCTCCTTAATGTACGCACGAT (nt# 258–237). Primers for glyceraldehyde phosphate dehydrogenase (GAPDH), the A-subunit of the succinate dehydrogenase complex (SDHA), and hypoxanthine phosphoribosyltransferase-1 (HPRT1) were obtained from *Macaca mulatta* orthologs (GenBank# AY624139 for GAPDH, BV209553 for SDHA, and BV166330 for HPRT1). GAPDH primer sequences were: forward – TGACCTGCCGTCTGGAAAA (nt# 194–212); and, reverse – CTCCGACGCCTGCTTCA (nt# 261–245). For SDHA, primers were: forward – CCAGGGAACGTGGCACTTAC (nt #261– 280); and, reverse – GCTGTGCCCAGTTTTATCATCTC (nt# 327–305). Finally, for HPRT1, primers were: forward – TGGAAACATCTGGAGTCCTATGG (nt# 47–69); reverse – CTGCTAAGCAGATGGCTACAGAAC (nt# 122–99). Primer sequences were identified using PrimerExpress software (Applied Biosystems) and all produced a single PCR product as determined by melting curve analysis and agarose gel electrophoresis. Melting curve analysis was performed on every reaction to ensure that a single product was produced in every sample. Primer efficiency was determined using the relative standard curve method (Floyd et al., 2004) with serial dilutions of a total RNA sample that was representative of several different forebrain regions (kindly provided by Dr. Steven Walker, WFUSM). Efficiencies of all primers used in this study were >83%.

mRNA expression levels for the different GABA_A subunits in each sample were standardized using a ‘normalization factor’ based on ubiquitous gene levels in that sample according to the method of Vandesompele et al. (Vandesompele et al., 2002) with the aid of “geNorm Add-in” for Microsoft Excel (PrimerDesign Ltd., Southampton UK). Briefly, the ubiquitous gene threshold-cycle (C_T) value in each sample was converted to a relative quantity by 1) calculating a ΔC_T between a given sample and the sample with the lowest C_T (highest expression) and 2) expressing this ΔC_T as a quantity (Q) defined as $Q = (\text{PCR Efficiency})^{-\Delta C_T}$. A normalization factor for each sample was then computed from the geometric mean of different ubiquitous gene Q values. C_T values for the various GABA_A subunit mRNAs were converted to a relative quantity as described above; and these Q_{GABA} values were normalized by dividing them by the sample’s normalization factor.

Statistics

All values are expressed as the mean $\pm$ SEM. Where indicated, standard student’s t-test (treatment) or two-way ANOVA (treatment X gender) were used for both electrophysiological and molecular biological studies. In all cases, results were considered significant if $P < 0.05$.

Result

Long-term Ethanol Self-administration Reduces the Flunitrazepam Sensitivity of Amygdala GABA_A Receptors

1 μM flunitrazepam greatly enhanced currents evoked by application of 3 μM GABA (Fig. 1A). In neurons isolated from control monkeys, flunitrazepam significantly increased GABA-gated current density from 6.7 ± 1.6 pA/pF to 9.3 ± 2.1 pA/pF ($P < 0.01$, paired t-test). Likewise, in amygdala neurons from ethanol exposed animals, the benzodiazepine increased apparent current density from 4.5 ± 0.8 pA/pF to 5.1 ± 0.9 pA/pF ($P < 0.01$, paired t-test). Note that the smaller apparent response in ethanol-exposed neurons, while not statistically different from control, is consistent our previous work demonstrating a decrease in GABA potency in macaque neurons following long-term self-administration (Floyd et al., 2004). Regardless,

when these data were expressed as a percent of the GABA-only response, chronic ethanol self-administration significantly reduced the facilitation of GABA current by flunitrazepam from $41.0 \pm 6.7\%$ in control neurons to $11.4 \pm 0.9\%$ in ethanol-exposed cells (Fig. 1B). This decrease in sensitivity to benzodiazepines was not dependent upon the gender of the animal from which neurons were isolated (Fig. 1C) with a significant main effect of ethanol ($F=16.31$, $P<0.01$, two-way ANOVA) in both males and females ($P<0.05$, Bonferroni posttest).

Long-term Ethanol Exposure Does Not Influence Direct Modulation by Ethanol

In addition to benzodiazepine sensitivity, we also examined GABA-current modulation by acute application of ethanol along with GABA. Similar to our previous observations in both rat (McCool et al., 2003) and mouse (DuBois et al., 2006) acutely isolated basolateral amygdala neurons, 100 mM ethanol produced a modest suppression of GABA-gated currents by $14.7 \pm 3.7\%$ in control neurons and $14.9 \pm 3.8\%$ in ethanol-exposed neurons (Fig. 2). This inhibition appears only modestly dose dependent since 20mM ethanol inhibited currents by $9.2 \pm 2.5\%$ and $6.7 \pm 5.0\%$ in control and chronic ethanol neurons, respectively. Regardless, this modest inhibition was not significantly influenced by drinking history ($F=0.087$, $P>0.05$). This suggests that long-term ethanol self administration by cynomolgus macaques does not alter the direct effects of acute ethanol on amygdala GABA_A receptors.

Long-term Ethanol Self-Administration Alters Amygdala GABA_A Receptor Subunit mRNA Expression

To examine possible subunit contributions to decreased benzodiazepine sensitivity, we examined GABA_A receptor subunit mRNA levels in samples from control and ethanol-drinking monkeys. However, given the limited number of tissue samples available for the current study ($n=6$ controls, 3 males and 3 females; and $n=9$ ethanol-drinking animals, 5 males and 4 females), we first established the most stable ubiquitous genes to which GABA_A subunit mRNAs should be normalized. β -actin, β_2 -microglobulin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase-1 (HPRT1), and the A-subunit of succinate dehydrogenase (SDHA) have been used as 'normalizers' in numerous studies but have never been examined in chronic ethanol-drinking monkeys. Quantitative RT-PCR revealed that β -actin and β_2 -microglobulin were not significantly affected by long-term ethanol self-administration (Table 2). Conversely, GAPDH ($F=10.4$, $P<0.01$), HPRT1 ($F=6.2$, $P<0.05$), and SDHA ($F=9.0$, $P<0.05$) mRNA expression was significantly down-regulated by long-term ethanol self-administration. While the down-regulation was apparent in both genders, only the lower expression of GAPDH and SDHA in male drinkers reached significance ($P<0.01$ and $P<0.05$ with Bonferroni posttests, respectively). Given these results, β -actin and β_2 -microglobulin mRNA levels in each sample were used to normalize the expression of GABA_A subunit mRNAs (see Methods).

As shown in Figure 3, long-term ethanol self-administration significantly reduced expression of β_1 (Fig. 3A; $P<0.05$, t-test) and γ_2 (Fig. 3B; $P<0.01$, t-test) subunits. In addition, there were trends towards decreases in γ_3 (Fig. 3B; $P<0.1$) and increases in δ (Fig. 3C, $P \approx 0.1$) subunit expression in samples from ethanol-drinking monkeys. When these expression data were analyzed using gender and ethanol-exposure as main factors (Table 3), the significant ethanol-dependent reduction in mRNA levels remained for the β_1 ($F=5.2$) and γ_2 subunits ($F=9.8$). For the γ_2 subunit, the down-regulation appeared to be largely due to the effects of ethanol in male macaques (56% reduction) compared to female monkeys (25% reduction). This may reflect the larger but non-significant quantities of ethanol consumed by males (2.5 ± 0.5 g/kg daily, $n=5$) relative to females (1.6 ± 0.4 g/kg daily, $n=4$, $P>0.05$ with t-test) in the current study. Interestingly, while there was no main effect of ethanol on γ_1 mRNA expression, there was a significant main effect of gender ($F=7.7$) that appeared to arise primarily from differences between control animals with subunit expression in control females ($n=3$) being 25–67% less

than in control males (n=3). We conclude that long-term ethanol self-administration reduces amygdala expression of β_1 , γ_2 , and perhaps γ_3 subunits and may increase expression of δ subunits.

Discussion

The current study has found that long-term ethanol self-administration by cynomolgus macaques reduces the benzodiazepine-facilitation of basolateral amygdala GABA_A receptors. These adaptations were specific since ethanol drinking did not alter the direct receptor modulation by acute ethanol application. Although our experimental approach would not have detected ethanol-dependent modulation of indirect mechanisms or synaptic GABAergic function, our findings compliment previous work describing reduced GABA potency and increased receptor desensitization kinetics in this model (Floyd et al., 2004). While the current study did not examine synaptic GABA_A receptor function, Weiner and co-workers (Roberto et al., 2006) have recently reported decreased GABA synaptic release without alterations in acute ethanol facilitation of macaque hippocampal GABAergic synaptic responses. Together with our studies, these findings may suggest an overall functional down-regulation of the GABAergic system following prolonged periods of ethanol self-administration in monkeys without significant alterations in acute ethanol sensitivity. There are several additional similarities between the GABA_A-related adaptations in the monkey amygdala following long-term ethanol drinking and more subchronic non-contingent ethanol exposures in rodents. In rat amygdala for example, there is an apparent lack of tolerance to the acute effects of ethanol on GABAergic synapses in the central nucleus (Roberto et al., 2004) and on GABA_A receptors in isolated basolateral amygdala neurons (McCool et al., 2003) following chronic ethanol. Chronic ethanol exposure is also associated with decreased potency of GABA_A receptor agonists measured in both isolated amygdala neurons (McCool et al., 2003) and membrane preparations (Papadeas et al., 2001).

It should be noted however that many of the GABA_A adaptations to chronic ethanol exposure in other brain regions are not always evident in the amygdala. These differences may be either model- or brain region-dependent. For example, the Olsen laboratory (Cagetti et al., 2003) has found reduced benzodiazepine modulation of CA1 hippocampal GABAergic synaptic currents following 55 days of once daily ethanol gavage. Similarly, subchronic ethanol-liquid diet exposure in mice reduced benzodiazepine modulation of GABA_A-mediated chloride-flux in membranes from cerebral cortex (Buck and Harris, 1990). In contrast, similar adaptations were not evident in isolated rat basolateral amygdala neurons following a 12-day exposure to ethanol liquid diet (McCool et al., 2003). While these findings might suggest a region-specific downregulation of benzodiazepine sensitivity following chronic ethanol, the reduced benzodiazepine sensitivity evident in isolated amygdala neurons following long-term self-administration in the current study might imply that particular GABA_A receptor adaptations to chronic ethanol follow distinct time- or 'severity of exposure'-dependent pathways. In the lateral/basolateral amygdala for example, shorter or less severe exposures may only decrease GABA potency (McCool et al., 2003) while longer/more robust treatments also seem to produce altered sensitivity to benzodiazepine allosteric modulators (current study). While we did not intend to differentiate such time- or 'severity'-related pathways, post-hoc analysis of subunit mRNA expression levels and ethanol drinking did not reveal any significant correlations between expression and the amount of ethanol consumed (not shown). This may suggest that variables like daily or total consumption contributed less to GABA_A molecular adaptations than did other variables, such as the length of time animals drank. We were unable to perform similar correlation analyses on the flunitrazepam electrophysiology data given that only six animals were included in the current study. Rodent inhalation models (Becker et al., 1997; Roberto et al., 2004) may offer enough flexibility to directly test the relative contributions of exposure length and severity. Finally, we can not ignore the possibility that the

benzodiazepine data may suggest rodent and primate adaptations to chronic ethanol exposure are simply distinct and species-specific. Given the overall similarities of the GABAergic systems as well as the parallel adaptations related to agonist potency, we do believe this is the case, but clearly additional studies are needed to more fully address this possibility.

Our electrophysiological findings led us to investigate possible subunit-dependent adaptations to long-term ethanol self-administration. We therefore examined β , γ , and δ subunit mRNA expression. However, it is customary to normalize the expression levels of a given subunit to a 'ubiquitous' gene in order to control for sample-to-sample variance. While searching for appropriate candidates, we found that long-term ethanol self-administration by cynomolgus macaques alters expression of several typical 'normalizer' mRNAs including decreased expression of GAPDH, SDHA, and HPRT1 mRNAs. Decreased GAPDH mRNA could suggest diminished capacity for NADH production in the monkey amygdala following chronic ethanol drinking while decreased SDHA mRNA might reflect or produce reduced mitochondrial oxidative phosphorylation. These findings together lend support to the idea of a general metabolic 'lesion' associated with chronic ethanol exposure (Cunningham and Bailey, 2001; Marcinkeviciute et al., 2000). Alternatively, increased GAPDH expression can initiate/stabilize cytotoxic pathways associated with apoptosis (Chen et al., 1999). Decreased mRNA during chronic ethanol might represent a compensatory adaptation down-regulating apoptotic pathways (Fukuhara et al., 2001).

The exact role of reduced guanine nucleotide metabolism coincident with decreased HPRT1 expression is less clear. HPRT mutations can lead to altered glial glutamate uptake (reviewed in (Deutsch et al., 2005)) and altered neuronal morphology (Mikolaenko et al., 2005). Perhaps ethanol-dependent reductions in HPRT1 expression cause similar effects. Finally, SDHA inhibition appears to disrupt mitochondrial metabolism in GABAergic neurons (Hassel and Sonnewald, 1995) and confers sensitivity to glutamate-mediated neurotoxicity (Massieu et al., 2001; Zeevalk et al., 1995). Thus, our findings might reflect the pronounced disruption of the balance between excitatory and inhibitory neurotransmitter during chronic ethanol exposure. Regardless of their ultimate physiological role in chronic ethanol, many of the 'ubiquitous' genes typically used to normalize quantitative RT-PCR data are substantially altered by long-term ethanol drinking in this non-human primate model, at least in our samples. These findings also serve to illustrate that the changes in gene expression for GABA_A receptor subunits are not necessarily unique to that neurotransmitter system.

In addition to alterations in gene products associated with metabolism, we also found that long-term ethanol self-administration by cynomolgus macaques significantly decreases expression of GABA_A β_1 and γ_2 subunits, along with more subtle decreases in γ_3 and increases in δ mRNA levels. Since γ subunits help form the benzodiazepine binding site on GABA_A receptors (Pritchett et al., 1989), our mRNA findings therefore suggest that down-regulation of γ_2 subunits is a potential mechanism mediating the functional decreases in benzodiazepine sensitivity in our electrophysiology experiments. Alternatively, since different γ subunits convey distinct absolute benzodiazepine sensitivities (Benke et al., 1996), decreased benzodiazepine efficacy in this model also suggests that chronic ethanol could merely alter the relative contributions of different γ subunits to the GABA_A complex. We have also previously found decreased α_2 , α_3 , and possibly α_1 mRNA expression in this brain region (Floyd et al., 2004). We must therefore interpret the current γ subunit findings with some caution given that both α and γ subunits contribute to benzodiazepine interactions with the GABA_A receptor (Pritchett and Seeburg, 1990; Pritchett et al., 1989). Regardless, these two studies suggest that, like rodent models of chronic ethanol exposure, GABAergic functional adaptations to long-term alcohol self-administration in this non-human primate model are associated with robust alterations in subunit gene expression.

The effects of long-term ethanol self-administration on β_1 subunit expression are more difficult to interpret in the context of potential functional contributions. Given that the limitations of real-time PCR, we have not provided a quantitative comparison between the different β subunits. Regardless, our data may suggest that the various β subunits are expressed at similar levels in the macaque amygdala. Therefore, since β_1 -containing receptors may have greater apparent GABA affinity (Fisher and Macdonald, 1997), the diminished expression of this subunit mRNA relative to other β subunits may help explain the decreased GABA potency following long-term ethanol drinking in macaques (Floyd et al., 2004). Since the β subunit-dependent allosteric modulator loreclezole has much greater apparent affinity for $\beta_{2/3}$ -containing receptors (Wafford et al., 1994), this hypothesis can be functionally tested by future experiments.

Another aspect of the current work that deserves some comment is apparent gender-specific effect on the expression of some subunits. For example, we found significant effects of ethanol drinking in males but not females on several genes, including GAPDH, HPRT1, SDHA, and GABA_A γ_2 subunit. We also found significant gender differences in GABA_A γ_1 subunit levels in the current dataset independent of ethanol drinking histories. These results are similar to our previous findings of gender-specific functional adaptations using this same model (Floyd et al., 2004). Despite this, there was no significant correlation between GABA_A subunit expression levels and the total amount of ethanol consumed by any given individual. This suggests that the gender-specific adaptations reported in the current work are not related to differences in total consumption between males and females. Although not extensively explored, gender-specific effects have also been reported in rodent studies using non-contingent exposure. For example, gender modulates the effects of ethanol exposure or withdrawal on α subunit peptide levels in the hypothalamus (Devaud et al., 1999) and cortex (Devaud et al., 1998). In addition, recent evidence suggests that the estrous cycle can modulate individual sensitivity to the acute behavioral (Bao et al., 1992; Cha et al., 2006; Ford et al., 2002) and neurobiological (Becker et al., 1985; Janis et al., 1998) effects of ethanol. Our findings therefore provide support for the notion that gender may modulate the neurobiological responses to chronic ethanol exposure.

In conclusion, our current results and those from previous studies (Floyd et al., 2004) are consistent with the hypothesis that chronic ethanol exposure in cynomolgus macaques is associated with a generalized down-regulation of the GABAergic system in the lateral/basolateral amygdala. While the behavioral consequences of these adaptations are currently not clear in the context of this nonhuman primate model, increased expression of anxiety-like behavior is a consequence of long-term exposure to ethanol in rodents (Gatch et al., 1999; Kliethermes et al., 2004) and humans (reviewed in (Kushner et al., 2000)). Diminished GABAergic function in the rodent amygdala increases expression of anxiety-like behaviors (Sanders and Shekhar, 1995); and, rodent genetic models with diminished GABA_A γ_2 subunit expression exhibit increased anxiety-like behavior (Chandra et al., 2005). These findings together suggest that the altered GABAergic function/expression described in the current work could provide evidence for ethanol-dependent adaptations in a nonhuman primate model that upset the balance between excitatory and inhibitory neurotransmitter systems. In the context of the amygdala, decreased inhibitory contributions could certainly underlie the increases in anxiety-like behavior that are frequently observed in both rodents and humans following chronic ethanol exposure.

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Figure 1.

Long-term ethanol self-administration reduces the sensitivity of amygdala GABA_A receptors to the benzodiazepine flunitrazepam. **(A)** Example traces from an ethanol-naïve neuron (top) and from a chronic ethanol-exposed neuron (bottom) showing the reduced modulation by flunitrazepam in neurons from chronic ethanol macaques. Bars above traces indicate the application of 3mM GABA alone (open bars) or along with 1 μ M flunitrazepam (closed bars). Calibration bars: y = 10pA, x = 1sec. **(B)** Summary of flunitrazepam modulation for neurons isolated from ethanol naïve controls ('Control') and from ethanol-drinking cynomolgus macaques ('CE'). Flunitrazepam modulation was significantly decreased (***) – $P < 0.001$, t-test) in neurons from chronic ethanol animals. **(C)** Gender did not influence the decrease in

flunitrazepam modulation caused by chronic ethanol exposure (* – $P < 0.05$, Bonferroni posttests following two-way ANOVA).

Figure 2.

Long-term ethanol self-administration does not alter acute ethanol sensitivity of amygdala GABA_A receptors. **(A)** Example traces of acute ethanol modulation of GABA (3mM, open bars) applied with either 20mM ethanol (hatched bar) or 100mM ethanol (closed bar) in a neuron isolated from a control animal (top) or an ethanol-drinking animal (bottom). Calibration bars: y = 20pA, x = 2sec. **(B)** Summary of acute ethanol modulation from control and chronic ethanol-exposed cynomolgus macaques. Note that chronic ethanol did not alter the modest inhibition of GABA-gated currents by acute ethanol.

Figure 3.

Long-term ethanol self-administration alters the expression of amygdala GABA_A subunit β and γ mRNAs. Summary of β subunit (**A**), γ subunit (**B**), and δ subunit (**C**) expression illustrates the specific down-regulation of the β_1 and γ_2 subunits (* – $P < 0.05$, t-test). More subtle alterations in γ_3 and δ subunit expression were also evident ('a' – $P < 0.1$, t-test).

Ethanol Consumption by Cynomolgus Macaques used in the Current Study

Table 1

Animal # ^a	Gender	Mean Daily g/kg ^b	Total g/kg ^b	Mean ± SEM (n)	BEC (n)	Range
6301 ^c	female	0.93±0.06	578.8	33 ± 9 (29)		0–184
4993	female	0.97±0.07	1111.4	15 ± 4 (32)		0–87
6304, ^{ce}	male	1.02±0.06	897.8	63 ± 13 (30)		0–271
6302 ^c	female	1.17±0.13	840.7	35 ± 11 (30)		0–286
6305, ^{ce}	male	1.49±0.08	1184.5	62 ± 10 (30)		0–242
6101, ^{cde}	male	1.85±0.12	1524.2	100 ± 9 (28)		0–260
5497 ^e	male	1.76±0.10	1670.8	105 ± 11 (33)		0–217
6102	female	2.08±0.09	1935.2	53 ± 9 (31)		0–168
5404 ^e	male	3.33±0.24	2289.8	188 ± 17 (33)		3–398
6306, ^{ce}	male	2.70±0.17	2757.7	173 ± 10 (35)		59–317

^a Animals are the same as those described in (Floyd et al., 2004).

^b Values represent consumption across a the last 12months of drinking (Mean Daily g/kg) or the entire 24-month period ending with euthanasia (Total g/kg).

^c Animals whose tissue was used in the electrophysiology experiments.

^d Animal not used in the molecular biology experiments.

^e Total g/kg data from these animals are the same as those that appear in (Hemby et al., 2006) and are reproduced here to facilitate comparisons between these works.

Table 2
Expression of 'Ubiquitous' Genes in LA/BLA Total RNA from Cynomolgus Macaques ^a

mRNA	Control	Treatment	Chronic Ethanol	Statistic ^b
β₂-microglobulin	1.96±0.17		1.98±0.34	
Male	2.02±0.35		2.21±0.62	ns
Female	1.90±0.11		1.70±0.14	ns
β-actin	1.34±0.16		1.12±0.17	
Male	1.20±0.32		1.04±0.24	ns
Female	1.48±0.08		1.20±0.26	ns
GAPDH	1.95±0.31		1.07±0.10	‡
Male	2.27±0.61		0.95±0.16	P<0.01
Female	1.63±0.11		1.22±0.05	ns
HPRT1	2.92±0.80		1.12±0.17	†
Male	3.87±1.41		1.41±0.35*	ns
Female	2.00±0.53		0.90±0.04	ns
SDHA	1.57±0.22		0.80±0.13	†
Male	1.68±0.44		0.73±0.24	P<0.05
Female	1.45±0.16		0.89±0.05	ns

^a mRNA levels were measured using the relative standard curve method (Floyd et al., 2004). Values are expressed here as 'ng equivalent' relative to whole-brain total RNA.

^b Two-way ANOVA (Treatment vs. Gender) using Bonferroni posttests.

†,‡ Significant main effect of ethanol (P<0.05 and P<0.01, respectively)

* HPRT1 expression was not detected in two male samples. These are excluded from the analysis.

Table 3

Gender-specific Effects of Long-term Ethanol Self-Administration on GABA_A Subunit mRNA Expression in Cynomolgus Lateral/Basolateral Amygdala

mRNA	Control	Treatment	Chronic Ethanol	Statistics
β_1				$\dagger$, P<0.05 ^a
Male	0.70±0.02		0.44±0.07	<i>n.s.</i> ^b
Female	0.50±0.06		0.43±0.09	<i>n.s.</i>
β_2				
Male	0.65±0.11		0.45±0.10	<i>n.s.</i>
Female	0.54±0.06		0.49±0.16	<i>n.s.</i>
β_3				
Male	0.62±0.13		0.50±0.12	<i>n.s.</i>
Female	0.60±0.06		0.55±0.11	<i>n.s.</i>
γ_1				$\ddagger$, P<0.05
Male	0.66±0.11		0.40±0.10	<i>n.s.</i>
Female	0.22±0.01		0.31±0.08	<i>n.s.</i>
γ_2				$\dagger$, P<0.01
Male	0.62±0.09		0.27±0.06	<i>P<0.01</i>
Female	0.40±0.03		0.30±0.07	<i>n.s.</i>
γ_3				
Male	0.62±0.13		0.39±0.09	<i>n.s.</i>
Female	0.50±0.05		0.39±0.10	<i>n.s.</i>
δ				
Male	0.51±0.07		0.67±0.13	<i>n.s.</i>
Female	0.68±0.02		0.79±0.15	<i>n.s.</i>

^aTwo-way ANOVA with “ethanol exposure” and “gender” as main variables;

$\dagger$ significant main-effect of ethanol exposure across both genders;

$\ddagger$ significant main-effect of gender but not ethanol exposure

^b Bonferroni posttests, *n.s.* = no significant post-hoc effect of ethanol exposure within a particular gender