THE EFFECTS OF CHRONIC ETHANOL ON GABA RECEPTORS IN THE
NONHUMAN PRIMATE BRAIN

BY

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## TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

ABSTRACT

CHAPTER 1: Introduction

CHAPTER 2: Chronic ethanol alters total GABA<sub>A</sub> receptor and α subunit binding density in monkey cortex

CHAPTER 3: Chronic ethanol alters GABA<sub>A</sub> receptor binding density and GABA receptor subunit gene expression in monkey cerebellum

CHAPTER 4: Discussion

CURRICULUM VITAE
LIST OF FIGURES AND TABLES

Chapter II

Figure 1: Whole cerebral cortical section showing location of measured fields.................................................................47

Figure 2: Laminar binding patterns of total GABA<sub>A</sub> receptor population........48

Figure 3: Chronic ethanol drinkers have less dense total GABA<sub>A</sub>, α1 and α4 subunit-containing receptor binding in the global cortex.................49

Table I: Receptor binding density and percent change for the total population of GABA<sub>A</sub> receptors.................................................................50

Table II: Receptor binding density and percent change for α1 subunit-containing receptors.................................................................51

Table III: Receptor binding density and percent change for α4 subunit-containing receptors.................................................................52

Figure 4: Main effects of chronic ethanol on total GABA<sub>A</sub>, α1 and α4 subunit-containing receptor binding density...............................53

Figure 5: Chronic ethanol drinkers have less dense total GABA<sub>A</sub>, α1 and α4 subunit-containing receptor binding in area 4..........................54

Figure 6: Chronic ethanol drinkers have less dense total GABA<sub>A</sub> and α1 subunit-containing receptor binding in area 3a..........................55

Figure 7: Chronic ethanol drinkers have less dense α1 subunit-containing receptor binding in area 3b.........................................................56

Figure 8: Chronic ethanol drinkers have less dense α1 subunit-containing receptor binding in area 1.................................................................57

Figure 9: Chronic ethanol drinkers have less dense α1 subunit-containing receptor binding in the rostral dysgranular insula...............................58
Figure 10: Chronic ethanol drinkers show less dense $\alpha_4$ subunit-containing receptor binding in both TEa and TE.

Figure 11: Chronic ethanol drinkers show less dense total GABA$_A$ and $\alpha_4$ subunit-containing receptor binding in the subiculum.

Table IV: $\alpha$ subunit-containing receptors percentage of total GABA$_A$ receptors.

Chapter III

Figure 1: Whole cerebellar sections showing location of measured fields.

Table I: Primers and probe sequences for GABA$_B$ subunits used in qPCR.

Table II: Receptor binding density and percent changes in the cerebellum.

Figure 2: Chronic ethanol drinkers have less dense total GABA$_A$ receptor binding globally in the cerebellum, in the hemisphere, and in the vermis.

Figure 3: Chronic ethanol drinkers have less dense $\alpha_6$ subunit-containing receptor binding in the combined layer analysis in the hemisphere.

Table III: $\alpha$ subunit-containing receptor percentage of total GABA$_A$ receptors.

Figure 4: Total GABA$_A$ receptor binding density correlates to average daily ethanol intake.

Figure 5: Chronic ethanol increases GABA$_A$ $\alpha_1$ subunit gene expression.

Figure 6: Chronic ethanol increases GABA$_B2$ subunit gene expression.

Figure 7: GABA$_A$ $\alpha_2$ subunit gene expression is correlated with average daily ethanol intake.
LIST OF ABBREVIATIONS

ACTB  beta actin
β2M  beta 2 microglobulin
CCAS  cerebellar cognitive affective syndrome
cDNA  complementary deoxyribonucleic acid
Ct  threshold cycle
EC  entorhinal cortex
GABA  γ-amino butyric acid
Id  dysgranular insula
IM  intramuscular
IV  intravenous
IPSC  inhibitory postsynaptic current
mIPSC  miniature inhibitory postsynaptic current
mRNA  messenger ribonucleic acid
NTC  no template control
PASB  parasubiculum
PKA  protein kinase A
PKC  protein kinase C
PSB  presubiculum
qPCR  quantitative real time polymerase chain reaction
R1  GABA$_{B1}$
R2  GABA$_{B2}$
ROI  region of interest
SDHA  succinate dehydrogenase complex, subunit A
sIPSC  spontaneous inhibitory postsynaptic current
SNP  single nucleotide polymorphism
Sub  subiculum
TPO  temporal parietal occipital association area
TTX  tetrodotoxin
The GABAergic system is a well-known molecular target of ethanol. Acute ethanol increases the function of the GABA_\alpha receptor, while chronic ethanol decreases function. Rodent studies have reported changes in gene and protein levels, receptor function, and receptor binding density in GABA_\alpha receptors and subunits after chronic ethanol, and changes in GABA_\beta gene and protein levels. However, human studies are somewhat scarce, and existing data is often conflicting. In a translational approach, we utilized a nonhuman primate model of chronic ethanol self-administration to investigate total GABA_\alpha, \alpha_1 and \alpha_4/6 subunit-containing receptor binding density in a variety of cerebral cortical regions and the cerebellum. Ethanol induces region and layer dependent receptor binding density alterations. In general, drinkers show less dense binding in both cerebral cortical regions and in the cerebellum. There was an upregulation of both the GABA_\alpha \alpha_1 and GABA_\beta_{2} subunit gene expression after chronic ethanol. These results indicate that both synaptic and extrasynaptic GABA_\alpha receptors are altered by ethanol, but that its effects are dependent on circuitry. Posttranslational modification may occur to account for differences seen between gene expression and receptor binding density results seen here.
CHAPTER I

INTRODUCTION

Alcohol abuse and dependence cause significant deleterious effects to our country’s health, finances, and social cohesion. An estimate from 2002 shows the current number of Americans 18 years old or older who abuse or depend on alcohol is 18 million, comprising 8.5% of the population (Grant et al. 2004). In 2003, alcohol abuse and dependence accounted for 1.4% of the total world disease burden, and more than half of Americans have a relative who has been or is currently dependent on alcohol (Grant et al. 2004). Alcohol abuse has been identified as the third leading preventable cause of death in the United States (Mokdad et al. 2004). Total financial costs of alcohol use were estimated at $148 billion in 1992, whereas costs in 1998 showed a 25% increase to $184 billion (Harwood 2000).

Alcohol has a pervasive, detrimental effect on the brain, affecting an array of neurotransmitter systems and virtually every brain region. The widely distributed GABAergic system is a well-known molecular target of ethanol. Human clinical studies have shown that baclofen, a GABA_B agonist, can reduce craving and the severity of withdrawal in alcoholics (Addolorato et al. 2000). Additionally, human genotyping shows a relationship between the GABA_A α6 subunit gene polymorphism and an increased risk for alcoholism (Schuckit et al. 1999). An α2 subunit polymorphism has been associated with impulsivity and lower subjective effects of ethanol, both of which are risk factors for alcoholism (Roh et al. 2010; Villafuerte et al. 2011).
Behavioral, electrophysiological, and other neurobiological approaches in animals also strongly support a role for GABA in mediating the effects of ethanol and being altered by chronic exposure to ethanol. Recently, studies with knockout mice with deletions of different GABA\textsubscript{A} subunits have been conducted (Boehm et al. 2004). The majority of these mice demonstrate alterations in behavioral effects of ethanol, providing evidence for the possible involvement of particular subunits in mediating the effects of ethanol.

**GABA\textsubscript{A} Receptors**

The brain contains ionotropic GABA\textsubscript{A} receptors and metabotropic GABA\textsubscript{B} receptors. GABA\textsubscript{A} receptors are inhibitory pentameric chloride channels that are typically composed of subunits from α, β, and γ families, though a δ subunit can be substituted for a γ subunit (Fritschy & Mohler 1995; Pirker et al. 2000). Subunit composition can affect pharmacology of the receptors (Ebert et al. 1997), which makes the study of these receptors and individual subunits critical in understanding the effects of ethanol on the GABAergic system. GABA\textsubscript{A} receptors mediate fast, phasic inhibition through synaptic receptors, and slower, tonic inhibition though extrasynaptic receptors (Farrant & Nusser 2005). GABA\textsubscript{B} receptors modulate the release of GABA when found presynaptically, and are linked to inwardly rectifying potassium channels when found postsynaptically.

**The Effects of Ethanol on GABA\textsubscript{A} Receptors in Rodents**

Several behavioral effects of ethanol are potentiated or attenuated by GABA\textsubscript{A} agonists and antagonists, respectively (Lister 1987; Becker & Anton 1990; Ho & Yu 1991). Additionally, GABA\textsubscript{A} agonists can facilitate acquisition of ethanol self-administration and can increase ethanol intake, while inverse agonists can decrease ethanol intake (Petry 1997). However, these effects can be slightly altered depending on the self-
administration paradigm used in the study. GABA<sub>A</sub> agonists can also be used to treat withdrawal symptoms.

Chronic ethanol decreases current flow through GABA<sub>A</sub> receptors, which led to the hypothesis that the number of GABA<sub>A</sub> receptors may be decreased by ethanol. Rodent studies have generally not found dramatic changes in GABA<sub>A</sub> receptor density after chronic ethanol, but instead report changes in subunit composition (Weiner & Valenzuela, 2006). Studies in whole cortex have reported an array of changes in subunit gene and protein expression. The α1 subunit gene expression and protein levels are decreased, as are the α2 and α3 peptides following chronic ethanol, but the α4/6 subunit gene expression and protein levels are increased, as is the β2/3 peptide. The γ2 peptide was not reported to show a change after chronic ethanol (Mhatre & Ticku 1992; Devaud et al. 1997a; Matthews et al. 1998). Chronic ethanol studies in rodents do not show changes in the α1, α2, α3, β2/3, or γ2 peptides in the hippocampus (Matthews et al. 1998). Additionally, studies in the cerebellum have demonstrated decreased α1 gene expression and protein levels and decreased β3 subunit gene expression (Mhatre & Ticku 1992; Wu et al. 1995). Other studies have demonstrated increased α6 subunit gene expression and protein levels, and increased β2 and γ2 subunit gene expression in the cerebellum after chronic ethanol (Mhatre & Ticku 1992; Wu et al. 1995). The δ subunit protein has been reported to decrease in the rat cerebellum after chronic ethanol (Ravindran et al. 2007). These data indicate region- and subunit-specific changes of GABA<sub>A</sub> receptor subunits in rodent models of chronic ethanol administration.

Interpreting receptor binding studies in rodents has been difficult because of conflicting findings. Receptor binding studies in rodents have demonstrated that total GABA<sub>A</sub>
receptor binding in the cerebral cortex and cerebellum either increased or did not change after chronic ethanol (Mhatre et al. 1988; Buck & Harris 1990). Another study has reported an increase in α6 subunit-containing receptor binding in mouse cerebellar membranes (Becker & Jarvis 1996). Additional studies in the rat did not report changes in α1 subunit-containing receptor binding in either the cerebral cortex or the cerebellum after chronic ethanol (Mehta & Ticku 1999). These variable findings suggest that methodology may modify the effects of ethanol and that ethanol affects brain regions differentially. Investigating a variety of brain regions is therefore important to our understanding of the effects of chronic ethanol on GABA_A receptors.

Electrophysiological studies have demonstrated that acute ethanol increases chloride flow through GABA_A receptors, while chronic ethanol decreases chloride flow (Grobin et al. 1998). Several studies have investigated the actions of acute ethanol at pre- and postsynaptic receptors. Additionally, studies reported no effect of ethanol on mIPSC or sIPSC amplitude in postsynaptic GABA_A receptors in the cerebellum (Carta et al. 2004; Hanchar et al. 2005; Mameli et al. 2005). These studies indicate that synaptic GABA_A receptors play an important role in mediating effects of ethanol. One mechanism that may explain the alterations in GABA_A receptor function after chronic ethanol would be a change in the number of receptors, which should be reflected by a change in receptor binding density. We are able to investigate the effect of chronic ethanol on α1 subunit-containing receptors, which make up the majority of synaptic GABA_A receptors, and α4 subunit-containing receptors, typically found extrasynaptically.

Ethanol can also affect extrasynaptic receptors. These GABA_A receptors desensitize slowly, and have a higher affinity for GABA than synaptic receptors (Merlo et al. 2000).
Data suggests that extrasynaptic receptors may be more sensitive to low concentrations of ethanol (Wallner et al. 2003). Ethanol increases tonic current in cerebellar slices; however, this effect is not seen in the presence of TTX, which indicates that spillover from action potential-induced release into the synapse is important for tonic current in the cerebellar granule cells (Carta et al. 2004). In summary, behavioral, electrophysiological, and molecular studies suggest that the effects of ethanol on GABA<sub>A</sub> receptors can depend on species, brain region, receptor subunit composition, protocol, and ethanol concentration. In order to address some of these questions about the effects of ethanol on GABA receptors, we used a nonhuman primate model of ethanol self-administration that closely mimics human drinking behavior; results from these studies could provide insights into what is happening to GABA<sub>A</sub> receptors in alcoholics.

**The Effects of Ethanol on GABA<sub>A</sub> Receptors in Nonhuman Primates**

GABA<sub>A</sub> receptor subunit gene expression has already been studied in the prefrontal cortex and amygdala of nonhuman primates after chronic ethanol self-administration. Chronic ethanol drinkers showed decreased α<sub>2</sub>, α<sub>4</sub>, β<sub>1</sub>, β<sub>3</sub>, γ<sub>1</sub> – 3 subunit gene expression in the orbitofrontal cortex, decreased β<sub>1</sub>, β<sub>2</sub>, γ<sub>1</sub>, and δ subunit gene expression in the dorsolateral prefrontal cortex, and no differences in the anterior cingulate cortex, and decreased gene expression for α<sub>2</sub>, α<sub>3</sub>, β<sub>1</sub>, and γ<sub>2</sub> subunits in the basolateral amygdala (Floyd et al. 2004; Hemby et al. 2006; Anderson et al. 2007). These studies indicate that chronic ethanol alters gene expression in the nonhuman primate cortex in a region and cortical field-dependent manner, again indicating that studies on several different brain regions are warranted.
The Effects of Ethanol on GABA Receptors in Humans

Currently, there are no studies describing the effects of ethanol on GABA$_B$ receptors in humans, and there are conflicting results about the effects of ethanol on GABA$_A$ receptors. Comorbid conditions like cirrhosis, nicotine use, diet, mental illness, and other health conditions can make results from human studies difficult to interpret. An early SPECT study reported reduced levels of total GABA$_A$ receptors in alcoholics without any gray matter atrophy. These reductions were found primarily in the parietal lobe, and further decreases with a small amount of atrophy were found in the orbitofrontal cortex, inferior medial frontal cortex, and dorsolateral prefrontal cortex (Lingford-Hughes et al. 1998). An additional SPECT study demonstrated lower distribution volume of total GABA$_A$ receptors in alcoholics in the frontal, anterior cingulate, and cerebellar cortices (Abi-Dargham et al. 1998). A PET study has reported decreased distribution volume of total GABA$_A$ receptors in the medial frontal lobes (Gilman et al. 1996).

Postmortem studies in alcoholics have reported that GABA$_A$ $\alpha1$ subunit gene expression increased or remain unchanged in the superior frontal cortex of alcoholics (Lewohl et al. 1997; Mitsuyama et al. 1998). Protein levels for this subunit are also unchanged, as were protein levels for the $\beta3$ subunit, and both gene and protein levels for the $\alpha4$ subunit in the superior frontal cortex in alcoholics (Mitsuyama et al. 1998). The $\beta3$ subunit gene expression has been reported to either increase or remain unchanged (Mitsuyama et al. 1998; Thomas et al. 1998; Buckley & Dodd 2004). Another study found no difference in $\alpha1, 2, 3, 5, \beta1$, or $\gamma2$ gene expression in the alcoholic cerebral cortex (Thomas et al. 1998).
There have been some reports of GABA<sub>A</sub> receptor binding studies in alcoholics, again with conflicting results. Alcoholics showed increased total GABA<sub>A</sub> receptor binding as measured by muscimol, but either no change or decreased total GABA<sub>A</sub> receptor binding as measured by flunitrazepam, and no change in α1 subunit-containing receptor binding (Tran et al. 1981; Dodd et al. 1996; Lewohl, Crane & Dodd 1997b). There is a report of decreased $K_d$ for GABA<sub>A</sub> binding in the cerebellum in alcoholics but no difference in binding density (Korpi et al. 1992). This literature demonstrates that alcohol affects gene expression in humans, as it does in rodents, though there are conflicting data about specific genes. Changes may be region specific, but that is difficult to discern because very few brain regions have been studied. The postmortem effect must be taken into account for human studies as well. These data indicate that it is important to study these effects in a more controlled condition. These human studies also indicate that ligand choice is important. Our autoradiographic studies use $[^3H]Ro15-4513$, which is an inverse agonist. It binds to GABA<sub>A</sub> receptors containing any α subunit (Sieghart 1995). This ligand allows us to distinguish subunits, while other ligands, such as muscimol, do not. Specifically, $[^3H]Ro15-4513$ can also be combined with zolpidem and diazepam to isolate α1 and α4/6 subunits, respectively.

**GABA<sub>B</sub> Receptors**

The metabotropic GABA<sub>B</sub> receptor is linked to the Gi/o protein, and consists of two subunits: GABA<sub>B1</sub> (R1) and GABA<sub>B2</sub> (R2) (Bettler & Tiao 2006). The GABA<sub>B</sub> receptor is a heterodimer, and both the R1 and R2 subunits are required for a functional receptor (Kaupmann et al. 1998; Jones et al. 1998; White et al. 1998). R1 has several splice variants, many of them without a well-established function or location. Two of the splice variants, however, have a known location. The GABA<sub>B1a</sub> (R1a) splice variant is found
predominantly as part of a presynaptic receptor whereas the GABA\textsubscript{B1b} (R1b) splice variant is found predominantly as a part of a postsynaptic receptor (Billinton et al. 1999; Mugnaini 2000). When found presynaptically, GABA\textsubscript{B} receptors help to control GABA release by limiting calcium influx; when found postsynaptically, GABA\textsubscript{B} receptors modulate inhibition through inward rectifying potassium channels (Bettler et al. 2004).

The Effects of Ethanol on GABA\textsubscript{B} Receptors
GABA\textsubscript{B} agonists such as baclofen have recently been found to reduce ethanol intake and reduce withdrawal signs, including seizures in dependent rats (Colombo et al. 2000). Clinical data has also demonstrated these effects in alcoholics.

Ethanol plays a role in the amount of GABA that is released. GABA\textsubscript{B} receptors limit ethanol’s potentiating effects on GABA\textsubscript{A} receptors in the hippocampus (Wan et al. 1996; Ariwodola & Weiner 2004). Ethanol may increase ambient GABA levels enough to activate the presynaptic GABA\textsubscript{B} receptor. When activated, this receptor limits calcium influx, resulting in less GABA release. Consequentially, there is less GABA to bind to postsynaptic GABA\textsubscript{A} receptors, thus limiting the potentiating effect ethanol can have on these receptors. Studies have demonstrated that ethanol increased the frequency of TTX-resistant miniature IPSCs (mIPSCs) and spontaneous IPSCs (sIPSCs), indicating an effect on presynaptic receptors (Sebe et al. 2003; Carta et al. 2004; Ariwodola & Weiner 2004). Carta et al. found ethanol increases the frequency of sIPSCs, but not mIPSCs in the cerebellum, suggesting there may be a mechanistic difference in GABA release in the cerebral cortex and the cerebellum (Carta et al. 2004). Like molecular studies, these electrophysiological studies indicate that the effects of ethanol may differ by brain region.
The effect of ethanol on GABA$_B$ receptor gene expression and protein levels has not been studied as extensively as effects of ethanol on the GABA$_A$ receptor. Only one study has investigated the molecular effects of chronic ethanol on GABA$_B$ receptors. Li et al. reported that both mRNA and protein levels of GABA$_{B1&2}$ are increased in cortex after 3 weeks of ethanol treatment, whereas only mRNA levels of R1 are increased in the hippocampus (Li et al. 2005). These changes indicate that chronic ethanol has an effect on the GABA$_B$ receptor, but it also appears that changes are regionally specific. The paucity of data indicates further studies are essential. The GABA$_B$ receptor system plays a role in GABAergic signaling, and is responsive to ethanol (Hahner et al. 1991; Ariwodola & Weiner 2004; Wu et al. 2005). Therefore, a study of the effects of ethanol on GABA$_B$ receptors seems warranted.

Previous studies have demonstrated that changes in GABA receptors are region specific. The cortex is incompletely studied, and requires a more systematic evaluation of differences in GABA receptors after chronic ethanol. We decided to investigate a variety of cortical areas involved in motor and sensory processing, interoception, multi- and unimodal integration, and limbic functions.

**Brain Region Functionality**

The cerebral cortex is responsible for higher cognitive functions, including sensory and motor processing, multimodal integration, and decision making, and it is made up of several dozen different cortical fields, which are distinguished from one another by cytoarchitectonic criteria, connections, and function. The majority of cortical regions are homotypical, meaning they have six layers. There are agranular and hypergranular cortical fields as well. Layers II, III, V, and VI all contain neurons that project to other
parts of the brain and their activity is modulated by GABAergic interneurons, which also modulate thalamic inputs into layer IV.

The most common type of inhibitory interneuron is the basket cell, which synapses on pyramidal cell dendrites in layer III. This interneuron is best described in the primary motor and somatosensory cortices (Jones & Hendry 1984). These interneurons are found in all layers that receive extrinsic afferents, including thalamic, callosal, and association inputs. This suggests that these interneurons play a large role in modulating synaptic integration at pyramidal cell dendrites, and previous research has suggested basket cells may have a role in lateral inhibition of cortical columns (Jones & Hendry 1984).

Martinotti cell axons ascend to layer I, providing the only source of cross-columnar inhibition in that layer (Fairen et al. 1984). Neurogliaform cell axons are heavily arborized and extend for long distances (Jones 1984). Neurogliaform cells, like basket cells, can receive thalamic input and could be responsible for some lateral inhibition. Double bouquet cells have axons extending to layers II – V with short collaterals in layers II, III, and sometimes V, and may synapse on pyramidal cell dendrites and other interneurons (Somogyi & Cowey 1984). Chandelier cells have vertically oriented axons that synapse on the initial segment of the pyramidal cell axon (Peters 1984).

We investigated 13 cortical fields found in frontal, parietal, insular, and temporal cortices. These included limbic and non-limbic agranular fields, one dysgranular field, one hypergranular field and 6 homotypical fields. Five of the areas we studied surrounded the central sulcus. Area 4, the primary motor cortex, is the primary source of motor commands to the brainstem and spinal cord motor neurons. Areas 3a, 3b, 1, and 2
make up the primary somatosensory cortex. Areas 3b and 1 receive inputs primarily from cutaneous receptors and project ventrally to the processing pathway that leads to memory formation for touch (Jones & Friedman 1982; Friedman et al. 1986). Areas 3a and 2 receive inputs predominantly from deep receptors and project caudally and dorsally to the processing stream that integrates somatosensory inputs into motor commands (Friedman & Jones 1981).

The insula plays a role in sensory processing, especially for interoceptive inputs. The insula in the monkey is made up of three different areas: the rostroventral agranular field, a transitional dysgranular field, and a posterior granular field (Augustine 1996). We focused on the dysgranular field. The agranular insula likely plays a role in integrating autonomic and visceral information into emotion or motivation. The dysgranular insula likely plays a role in somatosensory, vestibular, and perhaps auditory processing and projects to temporal lobe limbic areas important for memory formation (Mesulam & Mufson 1982; Mufson & Mesulam 1982; Augustine 1996). The granular insula likely has a role in higher order somatosensory processing (Naqvi & Bechara 2009). Cue-induced drug craving and rating of urge are related to activation of the insula in neuroimaging studies, and patients with insular lesions reportedly find it relatively easy to quit smoking without experiencing cravings (Naqvi & Bechara 2009; Naqvi & Bechara 2010). These studies indicate that interoception can play a role in the development and maintenance of addiction.

The fields within and surrounding the superior temporal sulcus are involved in higher order visual processing and multimodal sensory integration. TPO is a multimodal integration field (Baylis et al. 1987). TEa, in the lower bank of the superior temporal sulcus, is primarily a unimodal visual area responsible for higher order processing,
including facial recognition (Baylis et al. 1987). TE, in the inferior temporal gyrus, plays a role in visual discrimination tasks and visual short term memory and also projects to the amygdala (Baylis et al. 1987; Webster et al. 1991). Both TE and TEa primarily respond to stationary stimuli and are part of the ventral (“what”) visual pathway (Desimone & Gross 1979; Baylis et al. 1987).

We also studied two limbic areas, the subicular complex and the entorhinal cortex. The entorhinal cortex is the origin of the perforant path and the main input to the hippocampus, while the subiculum is the main output of the hippocampus. The subiculum also provides reciprocal feedback to the entorhinal cortex (Amaral et al. 1987; Kobayashi 1999). The subicular complex is composed of the subiculum, presubiculum, and parasubiculum, and has a role in spatial navigation and memory (O’Mara et al. 2001).

The cerebellum has been clearly linked to motor functions, but anatomical, functional, and behavioral data have recently suggested that the cerebellum participates in nonmotor functions, including cognition, affect, and attention (Fitzpatrick et al. 2008; Glickstein et al. 2009; Strick et al. 2009). Connectional studies in nonhuman primates reveal widespread cortical projections to the cerebellum via the pons (Schmahmann & Pandya 1997; Fitzpatrick et al. 2008), and the cerebellum, in turn, projects to widespread areas of the cerebral cortex, hippocampus, amygdala, and hypothalamus, including the mammillary bodies, through the deep nuclei (Anand et al. 1959; Dum & Strick 2003; Schmahmann 1997). Recently, imaging studies have found impaired function in the frontocerebellar executive loop in alcoholics, associated with deficits in verbal and spatial working memory tasks (Desmond et al. 2003; Chanraud et al. 2010). Decreases
in cerebellar volume in alcoholics have also been correlated with impairments in executive function (Sullivan et al. 2003; Oscar-Berman & Marinković 2007).

The cerebellum is made of three distinct layers: the outer cell sparse molecular layer, the single cell thick Purkinje cell layer, and the cell-dense granule cell layer. There are two types of inputs into the cerebellum. Climbing fibers arise from the inferior olivary complex and communicate directly with Purkinje cell dendrites. Mossy fibers carry information from the spinal cord, vestibular system, and brain stem (Fitzpatrick et al. 2008) and synapse on granule cell dendrites. The dendrites of granule cells and terminals of mossy fibers form a unique synaptic structure called the glomerulus. A third component of glomeruli are the axon terminals from GABAergic Golgi cells, which also synapse on granule cell dendrites (Rossi & Hamann 1998; Carta et al. 2004). This complex is enclosed by a glial sheath that inhibits diffusion of neurotransmitter from the glomerulus. This permits GABA to have influential extrasynaptic effects that modify granule cell excitability and synaptic transmission (Rossi & Hamann 1998; Farrant & Nusser 2005). The granule cells communicate with GABAergic Purkinje cell dendrites in the molecular layer. Purkinje cells are the output of the cerebellum.

Recent studies of human patients with cerebellar damage have led to the description of the cerebellar cognitive affective syndrome (CCAS). This syndrome includes problems in cognition, attention, visuospatial organization, visuospatial memory, affect, and language production (Schmahmann & Sherman 1998). Several impairments commonly seen in CCAS are also present in alcoholics, including visuospatial and working memory deficits. These data suggest that some cognitive and affective impairments seen in human alcoholics may in part be mediated by impaired cerebellar information processing.
GABA_\text{A} receptors in the cerebellum generally have the same subunit composition as those in the cortex, though an \( \alpha_6 \) subunit is present instead of an \( \alpha_4 \) subunit. The granule cell layer of the cerebellum contains the \( \delta \) subunit, which typically forms extrasynaptic receptors with the \( \alpha_6 \) subunit (Carta et al. 2004).

**Nonhuman Primate Model of Ethanol Self-Administration**

Animal models of drug self-administration are powerful because animals will readily self-administer most drugs of abuse, including ethanol, and animal models permit control over many variables that may potentially confound human research. Rodent models have been commonly used and have contributed greatly to our understanding of genetic and molecular changes resulting from chronic ethanol exposure. Nonhuman primates, however, more faithfully model human behavior than rodent models. Monkeys and humans also have very similar rates of ethanol metabolism (Green et al. 1999; Vivian et al. 2001). Moreover, the primate brain is more complex than the rodent brain, and there are many cortical fields that are present in monkeys and humans but not in rodents. Additionally, the widespread connections from the cerebellum to the frontal lobe of cerebral cortex are better elaborated in nonhuman primates than in rodents.

The studies reported here utilized the cynomolgus macaque to model the effects of excessive alcohol use in humans. These animals had *ad libitum* access to ethanol for 22 hours per day for either 12 or 18 months. This model is well established (Vivian et al. 2001; Grant et al. 2008) and the drinking histories of the monkeys for the proposed study have already been well characterized (Grant et al. 2008). Moreover, this model also
presents peripheral pathologies seen in alcoholics (Ivester et al. 2007; Williams et al. 2008; Cheng et al. 2010).

Total GABA$_A$, $\alpha 1$ subunit-containing, and $\alpha 4$ subunit-containing receptor binding densities were investigated in cortical areas 4, 3a, 3b, 1, and 2, at two levels of the dysgranular insula, TPO, TEa, TE, the entorhinal cortex, and the subicular complex. We investigated these cortical regions because they are representative of the various types of cortex and are involved in a wide array of higher cognitive functions that can be altered by ethanol. Total GABA$_A$, $\alpha 1$ subunit-containing, and $\alpha 6$ subunit-containing receptor binding density was investigated in the cerebellum. GABA$_A \alpha 1$-3, $\alpha 5$, $\alpha 6$, $\beta 1$-3, $\gamma 2$ and $\delta$ subunits and GABA$_B 1$ pan, GABA$_B 1a$, GABA$_B 1b$, and GABA$_B 2$ subunit gene expression were investigated in the hemisphere of the cerebellum.

We know that chronic ethanol decreases GABA$_A$ receptor function, and one possible explanation for this could be fewer receptors, reflected by less dense GABA$_A$ receptor binding, which is what we expected to find in various cortical fields and the cerebellum. Rodent literature has shown differential effects of chronic ethanol on GABA$_A$ and GABA$_B$ receptor subunits, but monkey data has demonstrated that chronic ethanol drinkers show decreased gene expression in several brain regions. We therefore expected to see decreased gene expression for many GABA$_A$ subunits in the cerebellum. We expect increased GABA$_B$ gene expression in the cerebellum, providing evidence of decreased GABAergic transmission.
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CHAPTER II

CHRONIC ETHANOL ALTERS TOTAL GABA\textsubscript{A} RECEPTOR AND $\alpha$ SUBUNIT BINDING DENSITY IN MONKEY CORTEX

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The following manuscript is in preparation to be submitted to Alcoholism: Clinical and Experimental Research. Stylistic variations are due to the requirements of the journal. Eugenia Dolson collected and analyzed data and prepared the manuscript. April Davenport performed the autoradiographic experiments. Kathleen Grant supplied the brain tissue. April Davenport, Kathleen Grant, and David Friedman acted in an advisory and editorial capacity.
ABSTRACT

Introduction: Chronic ethanol consumption alters GABAergic transmission and has been reported to alter the subunit composition and functional properties of GABA_A receptors in some brain regions. Previous studies in rodents and humans have presented conflicting data, however, suggesting that studies using a translational model with more complex brain structure than rodents and more controlled environment than human studies are necessary. To address this need we examined the changes in the densities of the total GABA_A receptor population, and α1-, and α4 subunit-containing receptors in 16 selected cortical fields using a nonhuman primate model of chronic ethanol self-administration.

Methods: Adult male cynomolgus macaques self-administered ethanol during 22 hour sessions in their home cage over 18 months. Control monkeys were exposed to the same environment and diet for eight months without an operant panel or ethanol. Brains were frozen in isopentane within 10 minutes of death and were processed for in vitro receptor autoradiography. The GABA_A receptor inverse agonist [³H]Ro15-4513 was used to estimate total GABA_A receptor density and co-incubation in the presence of 75nM Zolpidem or 100nM Diazepam allowed us to quantify the densities of the α1 or α4 subunit populations, respectively. Binding was measured in cortical areas from frontal, parietal, insular, and temporal lobes.

Results: Drinkers showed less dense total GABA_A receptor and α1 subunit-containing receptor binding globally and specifically in 7 of 16 cortical fields measured. Drinkers showed less dense α4 subunit-containing receptor binding globally and specifically in 5 of 16 cortical fields measured, and more dense binding in 1 cortical field. Results were also laminar specific in several of these fields.
**Conclusions:** Chronic ethanol affected the total population of GABA<sub>A</sub> receptors and α1, subunit-containing receptor binding densities equally, with α4 subunit-containing receptors showing 1 less affected field. There are discrete changes in different subpopulations of receptors that vary by field and layer, which suggest that the effects of ethanol likely depend on circuitry and are not just a direct effect of ethanol on GABA<sub>A</sub> receptors.

**INTRODUCTION**

GABA<sub>A</sub> receptors are pentameric ionotropic chloride channels typically made up of α, β, and γ or δ subunits. GABA<sub>A</sub> receptors are well known molecular targets of ethanol, and acute ethanol potentiates GABA<sub>A</sub> receptor function, whereas chronic ethanol attenuates function (Grobin et al. 1998). Rodent studies in which ethanol was delivered via the diet have found that α1 subunit gene expression and protein levels are decreased in cortical tissue, while α4 subunit gene expression and protein levels are increased in cortical tissue following chronic ethanol exposure (Devaud et al. 1995a; Devaud et al. 1997). GABA<sub>A</sub> gene and protein expression have also been studied in humans, but the data are conflicting. Two studies found no difference in α1 subunit gene and protein expression in the frontal cortex of alcoholics, whereas a third study found decreased expression of the α1 protein in the superior frontal cortex of uncomplicated alcoholics but no difference in cirrhotic alcoholics. A fourth study found increased gene expression of the α1 subunit in uncomplicated alcoholics but no difference in cirrhotic alcoholics (Lewohl et al. 1997; Mitsuyama et al. 1998; Thomas et al. 1998; Lewohl et al. 2001). While there has yet to be a comprehensive study of subunit gene expression, there is no reported difference in α1, α2, α3, α5, β1, β3, or γ2 mRNA (Thomas et al. 1998). One study demonstrated decreased α3 protein levels in the motor cortex of cirrhotic alcoholics (Lewohl et al. 1997).
2001). Human studies demonstrate both no difference and a significant increase in GABA_β3 gene expression without changes in protein levels (Mitsuyama et al. 1998; Thomas et al. 1998). One study reported no difference in either gene or protein expression for the α4 subunit (Mitsuyama et al. 1998). Studies of human alcoholics can be difficult to interpret and these findings suggest that factors other than alcohol, specifically cirrhosis, can play a role in GABA_α receptor gene and protein expression.

GABA_α receptor subunit gene expression has been investigated in the orbitofrontal cortex, dorsolateral prefrontal cortex, and anterior cingulate cortex of the same nonhuman primates used in this study. Chronic ethanol decreased specific subunit gene expression in a differential manner in two regions and anterior cingulate cortex was not affected (Hemby et al. 2006). The nonhuman primate model used in this study has several advantages. It offers a controlled environment that eliminates some confounds that can exist in human research. These animals self-administered ethanol, whereas other animal models used non-contingent ethanol administration. Additionally, the nonhuman primate cerebral cortex is highly analogous to humans and is more complex than in rodents, allowing for the study of brain regions not present in rodents.

In order to understand in more detail the effects of chronic ethanol on cortical GABA_α receptors, we sampled a variety of cortical areas in the nonhuman primate: areas 4, 3a, 3b, 1, and 2, the dysgranular insula, TPO, TEa, TE, the entorhinal cortex, and the subicular complex. These areas represent the major types of cerebral cortex and may therefore be representative of other cortical regions we did not directly sample. The sampled fields included limbic and nonlimbic agranular cortex, dysgranular cortex, konicortex, and homotypical cortex.
Regions surrounding the central sulcus that were measured in this study are areas 4, 3a, 3b, 1 and 2. Area 4 is the primary motor cortex, and areas 3a, 3b, 1, and 2 comprise the primary somatosensory cortex. Areas 4 and 3a are classic agranular cortex, area 3b is konicortex, and areas 1 and 2 are types of homotypical cortex.

The monkey insula contains three fields: a granular field, a dysgranular field, and an agranular field. We specifically measured the dysgranular field. This insular field plays an important role in interoceptive processing, and several imaging studies have linked insular activation to addiction (Naqvi & Bechara 2009; Naqvi & Bechara 2010).

The cortical areas surrounding the superior temporal sulcus are representative of higher order sensory processing fields and are all classified as homotypical cortex. TPO, in the upper bank of the superior temporal sulcus, is a multimodal association area that responds to somatosensory, visual, and auditory inputs (Baylis et al. 1987). TPO responds to the shape properties of stationary visual stimuli as well as movement and faces (Yeterian & Pandya 1991). TEa, in the lower bank of the superior temporal sulcus, is primarily a unimodal area responsible for higher order visual processing, including facial recognition (Baylis et al. 1987). TE, in the inferior temporal gyrus, plays a role in visual discrimination tasks and visual short term memory (Baylis et al. 1987). Both TE and TEa primarily respond to stationary stimuli and are part of the ventral (“what”) visual pathway (Desimone & Gross 1979; Baylis et al. 1987).

The subicular complex is a major output of the hippocampus, and also provides reciprocal feedback to the entorhinal cortex, which is the main input to the hippocampus and origin of the perforant path (Amaral et al. 1987; Kobayashi 1999).
The present study used a nonhuman primate model of chronic ethanol self-administration. This model allows for a long duration of experimental ethanol exposure with the animal voluntarily controlling the parameters of intake. A nonhuman primate model also allowed us to sample a variety of cortical fields that represent different types of cortex and different functions. We used autoradiography for precise anatomical resolution to study the total GABA\textsubscript{A} receptor population, \( \alpha 1 \), and \( \alpha 4 \) subunit-containing receptors.

**MATERIALS AND METHODS**

**Animal Model and Ethanol Self-Administration**

Adult male cynomolgus macaques (Macaca fascicularis), ages 66 – 78 months at the beginning of the study, were singly housed in quadrant racks. Four animals were trained to self-administer 4% (w/v) ethanol. Briefly, an operant panel that allowed access to all fluid and food requirements was attached to their home cage. The operant panel contained two drinking spouts, one lever, one pellet dispenser and stimulus lights associated with food and water or ethanol. Monkeys were induced to drink water for 30 days (1.5 g/kg) then subsequently increasing doses of ethanol: 0.5 g/kg, 1.0 g/kg, and 1.5 g/kg each over a 30 day period using a schedule-induced polydipsia technique. (Vivian et al. 2001; Grant et al. 2008). For 6 months, ethanol and water were available ad libitum and food was available in scheduled meals during daily 16-hour sessions. The monkeys then underwent a one year period of ethanol abstinence. The monkeys were again allowed ad libitum access to ethanol and concurrently available water, and food was available in scheduled meals from the panel in 22 hour sessions for 18 months. The total amount of ethanol consumed during the 18 month period ranged from 1210 to 2350 g/kg with daily averages ranging from 2.04 g/kg to 4.05 g/kg. Ethanol
intake correlated to blood ethanol concentrations (Vivian et al. 2001). Four different adult male cynomolgus macaques were housed in the same conditions with the same diet for 6 months. They did not have access to the drinking panels. All animal care and use was conducted in accordance with the National Institute of Health’s Guide for Care and Use of Laboratory Animals and under the supervision of Wake Forest University’s IACUC.

Necropsy

The average age of the ethanol drinkers at necropsy was 10.2 years, with a range of 9.8 to 11.4 years. The average age of the control monkeys at necropsy was 5.6 years, with a range of 5.5 to 5.83 years. Necropsies were scheduled to begin during the daily timeout period from food, water, and ethanol so that animals would not go into withdrawal. Animals were sedated with ketamine [15 mg/kg intramuscular (i.m.)] and brought into deep surgical plane of anesthesia with intravenous (i.v.) pentobarbital administered to effect (30–50 mg/kg). Following a complete craniotomy, each animal was perfused transcardially with ice-cold, oxygenated Krebs–Henseleit buffer for 1.5 minutes. The brain was then quickly removed from the skull and one hemisphere was blocked for autoradiography studies, and the other hemisphere was blocked for RT-PCR and electrophysiological studies (Anderson et al. 2007; Ariwodola et al. 2003; Floyd et al. 2004; Hemby et al. 2006; Sullivan et al. 2005). The tissue used for autoradiography was frozen within ten minutes of being removed from the skull.

Autoradiography

Brain blocks were sectioned on a cryostat at 20µm. Sections were thaw mounted onto plus-charged slides (Brain Research Laboratories, Newton, MA), desiccated under vacuum overnight at 4°C degrees and then stored at -80°C until use. Every tenth
section was Nissl stained. Seven serial sections were taken at four different levels for autoradiography. Total GABA<sub>A</sub> receptor binding was determined in the presence of 5nM [³H]Ro15-4513 (20Ci/mmol, Perkin Elmer Life Sciences, Boston, MA). The ligand was prepared in assay buffer (10mM KH₂PO₄, 100mM KCl, pH 7.4) and nonspecific binding was determined in the presence of 10µM unlabeled Ro15-4513. Binding to GABA<sub>A</sub> receptors containing the α1 subunit was determined in the presence of 5nM [³H]Ro15-4513 and 75nM zolpidem (Sullivan et al. 2005). This specific binding density was subtracted from the binding density of the total GABA<sub>A</sub> receptor population to determine the α1 subunit-containing receptor population. Binding of GABA<sub>A</sub> receptors containing the α4/6 subunit was determined in the presence of 5nM [³H]Ro15-4513 and 100nM diazepam (Sullivan et al. 2005). Ro15-4513, diazepam, and zolpidem and other reagents were obtained from Sigma (St. Louis, MO). Sections were preincubated in assay buffer for 10 minutes at 4°C then incubated in radioactive ligand (or unlabeled Ro15-4513 for nonspecific binding condition) for 90 minutes at 4°C and rinsed twice for 30 seconds each in assay buffer at 4°C and then dipped in ddH₂O. Slides were dried briefly under a stream of cold air and apposed to Fuji phosphorimaging plates for 5 days along with Amersham titrated RPA 506 and RPA 507 standards (Amersham Bioscience, Piscataways, NJ). The exposed plates were processed on a Fuji BAS 5000 phosphorimaging system (Fuji Medical Systems USA, Stamford, CT) and analyzed using MCID imaging software (InterFocus Imaging Ltd., Linton, United Kingdom). As each section was acquired, the image was exported to Adobe Photoshop 7 (Adobe Systems Inc., San Jose, CA). The same sections were then Nissl stained and Nissl images were captured and also downloaded into Photoshop; the images were then superimposed and regions of interest were defined on the overlaid image according to cytoarchitectonic criteria. A histogram of density throughout the region of cortex being measured was
used to determine which layers had similar densities; if there was no difference in density of adjacent layers, these layers were analyzed with a single region of interest (ROI), indicated by a slash between layers (for example, layers I/II). Layers analyzed independently are listed independently. See figure 1 for binding patterns of the total GABA<sub>A</sub> receptor population, α1, and α4 subunit-containing receptors. Nonspecific binding was not detectable under these binding conditions.

**Data analysis**

Sigma Stat (Systat Software, Inc., San Jose, CA) was used to perform two way ANOVA for a between group comparison of both field and layer.

**RESULTS**

**Laminar Distribution of GABA<sub>A</sub> Receptors**

We found three main laminar patterns of GABA<sub>A</sub> receptor distribution (Figure 2). Pattern 1 had the most dense binding in layer III with less dense binding in the other supragranular layers, and progressively less dense binding in the granular and infragranular layers. Pattern 2 had the most dense binding in layer I or layer I/II with binding density progressively decreasing through layer VI. Pattern 3 had the most dense binding in layers I-III, with progressively less dense binding through layer VI (pattern 3). Pattern 1 was seen in TPO, TEa, TE, dysgranular insula, and the majority of receptor binding conditions in areas 3a and 3b. Pattern 2 was found in the entorhinal cortex and subicular complex, while the majority of receptor binding conditions in areas 4, 1, and 2 corresponded to pattern 3. We did not detect any consistent relationship between the type of cortex and its binding pattern.
General Effects of Chronic Ethanol

When all cortical measurements were analyzed together, there was a significant main effect of less dense binding in the total GABA\textsubscript{A} receptor population ($p = 2.61 \times 10^{-8}$) and in the populations of $\alpha 1$ ($p = 5.82 \times 10^{-7}$) and $\alpha 4$ ($p = 7.32 \times 10^{-5}$) subunit-containing receptors in drinkers (Figure 3, Tables I, II, III). Specifically, there was less dense total GABA\textsubscript{A} binding in drinkers in areas 4, 3a, 3b, 1, 2, TE, the subicular complex, and the upper pyramidal cell layer of the subiculum. There was less dense $\alpha 1$ subunit-containing receptor binding in drinkers in areas 4, 3a, 3b, 1, 2, whole dysgranular insula, and rostral dysgranular insula. There was less dense $\alpha 4$ subunit-containing receptor binding in drinkers in areas 4, 1, TE\textsubscript{a}, TE, subicular complex, and the upper pyramidal cell layer of the subiculum. In every case where there was less dense binding of the total GABA\textsubscript{A} receptor population, there was also less dense $\alpha 1$ subunit-containing receptor binding, $\alpha 4$ subunit-containing receptor binding, or both. There was more dense $\alpha 4$ subunit-containing receptor binding in drinkers in the caudal dysgranular insula (Figure 4).

Chronic Ethanol Effects on Specific Cortical Fields

Areas 4, 3a, 3b, 1, and 2

There was a significant main effect in area 4 for less dense binding in drinkers of total GABA\textsubscript{A} receptors ($p = 2.78 \times 10^{-4}$), and a post hoc analysis showed a specific effect in the same direction for layer III ($p = 0.001$) and layer V/VI ($p = 0.012$). There was a significant main effect in area 4 for less dense binding of $\alpha 1$ subunit-containing receptors in drinkers, and a post hoc analysis revealed an effect in this same direction in layer III ($p = 0.024$). There was a significant main effect in area 4 for less dense binding of $\alpha 4$
subunit-containing receptors in drinkers, and a post hoc analysis showed less dense binding in layer III as well (p=0.03, Figure 5).

There was a significant main effect in areas 3a, 3b, 1, and 2 for less dense binding in drinkers of the total GABA\textsubscript{A} receptor population (p = 0.003, p = 0.031, p = 0.016, p = 0.041, respectively). A post hoc analysis revealed significantly less dense binding in the same direction in layer I/II of area 3a (p=0.045, Figure 6). There was a significant main effect in areas 3a, 3b, 1 and 2 for less dense binding of \(\alpha\)1 subunit-containing receptors in drinkers (p = 0.004, p = 0.001, p = 0.007, p = 0.034, respectively), and a post hoc analysis revealed less dense binding in layer I/II of areas 3a and 3b (p = 0.018, p = 0.002, respectively). Post hoc analyses showed an effect in this same direction in layer IV/V/VI of area 3b (p = 0.038, Figure 7), and in layer I/II/III (p = 0.026) and layer IV (p = 0.021) of area 1 (Figure 8). There was a significant main effect in area 1 for less dense binding of \(\alpha\)4 subunit-containing receptors in drinkers (p = 0.045).

*Dysgranular Insula*

The dysgranular insula was measured at two rostrocaudal levels. When data from both levels of the insula were analyzed together there was a significant main effect of less dense binding of \(\alpha\)1 subunit-containing receptors in ethanol drinkers (p = 0.01). There was a significant main effect at the more rostral level of the insula of less dense binding of \(\alpha\)1 subunit-containing receptors in drinkers (p = 0.003), and a post hoc analysis revealed a significant difference in the same direction in layer III (p = 0.047, Figure 9). There was a significant main effect of more dense binding of \(\alpha\)4 subunit-containing receptors in drinkers at the more caudal level of the insula (p = 0.032).
There was a significant main effect of less dense binding in drinkers of the total GABA\textsubscript{A} receptor population in TE ($p = 0.017$). There was a significant main effect in TEa and TE for less dense binding of $\alpha 4$ subunit-containing receptors in drinkers. A post hoc analysis revealed a specific effect in this same direction in layer V of TEa and TE ($p = 0.013$, $p = 0.02$, respectively, Figure 10). There were no significant differences in TPO.

**Entorhinal Cortex**

There was no significant difference between the ethanol group and the control group in the entorhinal cortex.

**Subicular Complex**

There was a main effect of less dense binding for total GABA\textsubscript{A} receptors and $\alpha 4$ subunit-containing receptors in the subicular complex ($p = 0.004$, $p = 0.003$, respectively). A post hoc analysis for both total GABA\textsubscript{A} receptors and $\alpha 4$ subunit-containing receptors revealed a specific effect in the same direction in the upper pyramidal cell layer ($p = 0.008$, $p = 0.01$, respectively, Figure 11). There were no significant differences in the presubiculum or the parasubiculum.

**Overview**

The total GABA\textsubscript{A} receptor is typically made up of either $\alpha 1$ or $\alpha 4$ subunits, though there are small populations of $\alpha 2$, $3$, and $5$ subunits throughout the brain. Our data indicate that changes in density of $\alpha 1$ subunit-containing receptors account for a large percentage of the differences seen after ethanol in the total population of GABA\textsubscript{A} receptors. The changes in $\alpha 1$ subunit-containing receptors in areas 3a, 3b, 1, and 2
account for nearly all the changes seen in the total GABA<sub>A</sub> receptor population in these areas. The percent change between controls and drinkers ranges from 0.2 – 24.5% (Tables I, II, III). The α1 subunit-containing receptors make up a larger portion of the total GABA<sub>A</sub> receptor population than α4 subunit-containing receptors. Receptors containing the α1 subunit account for 45% of the total GABA<sub>A</sub> receptor population globally in the cortex and α4 subunit-containing receptors account for 28%. Percentages for other cortical areas are in table IV.

**DISCUSSION**

Chronically self-administered ethanol induced a global decrease in total GABA<sub>A</sub> binding density, though the effect was moderate. Percent changes in fields measured range from 0.2% to 24.5%. Significant effects were seen in specific cortical fields and layers. Receptors containing α1 and α4 subunits were also altered differentially. There was less dense binding in drinkers for both the total population of GABA<sub>A</sub> receptors and α1 subunit-containing receptors in 7 of 16 fields measured. Less dense binding in drinkers of α4 subunit-containing receptors was seen in 5 of 16 fields measured, and one field showed more dense binding.

This is the first report of the effects of ethanol on GABA<sub>A</sub> receptor densities in nonhuman primates. Using a nonhuman primate model allowed us to sample significantly more cortical areas than have been reported in human studies, including fields that are not present in the rodent brain. We were also able to obtain greater anatomical resolution than has been reported in rat or human studies, and this is the first description of laminar distributions of the population of total GABA<sub>A</sub> receptors, α1, and α4 subunit-containing receptors in a variety of cortical areas in any species. This study also provided a
controlled method for chronic ethanol self-administration, and each animal had control of its own daily ethanol intake. Though the duration of drinking is short when compared to human studies, it is far longer than other animal studies. Techniques in this study also allowed us to examine subunits associated typically with both phasic ($\alpha_1$) and tonic ($\alpha_4$) inhibitory currents.

Chronic ethanol drinkers expressed lower densities of total GABA$_A$ receptors when all cortical data were analyzed together, and specifically in areas 4, 3a, 3b, 1, and 2, TE, and the subiculum. Chronic ethanol drinkers expressed lower densities of $\alpha_1$ subunit-containing receptors when all cortical data were analyzed together, and specifically in area 4, 3a, 3b, 1, and 2, global insula, and rostral level of the insula. They expressed lower densities of $\alpha_4$ subunit-containing receptors when all cortical data were analyzed together, and specifically in areas 4 and 1, TEa, TE, and the subiculum. They expressed more dense binding of $\alpha_4$ subunit-containing receptors in the more caudal level of the dysgranular insula.

Although we were able to measure differences between ethanol drinkers and control animals in many cortical fields, only four drinkers were included in this study. We may have seen greater differences or more layer-specific differences with a higher N. These animals also have a wide range of average daily ethanol intake (2.04 – 4.05 g/kg), but we saw no evidence that differences in GABA$_A$ receptor binding were dose related. Instead, it appears that there is a threshold effect since we were able to see significant differences even with lower ethanol consumption by some of the animals. The control animals also showed greater variability than drinkers in their receptor binding density measurements. A further technical limitation is that we were able to look at only two of
the subunits. Future studies should examine the effects of chronic ethanol on other GABA\textsubscript{A} subunit proteins, perhaps using Western blots.

Differences in both total GABA\textsubscript{A} and subunit-specific binding density after chronic ethanol can alter cortical information processing. Alterations in GABAergic current can occur through changes in receptor number, subunit composition of receptors, or the amount of GABA released. Less dense binding suggests a decreased receptor number and implies decreased current. We saw decreases in \( \alpha 1 \) subunit-containing receptors, which are more important in phasic inhibition and are typically found synaptically, as well as in the \( \alpha 4 \) subunit-containing receptors, which play a role in tonic inhibition and are typically found extrasynaptically. Both of these currents will likely be affected by differences in receptor and subunit binding density.

In this most comprehensive examination of the cerebral cortex to date, we saw overall less dense binding to the total GABA\textsubscript{A} receptor population, \( \alpha 1 \), and \( \alpha 4 \) subunit-containing receptors. Data in the literature are complex and contradictory, especially between species. Alcoholics are reported to have increased, decreased, or no difference in total GABA\textsubscript{A} receptor binding, and no difference in \( \alpha 1 \) subunit-containing receptor binding in what appears to be only 2 frontal cortical areas (Tran et al. 1981; Freund & Ballinger 1988). This is a clear indication of how factors other than ethanol can affect GABA\textsubscript{A} receptor density. Rodent studies report no change in total GABA\textsubscript{A} receptor or \( \alpha 1 \) subunit-containing binding in the cerebral cortex, though animals were receiving ethanol non-contingently and the duration of exposure was generally brief (Korpi et al. 1992; Devaud et al. 1995a). Our data also indicates field and layer-specific
differences, though it is difficult to compare to existing literature because of the variety of cortical areas studied here.

Previous studies have demonstrated that GABA\textsubscript{A} receptors play a role in determining the size and specificity of receptive fields of neurons in primary somatosensory, motor, and visual cortices as well as higher association areas. Inhibition of the GABA\textsubscript{A} receptors leads to larger, less specific receptive fields (Alloway & Burton 1991; Wang et al. 2002; Katzner et al. 2011).

The insula has three fields: the granular, dysgranular, and agranular fields. We were able to measure only the dysgranular field because of the rostral-caudal extent of the brain sections available for autoradiography. Therefore, our conclusions about GABA\textsubscript{A} receptors in the insula stem only from the apparently transitional dysgranular field. This field is known to project to temporal lobe limbic areas related to declarative memory and also likely plays a role in some somatosensory and vestibular processing (Augustine 1996).

TPO plays a role in multimodal integration, and both TE\textsubscript{a} and TE are responsible for higher order unimodal processing of visual information (Baylis et al. 1987; Hein & Knight 2008). Alterations in GABA\textsubscript{A} receptor binding in these areas will likely alter the way this information is integrated in these higher order processing areas.

The subicular complex is involved in spatial memory and navigation and a major output of the hippocampus (O’Mara et al. 2001). This area is affected by chronic ethanol, suggesting there may be an effect on spatial navigation and memory.
Studies of alterations of GABA<sub>A</sub> gene expression in the dorsolateral, orbitofrontal, and anterior cingulate cortex of the nonhuman primate showed field specific changes in gene expression. The orbitofrontal cortex showed decreased gene expression in α (including α4), β, and γ subunits. Dorsolateral prefrontal cortex showed decreased gene expression of β, γ, and δ. There were no changes in the anterior cingulate cortex (Hemby et al. 2006). It is not clear whether changes in gene expression will be reflected in protein expression, but these findings suggest the present data showing field-specific effects of ethanol.

There are several classes of GABAergic interneurons that are found in the cortex and whose terminal fields arborize in different layers. Basket cells are responsible for the majority of inhibitory input onto pyramidal cell dendrites and synapse primarily in layer III. These cells can also play a role in lateral inhibition between cortical columns because of several horizontal axon collaterals found in most layers of the cortex (Jones & Hendry 1984).

Martinotti cell axons extend to layer I where they inhibit pyramidal cell dendrites, providing the only source of cross-columnar inhibition in layer I (Markram et al. 2004). Neurogliaform cell axons are heavily arborized and extend for long distances (Jones 1984). Double bouquet cells have axons extending to layers II – V with short collaterals in layers II, III, and sometimes V. These cells may synapse on pyramidal cell dendrites, and may synapse on other interneurons (Somogyi & Cowey 1984). Chandelier cells have vertically oriented axons that synapse on the initial segment of the pyramidal cell axon (Peters 1984).
Chronic ethanol affects layers where interneuron axonal arborizations are seen. Basket, neurogliaform, and double bouquet cell axonal arborizations are likely affected in area 4, 3a, 3b, and 1, according to layer specific changes seen in those areas. Martinotti cells may be affected in areas 3a, 3b, and 1, where layer I shows less dense binding in drinkers. Chandelier cells may be affected in areas 4, 3b, and 1, where we demonstrated changes in layers III and V. Any alteration after chronic ethanol in specific layers of the primary motor or somatosensory cortices can affect cells found in that layer, but can also have an indirect effect through actions of these interneurons in other layers or in an overall region, thus affecting several classes of GABAergic inhibitory neurons that are responsible for inhibition either within or across cortical columns.

In conclusion, these data suggest that chronic ethanol decreases GABA_A receptor density in a region and layer specific manner. Effects were seen in the total population of GABA_A receptors, α1, and α4 subunit-containing receptors. The effects were seen in a variety of cortical areas, with functions that include visual, auditory, somatosensory, interoceptive, and motor processing, and memory. Studies of the functional implications of the changes in receptor density described here will need to focus on specific cortical areas where the changes occur.
REFERENCES


Figure 1 Whole cortical sections showing distribution of GABA\textsubscript{A} receptors and location of fields examined.  
A. Binding of \[^{3}\text{H}\]Ro15-4513 to visualize total GABA\textsubscript{A} receptors  
B. Binding of \[^{3}\text{H}\]Ro15-4513 and 75nM zolpidem to visualize \(\alpha\)1 subunit-containing receptors. Binding visible on the section represents all \(\alpha\) subunit-containing receptors except \(\alpha\)1.  
C. Binding of \[^{3}\text{H}\]Ro15-4513 and 100nM diazepam to visualize \(\alpha\)4 subunit-containing receptors. The approximate locations of the cortical fields measured in the current study are indicated in white numerals and letters.
Figure 2  Laminar binding patterns of total GABA_A receptor population. 

A. Pattern 1 shows most dense binding in layer III. This pattern was seen in TPO, TE, TEa, dysgranular insula, and the majority of receptor binding conditions in areas 3a and 3b. 

B. Pattern 2 shows most dense binding in layer I. This pattern was seen in entorhinal cortex and subicular complex. 

C. Pattern 3 shows most dense binding in layers I-III. This pattern was seen in majority of receptor binding conditions in areas 4, 1, and 2.
Figure 3  Chronic ethanol drinkers show less dense total GABA_\text{A}, \alpha_1 and \alpha_4 subunit-containing receptor binding globally in the cortex. *p ≤ 0.05, **p ≤ 0.01, actual P values are in text
Table I  Receptor Binding Density and Percent Change for the Total Population of GABA<sub>A</sub> Receptors

<table>
<thead>
<tr>
<th>Cortical Area</th>
<th>Control</th>
<th>Drinker</th>
<th>Percent Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global Cortex</td>
<td>431.01 ± 3.93</td>
<td>400.12 ± 3.6</td>
<td>7.2**</td>
</tr>
<tr>
<td>Area 4</td>
<td>437.16 ± 9.42</td>
<td>369.74 ± 9.42</td>
<td>15.4**</td>
</tr>
<tr>
<td>Area 3a</td>
<td>487.13 ± 9.98</td>
<td>434.1 ± 9.98</td>
<td>10.9**</td>
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<tr>
<td>Area 3b</td>
<td>521.75 ±14.91</td>
<td>470.34 ±14.91</td>
<td>9.9*</td>
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<td>Area 1</td>
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<td>371.08 ± 12.46</td>
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<tr>
<td>Area 2</td>
<td>408.77 ± 11.09</td>
<td>373.8 ± 11.09</td>
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<td>367.01 ± 9.57</td>
<td>3.6</td>
</tr>
<tr>
<td>TPO</td>
<td>420.56 ± 10.94</td>
<td>397.94 ± 9.48</td>
<td>5.4</td>
</tr>
<tr>
<td>TEa</td>
<td>427.2 ± 14.1</td>
<td>423.13 ± 12.21</td>
<td>1.0</td>
</tr>
<tr>
<td>TE</td>
<td>481.71 ± 15.06</td>
<td>430.83 ± 13.05</td>
<td>10.6*</td>
</tr>
<tr>
<td>EC</td>
<td>436.85 ± 15.31</td>
<td>438.31 ± 12.5</td>
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</tr>
<tr>
<td>Subiculum</td>
<td>391.19 ± 12.23</td>
<td>330.41 ± 10.59</td>
<td>15.5**</td>
</tr>
<tr>
<td>Upper Subiculum</td>
<td>415.31 ±32.79</td>
<td>363.6 ± 31.51</td>
<td>12.5**</td>
</tr>
<tr>
<td>Lower Subiculum</td>
<td>360.56 ± 13.3</td>
<td>324.13 ± 13.77</td>
<td>10.1</td>
</tr>
<tr>
<td>Presubiculum</td>
<td>374.0 ± 23.97</td>
<td>361.24 ± 20.76</td>
<td>3.4</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>462.62 ± 30.63</td>
<td>438.89 ± 21.66</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*Table I* Receptor binding density values (fmol/mg) and percent change between control and drinkers for the total population of GABA<sub>A</sub> receptors. *p ≤ 0.05, **p ≤ 0.01.  EC, entorhinal cortex
Table II  Receptor Binding Density and Percent Change for $\alpha_1$
Subunit-Containing Receptors

<table>
<thead>
<tr>
<th>Cortical Area</th>
<th>Control</th>
<th>Drinker</th>
<th>Percent Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global Cortex</td>
<td>195 ± 3.41</td>
<td>171.135 ± 3.12</td>
<td>12.2**</td>
</tr>
<tr>
<td>Area 4</td>
<td>216.22 ± 10.95</td>
<td>163.27 ± 10.95</td>
<td>24.5**</td>
</tr>
<tr>
<td>Area 3a</td>
<td>244.41 ± 11.68</td>
<td>185.81 ± 11.68</td>
<td>24**</td>
</tr>
<tr>
<td>Area 3b</td>
<td>273.53 ± 10.97</td>
<td>208.41 ± 10.97</td>
<td>23.8**</td>
</tr>
<tr>
<td>Area 1</td>
<td>213.64 ± 7.82</td>
<td>167.43 ± 7.82</td>
<td>21.6**</td>
</tr>
<tr>
<td>Area 2</td>
<td>206.13 ± 10.07</td>
<td>173.03 ± 10.07</td>
<td>16.1*</td>
</tr>
<tr>
<td>Insula</td>
<td>123.56 ± 6.13</td>
<td>98.79 ± 6.13</td>
<td>20.1**</td>
</tr>
<tr>
<td>Rostral Insula</td>
<td>119.09 ± 5.11</td>
<td>94.89 ± 5.11</td>
<td>20.3**</td>
</tr>
<tr>
<td>Caudal Insula</td>
<td>128.03 ± 11.99</td>
<td>102.68 ± 11.99</td>
<td>19.8</td>
</tr>
<tr>
<td>TPO</td>
<td>193.01 ± 10.37</td>
<td>181.96 ± 8.98</td>
<td>5.7</td>
</tr>
<tr>
<td>TEa</td>
<td>196.09 ± 10.55</td>
<td>197.79 ± 9.13</td>
<td>0.9</td>
</tr>
<tr>
<td>TE</td>
<td>213.76 ± 12.79</td>
<td>197.49 ± 11.08</td>
<td>7.6</td>
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<tr>
<td>EC</td>
<td>160.9 ± 9.32</td>
<td>139.09 ± 7.61</td>
<td>13.6</td>
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<tr>
<td>Subiculum</td>
<td>147.45 ± 14.95</td>
<td>135.47 ± 12.95</td>
<td>8.1</td>
</tr>
<tr>
<td>Upper Subiculum</td>
<td>134.02 ± 54.35</td>
<td>140.99 ± 13.45</td>
<td>5.2</td>
</tr>
<tr>
<td>Lower Subiculum</td>
<td>145.23 ± 46.73</td>
<td>140.99 ± 10.67</td>
<td>2.9</td>
</tr>
<tr>
<td>Presubiculum</td>
<td>170.12 ± 19.9</td>
<td>180.51 ± 17.23</td>
<td>6.1</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>169.31 ± 33.25</td>
<td>204.28 ± 23.51</td>
<td>13.9</td>
</tr>
</tbody>
</table>

Table II Receptor binding density values (fmol/mg) and percent change between control and drinkers for $\alpha_1$ subunit-containing receptor populations. *p ≤ 0.05, **p ≤ 0.01. EC, entorhinal cortex
Table III  Receptor Binding Density and Percent Change for α4 Subunit-Containing Receptors

<table>
<thead>
<tr>
<th>Cortical Area</th>
<th>Control</th>
<th>Drinker</th>
<th>Percent Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global Cortex</td>
<td>122.82 ± 0.98</td>
<td>117.46 ± 0.89</td>
<td>4.4**</td>
</tr>
<tr>
<td>Area 4</td>
<td>118.48 ± 4.51</td>
<td>102.85 ± 4.51</td>
<td>13.2*</td>
</tr>
<tr>
<td>Area 3a</td>
<td>129.16 ± 3.05</td>
<td>123.98 ± 3.05</td>
<td>4.0</td>
</tr>
<tr>
<td>Area 3b</td>
<td>129.47 ± 4.26</td>
<td>132.51 ± 4.26</td>
<td>2.3</td>
</tr>
<tr>
<td>Area 1</td>
<td>115.66 ± 2.54</td>
<td>107.84 ± 2.54</td>
<td>6.8*</td>
</tr>
<tr>
<td>Area 2</td>
<td>110.95 ± 3.18</td>
<td>104.92 ± 3.18</td>
<td>5.4</td>
</tr>
<tr>
<td>Insula</td>
<td>118.38 ± 2.22</td>
<td>123.58 ± 2.22</td>
<td>4.4</td>
</tr>
<tr>
<td>Rostral Insula</td>
<td>122.52 ± 2.78</td>
<td>122.7 ± 2.78</td>
<td>0.2</td>
</tr>
<tr>
<td>Caudal Insula</td>
<td>114.24 ± 3.13</td>
<td>124.45 ± 3.13</td>
<td>8.9##</td>
</tr>
<tr>
<td>TPO</td>
<td>114.46 ± 2.37</td>
<td>115.45 ± 2.05</td>
<td>0.9</td>
</tr>
<tr>
<td>TEa</td>
<td>121.34 ± 2.31</td>
<td>112.24 ± 2.0</td>
<td>7.5**</td>
</tr>
<tr>
<td>TE</td>
<td>132.79 ± 2.71</td>
<td>121.89 ± 2.35</td>
<td>8.2**</td>
</tr>
<tr>
<td>EC</td>
<td>151.96 ± 5.17</td>
<td>162.08 ± 4.22</td>
<td>6.7</td>
</tr>
<tr>
<td>Subiculum</td>
<td>129.63 ± 4.14</td>
<td>108.81 ± 3.58</td>
<td>16.1**</td>
</tr>
<tr>
<td>Upper Subiculum</td>
<td>138.91 ± 7.01</td>
<td>125.12 ± 9.98</td>
<td>9.9**</td>
</tr>
<tr>
<td>Lower Subiculum</td>
<td>117.77 ± 2.45</td>
<td>101.87 ± 7.2</td>
<td>13.5</td>
</tr>
<tr>
<td>Presubiculum</td>
<td>107.28 ± 9.23</td>
<td>85.43 ± 7.99</td>
<td>20.4</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>123.75 ± 4.34</td>
<td>116 ± 3.07</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Table III Receptor binding density values (fmol/mg) and percent change between control and drinkers for α4 subunit-containing receptor populations. *p ≤ 0.05, **p ≤ 0.01, #increased binding in drinkers. EC, entorhinal cortex
There was less dense binding for the total GABA<sub>A</sub> receptor population and the α<sub>1</sub> subunit-containing receptor population in 7 of the 16 fields measured. There was less dense binding of the α<sub>4</sub> subunit-containing receptor population in 5 of the 16 fields measured. Lightly shaded boxes indicate less dense binding in drinkers, black indicates more dense binding in drinkers. There was one region with more dense binding in drinkers for α<sub>4</sub> subunit-containing receptors. Regions of interest and number of fields are not equivalent because two of the regions of interest (whole cortex and subicular complex) contain multiple fields.

<table>
<thead>
<tr>
<th>Region</th>
<th>Total GABA&lt;sub&gt;A&lt;/sub&gt;</th>
<th>α&lt;sub&gt;1&lt;/sub&gt; subunit</th>
<th>α&lt;sub&gt;4&lt;/sub&gt; subunit</th>
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<tr>
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<tr>
<td>Area 3a</td>
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<tr>
<td>Area 3b</td>
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<tr>
<td>Area 1</td>
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<td></td>
</tr>
<tr>
<td>Area 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rostral Dysgranular Insula</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Caudal Dysgranular Insula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEa</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entorhinal Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subicular Complex</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Presubiculum</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Parasubiculum</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A. Area 4 Total GABA<sub>A</sub> Receptors

B. Area 4 α1 Subunit-Containing Receptors

C. Area 4 α4 Subunit-Containing Receptors

**Figure 5** Chronic ethanol drinkers have less dense total GABA<sub>A</sub>, α1, and α4 subunit-containing receptor binding in area 4. The first set of bars (area 4) represents the main effect for the whole area. A. There was less dense binding in total GABA<sub>A</sub> receptors in area 4, as well as layer III and V/VI. B. There was less dense binding of α1 subunit-containing receptors in area 4 as well as in layer III. C. There was less dense binding of α4 subunit-containing receptors in area 4 as well as in layer III. *p ≤ 0.05, **p ≤ 0.01
**Figure 6** Chronic ethanol drinkers have less dense total GABA$_A$ and $\alpha_1$ subunit-containing receptor binding in area 3a. The first set of bars (area 3a) represents the main effect. **A.** There was less dense binding in total GABA$_A$ receptors in area 3a, as well as layer I/II. **B.** There was less dense binding of $\alpha_1$ subunit-containing receptors in area 3a as well as in layer I/II. *$p \leq 0.05$, **$p \leq 0.01$
Chronic ethanol drinkers have less dense $\alpha_1$ subunit-containing receptor binding in area 3b. The first set of bars (area 3b) represents the main effect. There was less dense binding of $\alpha_1$ subunit-containing receptors in area 3b as well as in layer I/II and IV/V/VI. *p $\leq$ 0.05, **p $\leq$ 0.01

Figure 7 Chronic ethanol drinkers have less dense $\alpha_1$ subunit-containing receptor binding in area 3b. The first set of bars (area 3b) represents the main effect. There was less dense binding of $\alpha_1$ subunit-containing receptors in area 3b as well as in layer I/II and IV/V/VI. *p $\leq$ 0.05, **p $\leq$ 0.01
Figure 8 Chronic ethanol drinkers have less dense $\alpha_1$ subunit-containing receptor binding in area 1. The first set of bars (area 1) represents the main effect. There was less dense binding of $\alpha_1$ subunit-containing receptors in area 1 as well as in layer I/II/III and layer IV. *$p \leq 0.05$, **$p \leq 0.01$
Figure 9 Chronic ethanol drinkers have less dense $\alpha_1$ subunit-containing receptor binding in the rostral dysgranular insula. The first set of bars (insula) represents the main effect. There was less dense binding in $\alpha_1$ subunit-containing receptors in the rostral dysgranular insula and in layer III. *$p \leq 0.05$, **$p \leq 0.01$
Chronic ethanol drinkers have less dense α4 subunit-containing receptor binding in both TEa and TE. The first set of bars (TEa or TE) represents the main effect.

A. There was less dense binding in α4 subunit-containing receptors in TEa and in layer V.

B. There was less dense binding in α4 subunit-containing receptors in TE and layer V. *p ≤ 0.05, **p ≤ 0.01

Figure 10 Chronic ethanol drinkers have less dense α4 subunit-containing receptor binding in both TEa and TE. The first set of bars (TEa or TE) represents the main effect. A. There was less dense binding in α4 subunit-containing receptors in TEa and in layer V. B. There was less dense binding in α4 subunit-containing receptors in TE and layer V. *p ≤ 0.05, **p ≤ 0.01
Chronic ethanol drinkers show less dense total $\mathrm{GABA}_A$ and $\alpha_1$ subunit-containing receptor binding in the subiculum. The first set of bars (subiculum) represents the main effect. 

A. There was less dense binding in total $\mathrm{GABA}_A$ receptors in the subiculum as well as the upper level of subicular pyramidal cells. 

B. There was less dense binding in $\alpha_4$ subunit-containing receptors in the subiculum as well as the upper level of subicular pyramidal cells. 

$p \leq 0.05$, $**p \leq 0.01$
Table IV  α Subunit-Containing Receptors
Percentage of Total GABA<sub>A</sub> Receptors

<table>
<thead>
<tr>
<th>Cortical Area</th>
<th>α1 %</th>
<th>α4 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global Cortex</td>
<td>45</td>
<td>28</td>
</tr>
<tr>
<td>Area 4</td>
<td>49</td>
<td>27</td>
</tr>
<tr>
<td>Area 3a</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>Area 3b</td>
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<td>25</td>
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<tr>
<td>Area 1</td>
<td>51</td>
<td>28</td>
</tr>
<tr>
<td>Area 2</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>Insula</td>
<td>35</td>
<td>34</td>
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<tr>
<td>Rostral Insula</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>Caudal Insula</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>TPO</td>
<td>46</td>
<td>27</td>
</tr>
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<td>TEa</td>
<td>46</td>
<td>28</td>
</tr>
<tr>
<td>TE</td>
<td>44</td>
<td>28</td>
</tr>
<tr>
<td>Entorhinal Cortex</td>
<td>37</td>
<td>35</td>
</tr>
<tr>
<td>Subiculum</td>
<td>38</td>
<td>33</td>
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<tr>
<td>Upper Subiculum</td>
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<td>33</td>
</tr>
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<td>40</td>
<td>33</td>
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<tr>
<td>Presubiculum</td>
<td>45</td>
<td>29</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>37</td>
<td>27</td>
</tr>
</tbody>
</table>

Table IV  α Subunit-Containing Receptors Percentage of Total GABA<sub>A</sub> Receptors. The left column displays the percent of the total GABA<sub>A</sub> receptor population that is comprised of α1 subunit-containing receptors. The right column displays the percent of the total GABA<sub>A</sub> receptor population that is comprised of α4 subunit-containing receptors.
CHAPTER III

CHRONIC ETHANOL ALTERS GABAₐ RECEPTOR BINDING DENSITY AND GABA RECEPTOR SUBUNIT GENE EXPRESSION IN MONKEY CEREBELLUM

Eugenia Dolson, Scott Hemby, Brian McCool, April Davenport, Kathleen Grant, David Friedman

The following manuscript is in preparation to be submitted to Alcoholism: Clinical and Experimental Research. Stylistic variations are due to the requirements of the journal. Eugenia Dolson performed the experiments, collected and analyzed data, and prepared the manuscript. Brian McCool sequenced the primers and probes used in qPCR. April Davenport and Eugenia Dolson performed the autoradiographic experiments. Kathleen Grant supplied the brain tissue. Scott Hemby, Brian McCool, April Davenport, Kathleen Grant, and David Friedman acted in an advisory and editorial capacity.
ABSTRACT

Introduction: Chronic ethanol consumption attenuates GABAergic transmission and has been shown to alter gene expression and protein levels of GABA_A subunits in both the cerebral cortex and cerebellum. Very little is known about molecular effects of chronic ethanol on GABA_B receptors, especially in the cerebellum. Both GABA_A and GABA_B receptors are found within the cerebellum, and it is a structure that is particularly sensitive to ethanol. The cerebellum has traditionally been thought to play a role in motor learning and function, but it has recently been linked to cognitive function.

Methods: In this study we used a nonhuman primate model of chronic ethanol self-administration along with in vitro receptor autoradiography and qPCR to examine GABA receptor binding densities and subunit gene expression in the cerebellum.

Results: The total population of GABA_A receptors was less dense in ethanol drinkers in all layers of the hemisphere and in the combined layer analysis in the vermis. α6 subunit-containing receptors were less dense in the hemisphere. Both the GABA_A α1 gene and the GABA_B2 gene were upregulated in drinkers. There was also a significant negative correlation between average daily ethanol intake and the absolute expression of the GABA_A α2 gene.

Conclusions: These data suggest that chronic ethanol affects GABA_A receptor density and gene expression differentially. The effect on the total GABA_A receptor binding density is more widespread than the effect on α6 subunit-containing receptors. There is not a pervasive effect on gene expression, but rather a specific effect on a small number of genes.
INTRODUCTION

GABA_A receptors are inhibitory pentameric chloride channels that are well known targets of ethanol. GABA_A receptors are typically composed of subunits from α, β, and γ families, though a δ subunit can be substituted for a γ subunit (Pirker et al. 2000). Metabotropic GABA_B receptors are linked to the Gi/o protein, and consist of two subunits, GABA_B1 (R1) and GABA_B2 (R2) (Bettler & Tiao 2006). The two subunits must dimerize to form a functional receptor (Kaupmann et al. 1998; Jones et al. 1998; White et al. 1998). Both GABA_A and GABA_B receptors are densely distributed in the cerebellum. GABA_A receptors are located in all three layers of the cerebellum, while GABA_B receptors are found primarily in the molecular layer, with only a small number of receptors in the granule cell layer (Lu et al. 1999).

The cerebellum has classically been implicated in motor processing and motor learning. Damage to the cerebellum in alcoholics can result in ataxia, manifested by a broad, staggering gait (Nolte 2002). More recent findings implicate the cerebellum in higher cognitive functions more traditionally thought to be the province of the cerebral cortex. There is both functional and anatomical evidence that ethanol has a detrimental effect on cerebellar function. Recent imaging studies have found impaired function in the frontocerebellar executive loop in alcoholics, with deficits especially in verbal and spatial working memory tasks (Desmond et al. 2003; Chanraud et al. 2010). Decreases in cerebellar volume in alcoholics have also been correlated with impairments in executive function (Sullivan 2003; Oscar-Berman & Marinković 2007).

While there is ample evidence that ethanol alters GABA receptor function, few studies have found dramatic changes in GABA_A receptor density after chronic ethanol, instead reporting changes in subunit composition, which can alter receptor function (Weiner &
Studies in rodent cerebellum reported that $\alpha_1$ and $\beta_3$ gene expression were decreased after chronic ethanol, and $\alpha_6$, $\beta_2$, and $\gamma_2$ subunit gene expression were increased. $\alpha_3$ and $\alpha_5$ subunit gene expression did not change (Morrow et al. 1992; Grobin et al. 1998). Levels of $\alpha_1$ and $\delta$ subunit protein were decreased in the cerebellum, and there was an increase in $\alpha_6$ protein levels after chronic ethanol (Grobin et al. 1998; Ravindran et al. 2007). Currently, there are no primate studies that examine the effect of chronic ethanol on GABA receptor subunit gene or protein expression in the cerebellum. These findings suggest that ethanol has complex effects on the expression of GABA$_A$ receptor subunits that are regionally specific.

Even less is known about the effects of chronic ethanol on GABA$_B$ receptors. The majority of what we know comes from behavioral studies with baclofen, a GABA$_B$ agonist that is associated with reductions of alcohol craving in humans and ethanol intake in rodents (Colombo et al. 2000; Addolorato et al. 2000). Electrophysiological studies have demonstrated that the potentiating effects of ethanol on GABAergic transmission can be limited by ethanol effects on presynaptic GABA$_B$ receptors because these receptors decrease the release of GABA (Wan et al. 1996; Ariwodola & Weiner 2004). The only study of the effects of chronic ethanol on GABA$_B$ receptors reported that both mRNA and protein levels of R1 and R2 increased in cerebral cortex after 3 weeks of ethanol treatment, whereas in the hippocampus only mRNA levels of R1 are increased (Li et al. 2005). These changes indicate that chronic ethanol has an effect on the GABA$_B$ receptor expression, but that the effect may be regionally specific. It therefore appears that studies in other brain regions cannot predict what effect chronic ethanol will have on cerebellar GABA receptors.
We used a nonhuman primate model of chronic ethanol self-administration in this study. This model allows for a longer period of self-administration than rodent models, and also permits control over many of the variables that can confound human research. Moreover, the primate brain is more complex than the rodent brain and there are widespread connections from the cerebellum to prefrontal cortical fields in primates that do not exist in rodents. Monkeys and humans also have very similar rates of ethanol metabolism (Green et al. 1999; Vivian et al. 2001). The duration of drinking in this study, while long by animal standards, allows us to look at what are in effect relatively early stage molecular changes when viewed in the context of the duration of human alcoholism. Our results confirmed that ethanol’s effects on cerebellar GABA receptors are complex, as they are in other brain regions, but that there is an overall decrease in total GABAA receptor binding and increase in specific GABAA and GABAB subunit gene expression not seen in rodents.

MATERIALS AND METHODS

Animals and Ethanol Self-Administration

Two cohorts of adult male cynomolgus macaques (Macaca fascicularis) were used in this study. They were singly housed in a quadrant rack. The first cohort was aged 66 – 78 months at the beginning of the experiment. Six animals were trained to self-administer 4% (w/v) ethanol via an operant panel attached to their home cage that allowed access to all fluid and food requirements (Vivian et al. 2001; Grant et al. 2008). The operant panel contained two drinking spouts, one lever, one pellet dispenser and stimulus lights associated with food and water or ethanol. Monkeys were induced to drink water for 30 days (1.5 g/kg) then subsequently increasing doses of ethanol: 0.5 g/kg, 1.0 g/kg, and 1.5 g/kg each over a 30 day period using a schedule-induced polydipsia technique (Vivian et al. 2001; Grant et al. 2008). For 6 months, ethanol and water were available
ad libitum and food was available in scheduled meals during daily 16-hour sessions. The monkeys then underwent ethanol abstinence for 12 months. After the abstinence period, the monkeys were again allowed ad libitum access to ethanol and concurrently available water, and food was available in scheduled meals from the panel in 22 hour sessions for 18 months. The total amount of ethanol consumed by each animal during the 18 month period ranged from 593 to 2350 g/kg with daily averages ranging from 1.16 g/kg to 4.05 g/kg. Four adult male cynomolgus monkeys were housed in the same conditions with the same diet for 6 months. They did not have access to the drinking panels.

The second cohort was aged 50 – 62 months at the beginning of the experiment. Nine animals were trained to self-administer 4% (w/v) ethanol. This cohort had operant panels attached to their home cage that allowed access to all fluid and food requirements. These animals underwent the same training and induction period as the first cohort. Following the induction period, ethanol and water were available ad libitum and food was available in scheduled meals during daily 22 hour sessions for 12 months. The total amount of ethanol consumed during the 12 months period ranged from 499 – 1685 g/kg with daily averages ranging from 1.16 g/kg to 4.2 g/kg. Seven monkeys were housed in the same conditions with the same diet, but they did not have access to the operant panels. All animal care and use was conducted in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals and under protocols approved by Wake Forest University and Oregon Health and Science University’s IACUCs.
Necropsy

The average age of the first cohort of ethanol drinkers at necropsy was 10.5 years, while the average age of the second cohort was 7.65 years. The average age of the first cohort of control monkeys at necropsy was 6.5 years, while the average age of the second cohort was 15 years. Necropsies were scheduled to begin during the daily timeout period from food, water, and ethanol so that animals would not go into withdrawal. Animals were sedated with ketamine [15 mg/kg intramuscular (i.m.)] and brought into deep surgical plane of anesthesia with intravenous (i.v.) pentobarbital administered to effect (30–50 mg/kg). Following a complete craniotomy, each animal was perfused transcardially with ice-cold, oxygenated Krebs–Henseleit buffer for 1.5 minutes. The brain was then quickly removed from the skull and one hemisphere was blocked for autoradiography studies, and the other hemisphere was blocked for RT-PCR and electrophysiological studies (Anderson et al. 2007; Ariwodola et al. 2003; Floyd et al. 2004; Hemby et al. 2006; Sullivan et al. 2005). The cerebellum was removed from the brainstem by cutting through the peduncles and was then bisected through the midline.

Autoradiography

Brain blocks were sectioned sagittally on a cryostat at 20µm. Sections were thaw mounted onto plus-charged slides (Brain Research Laboratories, Newton, MA), desiccated under vacuum overnight at 4°C degrees and then stored at -80°C until use. Every tenth section was Nissl stained. The four most medial sections, which were part of the vermis, and the four most lateral sections, which were part of the cerebellar hemisphere, were used for these studies.
Total GABA<sub>A</sub> receptor binding was determined in the presence of 5nM \([^3\text{H}]\text{Ro15-4513}\) (20Ci/mmol, Perkin Elmer Life Sciences, Boston, MA). The ligand was prepared in assay buffer (10mM KH<sub>2</sub>PO<sub>4</sub>, 100mM KCl, pH 7.4) and nonspecific binding was determined in the presence of 10µM unlabeled Ro15-4513. Binding of GABA<sub>A</sub> receptors containing the α<sub>1</sub> subunit was determined in the presence of 5nM \([^3\text{H}]\text{Ro15-4513}\) and 75nM zolpidem (Sullivan et al. 2005). This specific binding density was subtracted from the binding density of the total GABA<sub>A</sub> receptor. Binding of GABA<sub>A</sub> receptors containing the α<sub>6</sub> subunit was determined in the presence of 5nM \([^3\text{H}]\text{Ro15-4513}\) and 100nM diazepam (Sullivan et al. 2005). Ro15-4513, diazepam, and zolpidem and other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Sections were preincubated in assay buffer for 10 minutes at 4°C then incubated in radioactive ligand (or unlabeled Ro15-4513 for nonspecific binding condition) for 90 minutes at 4°C and rinsed twice for 30 seconds each in assay buffer at 4°C and then dipped in ddH<sub>2</sub>O. Slides were dried briefly under a stream of cold air and apposed to Fuji phosphorimaging plates for 6 days along with Amersham titrated RPA 506 and RPA 507 standards (Amersham Bioscience, Piscataways, NJ). The exposed plates were processed on a Fuji BAS 5000 phosphorimaging system (Fuji Medical Systems USA, Stamford, CT) and analyzed using MCID imaging software (InterFocus Imaging Ltd., Linton, United Kingdom). As each section was acquired, the image was exported to Adobe Photoshop 7 (Adobe Systems Inc., San Jose, CA). Nissl images were captured and also downloaded into Photoshop; the images were then superimposed and molecular and granule cell layers were defined on the overlaid image according to cytoarchitectonic criteria.
**Quantitative Real Time Polymerase Chain Reaction**

Total RNA was isolated using Trizol (Sigma-Aldrich, St. Louis, MO) followed by chloroform extraction/isopropanol precipitation and stored at −80°C. Two micrograms of total RNA from each sample, as well as a pool of total RNA combined from all samples, was reverse transcribed in a 20µL reaction using SuperScript II kits (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Resulting cDNA product was diluted 1:150 with RNase-free water.

Sequences of the primers and probes of GABA$_A$α1 to α6, β1 to β3, γ2, and δ subunits and endogenous controls have been published previously (Floyd et al. 2004; Hemby et al. 2006; Anderson et al. 2007) and sequences of GABA$_B_1$ pan, GABA$_B_1a$, GABA$_B_1b$, and GABA$_B_2$ subunits and splice variants are described in Table 1. All probes were 5’-labeled with 6-FAM and 3’-labeled with BHQ1 (Integrated DNA Technologies, Coralville, IA). Quantitative PCR was performed using fluorescent probes in a 7900HTS real-time detector. 0.5 µL aliquots of primer mix (9µM), 0.5 µL of probe mix (2.5µM), 5 µL 2X Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, CA), and 3 µL diluted cDNA or water for a no template control (NTC) were mixed for each sample for each target gene, and an aliquot was placed into a single well of a 384-well PCR plate. A 2-fold dilution series of cDNA made from pooled samples (“standards”) was run on every plate for each gene of interest, as well as for β2 microglobulin (β2M), beta actin (ACTB), and succinate dehydrogenase complex, subunit A (SDHA), which were used as internal loading controls on every plate. Each sample, including NTC, was run in triplicate. Polymerase chain reaction conditions in the Applied Biosystems 7900HT were as follows: (1) 1 cycle 2 minutes at 50°C, (2) 1 cycle 10 minutes at 95°C, and (3) 40 cycles: 15 seconds at 95°C and 1 minute at 60°C. Fluorescence was measured during...
the 60°C step for each cycle. The reactions were quantified by the standard curve method (as described in User Bulletin #2, Applied Biosystems) using SDS2.1 software, where threshold cycle (Ct) for the target cDNA for each sample is selected and interpolated into a standard curve generated from the Ct values of the PCR product of interest in a 2-fold dilution series of cDNA standards. The Qty mean for each gene was calculated by the software using triplicate wells for each gene. The expression level of each gene of interest was normalized to the expression level of the endogenous reference in each sample.

Data analysis
Sigma Stat (Systat Software, Inc., San Jose, CA) was used to perform two way ANOVA which was used for a between group comparison of both field and layer for autoradiography. Data for each gene of interest was expressed as Qty mean for the gene of interest divided by the geometric average of the endogenous controls. Normalized values were then expressed as percent control. These values were used for subsequent statistical analysis. qPCR data were analyzed for each subunit (α, β, and the GABA<sub>B</sub> receptor) by two way ANOVA with group (control or drinker) and subunit (e.g. β1, β2, β3) as the main factors and mRNA level as the dependent measure. Data were analyzed by t-test for the δ, γ2, GABA<sub>B1</sub> pan, GABA<sub>B1a</sub>, GABA<sub>B1b</sub>, and GABA<sub>B2</sub> subunits.

RESULTS
Autoradiography
The first cohort was used for GABA<sub>A</sub> receptor binding. We independently measured both granule cell and molecular layers in both the vermis and hemisphere. We combined data for the vermis and hemisphere for a global analysis of ethanol’s effects
on the cerebellum, and combined data for the molecular and granule cell layer for the combined layer analysis (Table II).

**Chronic ethanol drinkers show less dense receptor binding in the cerebellum**

Chronic ethanol drinkers showed a main effect of less dense total GABA$_A$ receptor binding globally in the cerebellum ($p = 0.01$). Post hoc analysis showed less dense total GABA$_A$ receptor binding in the granule cell layer ($p = 0.038$) as well as in the combined layer analysis ($p = 0.01$, Figure 2A).

Chronic ethanol drinkers showed a main effect of less dense total GABA$_A$ receptor binding in the vermis ($p = 0.045$), manifested by less dense total GABA$_A$ receptor binding in drinkers in the combined layer analysis ($p = 0.045$, Figure 2B). Chronic ethanol drinkers also showed a main effect of less dense total GABA$_A$ receptor binding in the hemisphere ($p = 0.003$). Post hoc analysis showed less dense total GABA$_A$ receptor binding in drinkers in the granule cell layer ($p = 0.015$), the molecular layer ($p = 0.04$), and the combined layer analysis ($p = 0.003$, Figure 2C).

Ethanol drinkers showed a main effect of less dense $\alpha_6$ subunit-containing receptor binding in the hemisphere ($p = 0.027$), and a post hoc analysis revealed a significant difference in the same direction in the combined layer analysis in the hemisphere ($p = 0.027$, Figure 3). There were no significant differences for the $\alpha_1$ subunit-containing receptors.
Overview

The percent change between controls and drinkers ranges from 3.6% - 13.1% (Table II). $\alpha_1$ and $\alpha_6$ subunit-containing receptors made up similar proportion of the total population of GABA$_A$ receptors in every region of the cerebellum (Table III). $\alpha_1$ subunit-containing receptors can account for 22 – 31% of the change in the total GABA$_A$ receptor population, and $\alpha_6$ subunit-containing receptors can account for 13 – 35% of the change.

Average daily intake correlates to receptor binding density

The average daily ethanol intake in g/kg negatively correlated to total GABA$_A$ receptor binding density (fmol/mg) for the molecular layer in both the vermis ($p = 0.02, r^2 = 0.76$) and globally in the cerebellum ($p = 0.01, r^2 = 0.83$, Figure 4). Average daily intake ranged from 1.16 g/kg to 4.05 g/kg. Receptor binding density ranged from 353 fmol/mg to 534 fmol/mg in the molecular layer of the vermis, and 371 fmol/mg to 538 fmol/mg in the molecular layer of the global cerebellum.

Gene Expression

Chronic ethanol affected only two genes, one GABA$_A$ receptor subunit and one GABA$_B$ receptor subunit. Absolute expression for both of these genes was increased. There was also a significant correlation between average daily ethanol intake and absolute gene expression.

GABA$_A$ receptor subunit gene expression

There was a significant main effect of greater $\alpha$-family gene expression in drinkers ($p = 0.0003$), but there was no significant main effect for $\beta$-family genes. There was
significantly increased GABA_α1 gene expression (p = 0.001) in drinkers (Figure 5). There was no significant difference other GABA_α subunits (α2, α3, α5, α6, β1-3, γ2, and δ).

GABA_β receptor subunit gene expression
There were no significant main effects for all GABA_β genes or for GABA_β1 splice variants. There was no significant difference in GABA_β1, GABA_β1a, or GABA_β1b subunits and splice variants. There was, however, significantly increased expression of GABA_β2 subunit mRNA in the drinkers (p = 0.006, Figure 6).

Average daily ethanol intake correlates to gene expression
There was a significant negative correlation between average daily ethanol intake and gene expression for the GABA_α α2 subunit (p = 0.004, r^2 = 0.782, Figure 7).

DISCUSSION
Chronic ethanol self-administration had a moderate effect on GABA_α receptor binding density and only a small effect on GABA_α and GABA_β subunit gene expression in this first study in monkeys. Effects were primarily manifested on the total population of GABA_α receptors and on the gene expression of two subunits.

Chronic ethanol drinkers showed less dense total GABA_α receptor binding in the vermis and hemisphere, though the percent change was small. Only the hemisphere showed less dense α6 subunit-containing receptor binding in drinkers. There are significant negative correlations between average daily intake and total GABA_α receptor binding density in the molecular layer of both the vermis and globally in the cerebellum. There is
increased absolute gene expression for both the GABA_{\alpha} \alpha_1 subunit and the GABA_{\beta} \beta_2 subunit. There is a significant negative correlation between absolute gene expression of the GABA_{\alpha} \alpha_2 subunit and average daily ethanol intake.

This is the first report of the effects of chronic ethanol self-administration of GABA receptors in the cerebellum of nonhuman primates and is therefore an important translational advance in understanding ethanol’s effects. We report findings with greater neuroanatomical resolution than has been available to date in rat or human studies. The use of monkeys permitted control over many environmental variables and each animal drank its preferred amount during 22 hours of daily ethanol access. Though the duration of drinking is short when compared to the duration of drinking of humans who have been studied, it is far longer than other animal studies. This study also allowed for a comprehensive investigation of both GABA_{\alpha} and GABA_{\beta} receptor subunits using probes and primers specifically sequenced for the cynomolgus macaque. This is the first study to examine both receptor binding density and gene expression after chronic ethanol in a primate cerebellum.

Despite these strengths, this study has some methodological limitations. Two different cohorts were used for these experiments. One cohort went through two drinking periods, with an abstinence period of one year before finally drinking for 18 months prior to necropsy. Abstinence may affect receptor binding density findings, though it seems reasonable that the final 18 months of drinking had a far more profound effect, and we saw significant negative correlations between receptor binding density and average daily ethanol intake in this cohort. Due to the way tissue was processed at necropsy for the second cohort, it was not possible to assess both hemisphere and vermis for gene
expression. There is evidence in human literature that there may be a difference in sensitivity to ethanol between these regions, and we did see a difference in $\alpha_6$ subunit-containing receptors in the hemisphere, but not the vermis.

Previous studies in rodents and humans have provided conflicting data about the effects of chronic ethanol exposure on the cerebellum. In contrast to the findings reported here, ethanol administered chronically to rats reportedly decreased GABA$_A$ $\alpha_1$ subunit gene expression and protein levels and increased GABA$_A$ $\alpha_6$ subunit gene expression and protein levels (Morrow et al. 1992). Rodent studies using receptor binding techniques reported no differences in the density of total GABA$_A$ or $\alpha_1$ subunit-containing receptor binding and reported an increase in the density of $\alpha_6$ subunit-containing receptors (Becker & Jarvis 1996; Mehta & Ticku 1999). There is one report of decreased $K_d$ for GABA$_A$ binding in the cerebellum of alcoholics, with no change in overall or $\alpha_6$ binding (Korpi et al. 1992). In addition to species, factors like means and duration of ethanol exposure in rodents and potential comorbidities and polydrug abuse in the human population may explain such differences.

We report less dense binding in $\alpha_6$ subunit-containing receptors in the hemisphere of the cerebellum. This subunit, along with the $\delta$ subunit, is typically found extrasynaptically in the granule cell glomerulus. These extrasynaptic receptors are activated by ambient GABA and GABA spillover, which are contained in the glomerulus, and these receptors mediate tonic inhibition in granule cells (Carta et al. 2004). Less dense receptor binding likely translates to fewer receptors, which could lead to decreased inhibitory currents. This would allow increased information flow throughout the cerebellum, and this dysregulation may impair cerebellar functioning. Since we did
not report any differences in α1 subunit-containing receptors, it is likely that synaptic receptors are not significantly affected. Combined with less dense binding of total GABA\textsubscript{A} receptors, this may indicate that the primary effect is on extrasynaptic receptors that mediate tonic inhibition, which accounts for the majority of inhibition in the cerebellum (Hamann et al. 2002; Carta et al. 2004).

Our GABA\textsubscript{A} subunit gene expression and receptor binding density studies present divergent findings, indicating a dissociation between gene transcription and receptor expression. This suggests that that posttranslational modification, receptor trafficking and input from other signal transduction mechanisms may be affected by ethanol. Protein modifications like phosphorylation, palmitolation, and ubiquitination are all important for cell surface expression, receptor function, and receptor trafficking, and may possibly be altered by ethanol. Phosphorylation plays a role in maintaining receptor structure and function (Kumar et al. 2004). Palmitolation is involved in membrane targeting of receptors and subcellular trafficking (Arancibia-Cárcamo & Kittler 2009). Ubiquitination is also important for receptor expression at the cell surface (Arancibia-Cárcamo & Kittler 2009).

Ethanol is known to interact with several protein kinases, including PKC, which can alter transcription factors (Hu et al. 2008). There are several isoforms of PKC, some of which have been shown to interact with GABA\textsubscript{A} receptors (Kumar et al. 2004). For example, PKC\textsubscript{γ} interacts with GABA\textsubscript{A} α1 and α4 subunits in the cerebral cortex, and this isoform has been reported to modulate the effects of chronic ethanol (Kumar et al. 2004). Ethanol may also modulate signal transduction factors that interact with α6 subunits. Any effect of chronic ethanol on phosphorylation, ubiquitination, or palmitolation sites or
associated proteins could alter cell surface expression, possibly explaining the discrepancy we see between receptor binding data and gene expression data.

Preclinical and clinical trials have reported that baclofen reduces ethanol intake in rodents and humans, and also suppresses cravings in humans. Baclofen is a GABA$_B$ agonist that binds to the GABA$_B_1$ subunit. We found that the GABA$_B_2$ subunit shows increased gene expression after chronic ethanol. This subunit is coupled to the Gi/o protein and activates the second messenger signaling cascade, and is also responsible for trafficking the GABA$_B_1$ subunit to the cell surface. In order for the GABA$_B$ receptor to be functional, these two subunits must form a heterodimer. These data may suggest that while one of the subunits is upregulated after chronic ethanol, this may not lead to a change in the number of functional receptors.

A polymorphism of the GABA$_A$ $\alpha_2$ subunit has been correlated with increased risk for alcohol dependence in humans, particularly due to decreased responsiveness to ethanol (Roh et al. 2011). This subunit has also been correlated with impulsivity, another risk factor for alcoholism, and activation of the insula, a brain region that has been associated with cue-induced drug craving (Villafuerte et al. 2011).

In conclusion, chronic ethanol self-administration decreases total GABA$_A$ receptor binding globally in the cerebellum, and in the vermis and hemisphere. There is no difference in $\alpha_1$ subunit-containing receptors, but there is less dense binding in $\alpha_6$ subunit-containing receptors in the hemisphere, which suggests that extrasynaptic receptors may be more affected in the cerebellum. There is increased gene expression of the GABA$_A$ $\alpha_1$ subunit and of the GABA$_B_2$ subunit in drinkers. The discrepancy
between our gene expression and receptor binding data suggest posttranslational modification may occur after chronic ethanol.
REFERENCES


Figure 1 Whole cerebellar sections showing distribution of GABA<sub>A</sub> receptors and location of layers examined. 

A. Binding of [<sup>3</sup>H]Ro15-4513 to visualize total GABA<sub>A</sub> receptors

B. Binding of [<sup>3</sup>H]Ro15-4513 and 75nM zolpidem to visualize α<sub>1</sub> subunit-containing receptors. Binding visible on the section represents all α subunit-containing receptors except α<sub>1</sub>

C. Binding of [<sup>3</sup>H]Ro15-4513 and 100nM diazepam to visualize α<sub>6</sub> subunit-containing receptors. GC, granule cell layer; ML, molecular layer
Table I  Primer and Probe Sequence for GABA<sub>B</sub> Subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA&lt;sub&gt;B1&lt;/sub&gt; pan</td>
<td>CTACGACCCTTTCTATCAACTGC</td>
<td>GGGATGTCATGTGGAAATGC</td>
<td>TGTGATGCTGAATCTGGCAATACCC</td>
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<tr>
<td>GABA&lt;sub&gt;B1a&lt;/sub&gt;</td>
<td>GCCAGTGCACTATGGAGATTGAG</td>
<td>ATAAGACTTTGGAGACAGATTCGG</td>
<td>ACAGATATGGACACACCCCAGCCG</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B1b&lt;/sub&gt;</td>
<td>CTCCCAAGAAAAAGTGTCG</td>
<td>AACCAGAAAGCGGCAGTG</td>
<td>CCTAACGCTCCCCAACAGCTACC</td>
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<tr>
<td>GABA&lt;sub&gt;B2&lt;/sub&gt;</td>
<td>CCAAGGTCCGCAAGTG</td>
<td>CGTTCTGAGTGTGGCGTTCGAT</td>
<td>ACAGATATGGACACACCCAGCCG</td>
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Table II  Receptor Binding Density and Percent Changes in the Cerebellum

<table>
<thead>
<tr>
<th>Cerebellar Area</th>
<th>Control</th>
<th>Ethanol</th>
<th>Percent Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total GABA\textsubscript{A} Receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td>502.61 ± 15.86</td>
<td>443.17 ± 12.95</td>
<td>11.9**</td>
</tr>
<tr>
<td>Vermis</td>
<td>496.30 ± 18.56</td>
<td>444.12 ± 15.16</td>
<td>10.5*</td>
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<tr>
<td>Hemisphere</td>
<td>508.93 ± 14.72</td>
<td>442.22 ± 12.02</td>
<td>13.1**</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Cerebellar Area</th>
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<th>Percent Change</th>
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<tbody>
<tr>
<td><strong>α1 Subunit-Containing Receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td>216.33 ± 10.58</td>
<td>200.15 ± 8.64</td>
<td>7.5</td>
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<tr>
<td>Vermis</td>
<td>213.31 ± 12.18</td>
<td>197.1 ± 9.94</td>
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<tr>
<td>Hemisphere</td>
<td>219.34 ± 12.20</td>
<td>203.2 ± 9.96</td>
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<table>
<thead>
<tr>
<th>Cerebellar Area</th>
<th>Control</th>
<th>Ethanol</th>
<th>Percent Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α6 Subunit-Containing Receptors</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td>191.72 ± 7.57</td>
<td>176.54 ± 6.18</td>
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<tr>
<td>Vermis</td>
<td>189.09 ± 9.15</td>
<td>182.23 ± 7.47</td>
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<tr>
<td>Hemisphere</td>
<td>194.34 ± 7.48</td>
<td>170.85 ± 6.11</td>
<td>12.1*</td>
</tr>
</tbody>
</table>

Table II  Receptor binding density and percent changes in the cerebellum.  
A. Receptor binding density values (fmol/mg) and percent change between control and drinkers for the total population of GABA\textsubscript{A} receptors.  
B. Receptor binding density values (fmol/mg) and percent change between control and drinkers for \( \alpha \text{1} \) subunit-containing receptors.  
C. Receptor binding density values (fmol/mg) and percent change between control and drinkers for \( \alpha \text{6} \) subunit-containing receptors.  
*\( p \leq 0.05 \), **\( p \leq 0.01 \)
Figure 2  Chronic ethanol drinkers have less dense total GABA\(_A\) receptor binding globally in the cerebellum, in the hemisphere, and in the vermis A. There was less dense total GABA\(_A\) receptor binding globally in the cerebellum in the granule cell layer and combined layer analysis. B. There was less dense total GABA\(_A\) receptor in the molecular, granule cell, and combined layers in the hemisphere. C. There was less dense total GABA\(_A\) receptor in the combined layer in the vermis. * \(p \leq 0.05\), **\(p \leq 0.01\), actual P values are in text
Chronic ethanol drinkers have less dense $\alpha_6$ subunit-containing receptor binding in the combined layer analysis in the hemisphere. * $p \leq 0.05$

**Figure 3** Chronic ethanol drinkers have less dense $\alpha_6$ subunit-containing receptor binding in the combined layer analysis in the hemisphere. * $p \leq 0.05$
Table III  \( \alpha \) Subunit-Containing Receptors  
Percentage of Total GABA\( _A \) Receptors

<table>
<thead>
<tr>
<th>Cerebellar Area</th>
<th>( \alpha 1 ) %</th>
<th>( \alpha 6 ) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>Vermis</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>Hemisphere</td>
<td>43</td>
<td>38</td>
</tr>
</tbody>
</table>

**Table III** \( \alpha \) Subunit-Containing Receptors Percentage of Total GABA\( _A \) Receptors. The left column displays the percent of the total GABA\( _A \) receptor population that is comprised of \( \alpha 1 \) subunit-containing receptors. The right column displays the percent of the total GABA\( _A \) receptor population that is comprised of \( \alpha 6 \) subunit-containing receptors.
Figure 4 Total GABA_A receptor binding density correlates to average daily ethanol intake.  

A. Total GABA_A receptor binding in the molecular layer globally in the cerebellum correlates to average daily ethanol intake, p = 0.01, r^2 = 0.83.  

B. Total GABA_A receptor binding in the molecular layer of the vermis correlates to average daily ethanol intake, p = 0.02, r^2 = 0.76.
Figure 5 Chronic ethanol drinkers show increased GABA$_A$ $\alpha_1$ subunit gene expression, $^{**}p \leq 0.01$
Figure 6 Chronic ethanol drinkers show increased GABA$_B$ subunit gene expression, **p ≤ 0.01
Figure 7 GABA_A α2 subunit gene expression is correlated with average daily ethanol intake, p = 0.0036, $r^2 = 0.782$
CHAPTER IV

DISCUSSION

These studies have demonstrated that the effects of chronic ethanol on GABA_A receptors in the nonhuman primate brain are field, and often layer, specific. There is a moderate effect on the total population of GABA_A receptors, and on the populations of α1 and α4 subunit-containing receptors in the cerebral cortex, a sizable effect on the total population of cerebellar GABA_A receptors and a small effect on cerebellar α6 subunit-containing receptors. There was little effect on GABA_A and GABA_B receptor subunit gene expression in the cerebellar hemisphere.

Chronic ethanol affected the total GABA_A receptor population and the population of α1 subunit-containing receptors in an equal number of cortical fields, whereas the α4 subunit-containing receptors were affected in slightly fewer cortical fields. The total GABA_A receptor population and α1 subunit-containing receptor population each showed less dense binding in 7 of 16 fields, and the α4 subunit-containing receptor population showed less dense binding in 5 of 16 fields, with more dense binding in 1 field.

The most common effect of chronic ethanol on GABA_A receptor binding in the cerebellum was on the total population of GABA_A receptors, with only a small, specific effect of less dense α6 subunit-containing receptors in the hemisphere. The granule cell layer showed less dense binding more frequently than the molecular layer, and the hemisphere was more affected than the vermis.
There was very little effect of chronic ethanol on GABA\textsubscript{A} and GABA\textsubscript{B} receptor subunit gene expression in the posterior cerebellum, with only the GABA\textsubscript{A} \(\alpha1\) subunit and the GABA\textsubscript{B2} subunit showing upregulation.

There was a significant negative correlation between total GABA\textsubscript{A} receptor binding and average daily ethanol intake in the molecular layer of both the vermis and the global cerebellum. There was a significant negative correlation between GABA\textsubscript{A} \(\alpha2\) subunit gene expression and average daily ethanol intake.

**Study Design Strengths**

This study presents data on GABA receptors that have not previously been available. The nonhuman primate self-administration model employed here has been developed relatively recently, and it has been shown to reliably model human drinking patterns in ways that rodent models do not. Monkey self-administration therefore represents an important translational approach to the study of alcohol. The duration of drinking, while short by the standards of the human alcoholics, is quite long by the standards of animal research. To the extent that duration of exposure has an effect on GABA receptor density and gene expression, this study has an important advantage over previous studies in rodents.

The use of *in vitro* autoradiography in monkeys allowed us to examine a broad array of cortical fields, some of which are not present in the rodent, and none of which have yet been studied in humans. The laminar analysis used here supplied a level of resolution that has so far not been available in the literature and has allowed us to present the first
description of normal GABA\textsubscript{A} receptor distribution in monkey cortex. The combination of broad sampling and high anatomical resolution is a noteworthy advance in the literature.

The monkeys also lived in a controlled environment that eliminated many of the confounding factors typically seen in human studies. This makes it likely that the effects reported here are due to the effects of ethanol.

**Study Limitations**

Despite the strengths of the current design, these studies do have some limitations. Due to tissue sharing with other investigators, we used tissue from two different cohorts, with different lengths of self-administration. One cohort had 16 hours/day of access to ethanol for six months, followed by one year of abstinence and then 18 months of self-administration, when they had access to ethanol for 22 hours/day. The second cohort had access to ethanol 22 hours/day for 12 months and did not go through an abstinence period.

**Cross species analysis**

The data presented here is in partial agreement with findings already present in the literature, but is discrepant with other findings from both rodents and humans. Differences in how ethanol was administered, species differences, effective blood ethanol concentrations, and length of ethanol exposure may explain some of the differences, both among the rodent studies and between the monkey and rodent studies. Human studies present their own inherent difficulties, which include polydrug abuse, comorbid physical and psychiatric conditions, poor nutrition and factors related to brain preservation.
Chronic Ethanol Affects Rodents, Nonhuman Primates, and Humans Differentially

Discrepancies between the rodent and human literature are not uncommon, and our studies only partially resolve these species differences. Rodent studies have demonstrated decreased $\alpha_1$ gene expression and protein levels in the whole cortex and cerebellum following chronic ethanol (Mhatre & M. Ticku 1992; Devaud et al. 1997). We saw a similar decrease in some cortical fields, including the areas surrounding the central sulcus and in the insula. Some human studies have reported that there is no difference in $\alpha_1$ subunit gene expression or protein levels in alcoholics (Mitsuyama et al. 1998), and we similarly saw no differences in TPO, TEa, TE, entorhinal cortex, the subicular complex, and both vermis and hemisphere of the cerebellum. This suggests that one source of the previous discrepancies may have been the result of combining cortical fields or studying only a limited number of fields. We have attempted to address this issue by examining 18 different brain regions in our study.

Rodent studies have reported increased $\alpha_4/6$ gene expression and protein levels in the whole cortex and cerebellum following chronic ethanol (Mhatre & Ticku 1992; Devaud et al. 1997). We found such an increase in only in the caudal dysgranular insula, suggesting that the $\alpha_4$ gene expression and protein level increases seen in rodents may be specific to rodents. Mitsuyama et al. did not report any differences in $\alpha_4$ subunit protein levels in human superior frontal cortex, and in this study $\alpha_4$ subunit-containing receptor binding density was unchanged in areas 3a, 3b, 2, TPO, entorhinal cortex, presubiculum, and parasubiculum. There is no difference in $\alpha_6$ subunit-containing receptor binding density in the cerebellar vermis. Unlike both the rodent and human literature, we saw less dense $\alpha_4$ subunit-containing receptor binding in areas 4 and 1, the insula, TE, TEa, and the subicular complex. We saw less dense $\alpha_6$ subunit-
containing receptor binding in the cerebellar hemisphere. Studies of additional fields in humans would be needed to determine if ethanol induced changes in α4 subunit-containing receptor binding densities in the human brain. There do not seem to be disagreements among findings from rodent studies, so there may be true species differences between rodents on the one hand and monkeys and humans on the other. A monkey model is advantageous translationally to address the possible rodent-primate species difference, and can indicate whether there may a nonhuman primate-human species difference as well.

Even though the majority of human studies have focused on changes in the superior frontal cortex of alcoholics, the receptor binding data from these studies are not consistent. Different studies found increased, decreased, or no change in total GABA_A receptor binding density in this general brain region, and no difference in α1 subunit-containing receptor binding (Tran et al. 1981; Freund & Ballinger 1988; Lewohl et al. 1997). These receptor binding studies may reflect some of the well known difficulties in using in human tissue.

There are reports of decreased K_D for GABA_A receptor binding in the cerebellum of alcoholics, with no change in the density of the total GABA_A population or in α6 subunit-containing receptors (Korpi et al. 1992). We also saw no difference in α6 subunit-containing receptor binding density in the vermis, though we saw a decrease in the cerebellar hemisphere.

Rodent studies have also examined total GABA_A receptor binding density in both the cortex and cerebellum. There are reports of no differences or increased total GABA_A
receptor binding density and no differences in $\alpha_1$ subunit-containing receptor binding density in either cortex or cerebellum, but an increase in $\alpha_6$ subunit-containing receptor binding density in mouse cerebellum after chronic ethanol (Buck & Harris 1990; Becker & Jarvis 1996; Mehta & Ticku 1999). There is therefore some agreement between rodent and human studies about the effects of chronic ethanol on the density of the total population of GABA$_\alpha$ receptors, but discrepancies concerning $\alpha_6$ subunit-containing receptors. Our data agree with portions of both the human and rodent data. This indicates that the effects of ethanol may be due to possible species differences as well as some differences in methods.

We found that there was less dense binding of the total population of GABA$_\alpha$ receptors throughout the cerebellum, and less dense $\alpha_6$ subunit-containing receptor binding in the hemisphere of the cerebellum, which disagrees with both human and rodent literature in the cerebellum. We showed less dense total GABA$_\alpha$, $\alpha_1$, and $\alpha_4$ subunit-containing receptor binding in several cortical fields. This agrees with a portion of the human literature that investigated total GABA$_\alpha$ receptor binding, though our $\alpha_1$ subunit-containing receptor results disagree. Our results disagree with existing rodent literature about the effects of chronic ethanol exposure on total GABA$_\alpha$ and $\alpha_6$ subunit-containing receptor binding, though there is agreement among the $\alpha_1$ subunit-containing receptor results.

**Mechanism changes in GABA receptor expression**

Some human studies reported that $\alpha_1$ gene expression increased in the cortex of alcoholics, but saw no difference in receptor binding density (Lewohl, Crane & Dodd 1997a; Lewohl, Crane & Dodd 1997b). We saw a similar result in the posterior lobe of
the nonhuman primate cerebellum. The failure of gene expression data to simply predict receptor binding data is not unusual. Such a discrepancy suggests that posttranslational modifications may occur after chronic ethanol. Posttranslational modification can include mechanisms like phosphorylation, ubiquitination, and palmitololation, all of which may possibly be altered by ethanol. There is also evidence that ethanol can modulate several components of second messenger signaling cascades that are responsible for gene transcription and receptor expression at the cell surface.

Phosphorylation can affect the availability of the binding site necessary for endocytosis (Arancibia-Cárcamo & Kittler 2009; Vithlani et al. 2011), and GABA_A endocytosis can be increased after chronic ethanol. This is thought to be dependent on PKA signaling (Kumar et al. 2003). Both PKA and PKC activation can affect gene transcription, depending on which subunit is phosphorylated (Hu et al. 2008; Arancibia-Cárcamo & Kittler 2009). PKC has several isoforms, some of which are known to mediate the effects of chronic ethanol. These isoforms can have opposing effects on subunit regulation and on behavior induced by ethanol, including anxiolytic, ataxic, and sedating effects (Messing et al. 1991; Ueno et al. 2001; Kumar et al. 2004; Kumar et al. 2009).

Ubiquitination can serve as a signal for endocytosis or degradation (Vithlani et al. 2011), and there is some evidence that ubiquitination proteins may be targeted by ethanol. If receptor activity is decreased, perhaps through decreased function, this can result in increased ubiquitination levels, leading to reduced receptor expression levels (Arancibia-Cárcamo & Kittler 2009). Reduced receptor expression at the cell surface may translate to less dense receptor binding, without affecting levels of gene transcription.
Palmitolation can enhance membrane association of a receptor, and can be involved in postsynaptic clustering and subcellular trafficking (Arancibia-Cárcamo & Kittler 2009; Vithlani et al. 2011). GABA\textsubscript{A} receptor associated proteins can also be involved in trafficking and synaptic clustering (Farb et al. 2007). Although a direct action of ethanol on several receptor associated proteins and proteins necessary for posttranslational modification has not often been studied, it is likely that ethanol can have some effect on these proteins through an indirect mechanism.

We saw upregulated expression of the GABA\textsubscript{B2} subunit. This subunit is coupled to the G protein and activates the second messenger system, while GABA\textsubscript{B1} contains the binding site for GABA, agonists, and antagonists (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998). Without an upregulation of the GABA\textsubscript{B1} subunit, it is difficult to conclude that this difference in the GABA\textsubscript{B2} subunit could result in increased receptor number.

**GABAergic Cortical Interneurons**

The binding patterns reported here likely match the axonal arborizations of various GABA interneurons. Known location of these interneurons in the primate brain could provide a suggestion of their function in a control condition, and we may be able to make some inferences about how effects of ethanol in specific layers may affect specific types of interneurons. Several types of GABAergic interneurons have been identified, including basket cells, chandelier cells, Martinotti cells, double bouquet cells, and neurogliaform cells (Markram et al. 2004). Their axon terminals are generally located in different layers and may synapse on either cell bodies and initial axon segments or dendrites. When interneurons target axons they can have a potent effect on the generation and timing of action potentials, therefore playing a role in synchronizing
neuronal activity. When dendrites are the targets of interneurons, they can affect integration of synaptic activity and modify signal to noise ratio (Markram et al. 2004).

Basket cells account for a large percentage of the inhibition impinging on pyramidal cells. Their axons extend horizontally and synapse on somata of nearby pyramidal cells in layer III to sharpen cortical processing by inducing lateral inhibition of cortical columns (Jones & Hendry 1984). Basket cells are found in layers that receive extrinsic afferents, including thalamic, association, and callosal input (Jones & Hendry 1984). Studies have shown that these cells are important for entraining oscillations involved in attention, sensory integration, sleep, and memory (Méndez & Bacci 2011).

Chandelier cell axons can ascend or descend, and target the initial segment of pyramidal cell axons (Peters 1984). Chandelier cells may receive callosal afferents. Martinotti cells axons project to layer I, targeting pyramidal cell dendrites, and run horizontally across cortical columns to provide an additional source of lateral inhibition (Markram et al. 2004). Double bouquet cell axons typically descend from layers II or III to V and give off collaterals in layers III and V that may synapse on pyramidal cell dendrites and play a role in intracolumnar inhibition. These cells may also synapse on other interneurons (Somogyi & Cowey 1984). Neurogliaform cells likely receive thalamic input and may be important for lateral inhibition, at least in layer IV. Their axons are heavily arborized and may synapse on dendrites of target cells (Jones 1984; Markram et al. 2004).

Many of the regions we measured showed laminar-specific effects. Based on the layers showing significantly less dense total GABA\textsubscript{A}, \(\alpha_1\), and \(\alpha_4\) subunit-containing receptors, chronic ethanol can have a direct effect on neurons postsynaptic to basket,
neurogliaform, and double bouquet cells in areas 4, 3a, 3b, and 1. Martinotti cells may be affected in areas 3a, 3b, and 1, where layer I shows less dense binding in drinkers. Chandelier cells may be affected in areas 4, 3b, and 1, where we demonstrated changes in layers III and V. Alterations in one layer of the cortex will affect cells in other layers, in turn affecting regions beyond the one in which we saw significantly less dense binding.

Although our data showed labeling for the total population of GABA<sub>A</sub> receptors, along with α1, and α4 subunit-containing receptors in every layer of each cortical region measured, we were able to establish three different binding patterns. Pattern 1, showing most dense binding in layer III, was found in the majority of binding conditions in areas 3a and 3b, dysgranular insula, TPO, TE<sub>a</sub>, and TE. Pattern 2, where the most dense binding was in layer I, was found the entorhinal cortex and subicular complex. Pattern 3, where most dense binding was seen homogeneously in layers I-III, was found in the majority of receptor binding conditions in areas 4, 1, and 2.

GABA<sub>A</sub> receptors have been demonstrated to play a role in determining the size of neuronal receptive fields in the primary motor, visual and somatosensory cortices and in higher visual processing areas. Blocking GABA<sub>A</sub> receptors will enlarge receptive fields, making them less specific (Alloway & Burton 1991; Wang et al. 2002; Katzner et al. 2011). Similar effects may apply to other areas that have not yet been investigated. Any alteration in neuronal sensitivity will affect how information is processed in a particular region, and thus what information is available to be communicated to other regions for further processing.

It is difficult to draw conclusions about the effect that differences in GABA<sub>A</sub> receptor binding density may have in most of the cortex because the role of GABA<sub>A</sub> receptors has
not yet been well investigated. We can conclude, however, that decreasing GABA$_A$ receptor density likely translates to fewer receptors, which may well lead to decreases in inhibitory current, affecting information processing.

We demonstrated less dense total GABA$_A$ receptor binding globally in the cerebellum, in the vermis, and in the hemisphere. Our data suggest that the granule cell layer is more affected by chronic ethanol than the molecular cell layer, and the hemisphere is more affected than the vermis. GABA$_A$ receptors in the granule cell layer mediate both phasic and tonic inhibition, particularly within the glomerulus (Carta et al. 2004). We did not see a difference in $\alpha$1 subunit-containing receptor binding, but we did find less dense $\alpha$6 subunit-containing receptor binding. This subunit is typically found extrasynaptically, which suggests that extrasynaptic receptors may be more affected than synaptic receptors that contain the $\alpha$1 subunit. Extrasynaptic receptors mediate tonic inhibition, which accounts for the majority of the inhibition seen in the granule cell layer.

Alcoholics can show impaired cognitive function that closely resembles the cerebellar cognitive affective syndrome (CCAS) produced by other disease processes. Patients with CCAS show deficits in executive functioning, particularly deficits in planning, abstract reasoning, working memory, and decreased verbal fluency (Schmahmann & Sherman 1998). These patients also showed impaired visuospatial memory and organization as well as problems with anomia and agrammatism (Schmahmann & Sherman 1998). Patients with damage to the vermis showed severe mood swings, blunted emotion, or inappropriate behavior (Schmahmann & Sherman 1998). Long-term alcoholics similarly show impaired verbal fluency and visuospatial processing, blunted affect, memory, and also show impaired activation of the cerebellum and frontal cortices
during cognitive tasks (Desmond et al. 2003; Oscar-Berman & Marinković 2007; Chanraud et al. 2010). Alcoholics also show impairments in verbal and spatial learning, memory, executive functions, decision making, and information processing (Sullivan & Pfefferbaum 2005; Dom et al. 2006; Cacace et al. 2011). It is possible that some of these deficits may be due to altered GABAergic function in the cerebellum as well as in the cerebral cortex.

Gene Expression and Ethanol Intake Correlations

We saw a significant negative correlation between average daily ethanol intake in our animals and \( \text{GABA}_\alpha \text{2} \) gene expression. A single nucleotide polymorphism (SNP) of the GABRA2 gene, coding for the \( \alpha2 \) subunit, has been associated with lower subjective effects of ethanol, increased impulsivity scores, and greater activation of the insula during anticipation of both reward and loss (Villafuerte et al. 2011). This SNP has been identified as being associated with a risk for alcoholism, as well as risk for relapse in an alcoholic. The study identifying the association between this SNP and insula activation suggests that this subunit could be involved in the interoceptive process, and may play some role in drug craving. Our data, however, show no significant difference in \( \alpha2 \) subunit gene expression, and a study by Haughey et al. corroborate our findings. That study demonstrated that there was no significant difference \( \alpha2 \) subunit gene expression or protein levels in the prefrontal cortex of alcoholics (Haughey et al. 2008). However this group did report a significant difference in \( \alpha2 \) subunit gene expression between GABRA2 genotype groups. This provides evidence for the involvement of the GABRA2 gene in alcoholism, even though there is not necessarily a change in its expression in alcoholics or in our nonhuman primate model of chronic ethanol self-administration.
GABA<sub>B</sub> Receptors and Ethanol

There is little data on the effects of ethanol on GABA<sub>B</sub> receptor gene or protein expression. Li et al. reported increases in GABA<sub>B1</sub> and GABA<sub>B2</sub> subunit gene expression and protein levels in the cerebral cortex due to chronic ethanol exposure, but only increased GABA<sub>B1</sub> mRNA in the hippocampus (Li et al. 2005). We found that GABA<sub>B2</sub> mRNA was increased in the cerebellum, as is seen in the rat cerebral cortex. These data once again suggest regional differences.

GABA<sub>A</sub> and GABA<sub>B</sub> Receptor Interactions

The GABA<sub>B</sub> receptor can be both an autoreceptor and a heteroreceptor, implying that this receptor could play a significant role in mediating excitation and inhibition throughout the brain (Billinton et al. 2001). Electrophysiological studies have revealed interactions between the GABA<sub>A</sub> and GABA<sub>B</sub> receptors that can modulate the effects of chronic ethanol (Wan et al. 1996; Ariwodola & Weiner 2004). These studies have been conducted in the hippocampus, though this relationship may exist in other brain regions. An additional interaction between the GABA receptors has been discovered. The GABA<sub>A</sub> γ2 subunit can traffic the GABA<sub>B1</sub> subunit to the cell surface, a role that has previously been thought to be performed solely by the GABA<sub>B2</sub> subunit (Balasubramanian et al. 2004). Despite the method of trafficking, both GABA<sub>B</sub> subunits are required to assemble a functional receptor. Investigating both GABA<sub>A</sub> and GABA<sub>B</sub> receptors could provide data that may aid in a more complete description of independent and cooperative roles for these receptors.

Translational Considerations

Though our data do not reconcile many of the existing species differences, there is some congruence between our data and previous findings from both rodent and human
Most importantly, we report a global decrease in GABA<sub>A</sub> receptor binding that supports human PET and SPECT findings that also showed decreased GABA<sub>A</sub> binding potential. Our model featured a greater length of ethanol self-administration than other animal models and also allowed us to control most of the potentially confounding variables seen in human literature. We have also conducted the most comprehensive study to date of GABA<sub>A</sub> receptor binding density in any species, and our findings of regional and laminar differences in ethanol's effects of GABA<sub>A</sub> receptor density support the need for such comprehensive analyses. Our GABA<sub>A</sub> and GABA<sub>B</sub> receptor subunit gene expression findings from in the cerebellum suggest that posttranslational mechanisms may be playing an important role in the effects of chronic ethanol. We have not yet been able to study GABA receptor gene expression throughout the cortex, but our findings suggest that there will be discrepancies between the gene and protein expression results and should trigger the study of posttranslational mechanisms in the control of GABA receptor density.

Conclusions

These studies report data that support the idea that ethanol affects both GABA receptor and gene expression in nearly every brain region. There was a global decrease in the total GABA<sub>A</sub> receptor binding density, with significant decreases in 9 of 18 fields measured. α<sub>1</sub> subunit-containing receptor binding density was decreased in 7 of 18 fields, and α<sub>4/6</sub> subunit-containing receptor binding density was decreased in 6 of 18 fields while increasing in 1 field. There was little effect of chronic ethanol on gene expression of either GABA<sub>A</sub> or GABA<sub>B</sub> subunits. The GABA<sub>A</sub> α<sub>1</sub> subunit and the GABA<sub>B<sub>2</sub></sub> subunit gene expression were upregulated in drinkers. There were also significant negative correlations for both gene expression and receptor binding density.
with average daily ethanol intake. Our gene expression findings did not predict receptor binding data for the cerebellum, suggesting posttranslational modification may occur. These studies demonstrated region and layer specific effects of chronic ethanol on total GABA\textsubscript{A} receptor, \(\alpha1\), and \(\alpha4\) subunit-containing receptor binding density, and information processing is likely affected in nearly every region we analyzed.
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CURRICULUM VITAE

Education:
M.S. Wake Forest University Graduate School of Arts and Sciences
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B.S. Rhodes College
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Major Concentration: Neuroscience
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2007 – 2008: St Jude Children’s Research Hospital
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2003 – 2004: Rhodes College, student assistant in the Digital Media Lab

Research Experience:
Wake Forest University School of Medicine, Department of Physiology and Pharmacology
Thesis topic: Effects of chronic ethanol self-administration on GABA_A & GABA_B receptors in the nonhuman primate brain
Advisor: Dr. David Friedman, Ph.D., June 2009 – present
• Gained expertise in drug administration by both a nasogastric tube and an intravenous catheter
• Developed surgical skills to harvest live brain tissue during a necropsy
• Sedated and transported animals for MRI scans
• Used shaping techniques to train nonhuman primates to self-administer food and water
• Sectioned tissue on a cryostat for autoradiography
• Visualized cell bodies using a Nissl stain on tissue sectioned on the cryostat
• Analyzed GABA_A receptor binding density in areas 4, 3a, 3b, 1, 2, dysgranular insula, TPO, TEa, TE, entorhinal cortex, subicular complex, vermis and hemisphere of the cerebellum
• Performed qPCR of the posterior lobe of cerebellum to assess the effect of chronic ethanol on GABA_A and GABA_B receptor subunits
• Tested new autoradiography protocols

Wake Forest University School of Medicine, Department of Physiology and Pharmacology
Changes in the BDNF receptor after limited and extended access to cocaine
Rotation Advisor: Dr. Scott Hemby, Ph.D., January 2009 – May 2009
• Used qPCR to determine differences in gene expression for splice variants of BDNF
• Tested a BDNF-sensitive antibody using Western blot

Wake Forest University School of Medicine, Department of Physiology and Pharmacology
Effects of chronic ethanol and early life stress on 5HT$_{1A}$ receptor density
Rotation Advisor: Dr. David Friedman, Ph.D., August 2008 – December 2008
• Determined and analyzed receptor density using autoradiography
• Learned to draw blood from awake nonhuman primates

St. Jude Children’s Research Hospital, Developmental Neurobiology
Changes in dopamine neuron counts in the substantia nigra of a mouse model of Parkinson’s disease
Advisor: Dr. Richard Smeyne, Ph.D., June 2007 – May 2008
• Cut rodent brain tissue on a microtome
• Learned immunohistochemical techniques to visualize dopamine neurons
• Stereological counting of dopamine neurons in the substantia nigra

St. Jude Children’s Research Hospital, Developmental Neurobiology
Effects of chronic methylphenidate on motor and somatosensory neurons
Advisor: Dr. Richard Smeyne, Ph.D., June 2007 – May 2008
• Golgi stained brain sections
• Traced somatosensory and motor neurons using Neurolucida
• Analyzed both volume and number of branch points of dendrites

St. Jude Children’s Research Hospital, Radiation Oncology
Predicting behavioral problems in craniopharyngioma survivors after conformal radiation therapy
Advisor: Dr. Thomas Merchant, D.O, Ph.D., May 2005 – August 2006
• Saw patients twice per week to understand physical and neuroanatomical changes that occur with pediatric brain tumors
• Analyzed data from the Child Behavior Checklist and other psychological tests that were given annually up to five years after treatment to patients treated at St. Jude
• Contoured frontal lobes from co-registered MRI and CT scans to assess whether changes in frontal lobe volume were correlated to changes in psychological test scores
• This project resulted in a first author publication in Pediatric Blood and Cancer

Professional Memberships:
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**Professional Meetings:**
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- Research Society on Alcoholism, San Antonio TX, June 2010
- Society for Neuroscience, San Diego CA, November 2010
- Research Society on Alcoholism, Atlanta GA, June 2011
- Society for Neuroscience, Washington DC, November 2011

**Honors and Awards:**
- 2007  Hunter Award for Excellence in Neuroscience
- 2006  Undergraduate Research and Creative Activities Symposium Presentation
  Rhodes College, winner of first place in the session
- 2003 – 2007  Cambridge Scholarship, worth $16,500 annually

**Professional Interests:**
- Utilizing a nonhuman primate model of chronic ethanol self-administration to investigate neurobiological effects and endocrine changes that occur with chronic ethanol
- Using molecular techniques, including qPCR and autoradiography, to investigate effects of chronic ethanol intake on GABA receptors in the nonhuman primate brain

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**Journal Articles:**

EP Dolson, HM Conklin, C Li, X Xiong, TE Merchant “Predicting behavioral problems in craniopharyngioma survivors after conformal radiation therapy” Pediatric Blood and Cancer 2009 Jul;52(7):860-4

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EP Dolson, SE Hemby, BA McCool, KA Grant, DP Friedman. *Chronic Ethanol Alters GABA<sub>A</sub> and GABA<sub>B</sub> Receptors in the Monkey Posterior Cerebellum.* [In preparation]

**Abstracts:**


**EP Dolson**, AT Davenport, KA Grant, DP Friedman. GABA_\text{A} receptor and alpha subunit densities are altered in the cerebellum in a monkey model of chronic ethanol self-administration. *Society of Neuroscience Annual Meeting, San Diego, CA, 2010*


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"Implications of Radiation Therapy on Memory in Craniopharyngioma Patients" Undergraduate Research and Creative Activities Symposium, Rhodes College 2006

**Community Activities and Service:**

- Station Development for Brain Awareness Council June 2009 - present
- Graduate Student Association (GSA) representative to the GPSA, June 2009 – May 2010
- GSA Treasurer, June 2009 – May 2010
GSA co-chair December 2008 – May 2009

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Member of Brain Awareness Council September 2008 – present