CANDIDATE GENE ANALYSIS OF THE $GABA_A$ RECEPTOR SUBUNIT CLUSTER ON MOUSE CHROMOSOME 11 FOR ACUTE ETHANOL WITHDRAWAL SEVERITY

by

Heather M. Hood

A DISSERTATION

Presented to the Department of Behavioral Neuroscience

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

December 2003

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Heather M. Hood

has been approved

[redacted]

Professor in charge of thesis

[redacted]

Member

[redacted]

Member

[redacted]

Member

[redacted]

Member

TABLE OF CONTENTS

List of Tables and Figures

Tables

Figures

ACKNOWLEDGEMENTS

What an interesting journey this has been! I would never have made it this far without the help and guidance of so many wonderful people – each and everyone one you have affected me deeply both at the scientific and personal level. First, I would like to thank my mentor, Kari J. Buck. What an accomplishment - you helped a psychology major transform into a molecular biologist/behavioral geneticist! I did not even know what PCR was when I arrived in your lab (you've said that you like a challenge)! The other day I found a flow-chart of my rotation project that you drew which made me realize how much I have learned during these past few years (and with writing this thesis, how much I still don't know)! I must say, it has also been great watching you grow scientifically (Associate Professor Buck!). On the personal side, thanks for hanging in there with me when times got rough – your empathy helped me get through it.

John Belknap and John Crabbe, my "co-mentors", have helped me become a much better scientist. When I would pop my head into John B's office and say "Got a minute?" he had more than just one to give. Thanks for all the great statistical and experimental design advice (and the refrigerator)! My writing and professional skills have dramatically improved with John Crabbe's guidance and suggestions. My whole advisory committee- David Grandy, chair, Kari Buck, thesis advisor, John Belknap, John Crabbe and Robert Hitzemann - has helped me scientifically mature.

The current and past members of the Buck Lab have been one of my greatest resources – both scientifically and personally. Matthew Reilly, my pen throwing target (like you don't deserve it!!), your sarcastically dry sense of humor has

stopped me from throwing a $50,000 piece of equipment (or myself) out the window! More importantly, thanks for being an awesome friend and listening when experiments and life-related stuff just were not going well. Although that #$%^ antibody is still not cooperating, Renee Shirley gives great experimental advice. But even more relevant, Renee has told me some secrets of getting of out Graduate School alive. My QRT-PCR experiments – the main focus of my dissertation (i.e., expression) were significantly improved with tremendous help from Nikki Walter (I am now confident that the "Q" represents quantitative)! Other lab members, Nguyet Pham, Dimitri Boss, and Nate Spofford gave me excellent technical assistance.

Members of the Souse Mouse lab helped me with several experiments. Most importantly Pam Metten and her data facilitated the D1D2 haplotype mapping described in this thesis. Statistically, Pam is a whiz as well and has given me awesome suggestions on how to analyze my data. John Belknap's Lab have "leant" me mice more than once to get me through a pinch (how can Jax not have D2 mice?). Laurie O'Toole and Melinda Helms have also got me rolling with laughter more times than I can count.

On a personal note, there are those "non-science" people who have impacted my life the most. My parents, Ed and Judy Hood, encouraged my education. But more importantly had faith in me when I did not, which carried me over many rough spots. This dissertation is dedicated to my Dad, who taught me never to quit (especially after that cheerleading incident in 5th grade). Lastly, I thank my partner Ramsin Eiwazpour-Adeh, your nature has given me a different perspective in which to view and appreciate the universe with.

ABSTRACT

Alcoholism is complex disease that is influenced by many biological and environmental factors. Although, many twin and adoption studies show a genetic role in alcoholism, the particular genes have yet to be elucidated. Genetic heterogeneity and environmental differences in the human population makes the identification of genes influencing alcoholism a complicated undertaking. Therefore, we use well-defined preclinical (mouse) models to identify genomic loci that contain genes that may be relevant to alcoholism. Although we cannot completely mimic this disease in mice, we assess phenotypes that are thought to be related to this disorder, for instance, alcohol (ethanol) withdrawal severity. DBA/2J (D2) and C57BL/6J (B6) mice are well-characterized mouse strains that display severe verses mild ethanol withdrawal reactions. Our laboratory detected three chromosomal regions or quantitative trait loci (QTL) that modulate acute ethanol withdrawal using mice derived from the D2 and B6 progenitors strains. These loci map to Chromosome 1 (*Alcw1*), 4 (*Alcw2*) and 11 (*Alcw3*).

The next logical step following QTL identification is to determine which gene(s) is *the* quantitative trait gene (QTG). There are many valid methods that can be used to identify QTG such as functional complementation tests (e.g., gene knock-in studies). Here, we took a multidisciplinary approach to identify the most promising candidate gene(s) that underlie *Alcw3*. We fine-mapped this QTL by assessing Chromosome 11 haplotype structure between genetically informative inbred mouse strains, DBA/1J and DBA/2J mice. We narrowed the genomic interval to ~ 4 centimorgans and eliminated several false candidate genes while retaining a cluster of $GABA_A$ receptor subunit genes that includes $\gamma 2$

(*Gabrg2*), α1 (*Gabra1*), α6 (*Gabra6*) and β2 (*Gabrb2*). Next, we elected to focus on this gene cluster as potential candidates. We sequenced the coding region of *Gabrg2* in D2 and B6 mice and detected three single nucleotide polymorphisms (SNPs). One SNP alters amino acid sequence; D2 mice have a threonine at residue 11 of the mature γ2 peptide, whereas B6 mice possess an alanine. Other sequencing results show that α1, α6, and β2 peptides do not differ between D2 and B6 mice. Functional differences in ethanol action were not detected in a recombinant receptor system expressing γ2 variation. These SNPs may instead be a molecular marker for polymorphism within a regulatory region that alters expression of *Gabrg2* or another closely linked gene. We therefore measured *Gabrg2*, *Gabra1*, *Gabra6*, and *Gabrb2* expression in inbred mouse strains that differ in acute ethanol withdrawal severity. Cortical *Gabrg2* expression is greater in the D2 strain compared to B6 mice. However, we did not detect a difference in *Gabra1*, *Gabra6*, and *Gabrb2* expression. We assessed γ2 heterozygous null mutant mice for acute ethanol withdrawal severity and show that decreased γ2 peptide is associated with increased withdrawal magnitude. Our mapping, sequencing, expression, and behavioral data converge upon *Gabrg2* as the strongest candidate gene to date to underlie *Alcw3*. Thus, the work presented here is an illustration of how one may go from QTL to QTG. Further characterization of *Gabrg2* is however warranted to say definitively that this is *the* gene.

I. Introduction

I.A. Alcohol Dependence

Alcoholism is a complex disease that has environmental, psychological, and biological determinants. It is characterized by compulsive behavior to consume alcohol that can impair social, mental, and physical well-being (DSM IV, 1994). Many twin (*e.g.*, Heath et al., 1997; Prescott and Kendler, 1999) and adoption studies (*e.g.*, Sigvardsson et al., 1996) indicate that there is a genetic contribution to alcoholism. Overall, it is estimated that between 40 and 60% of the phenotypic variance is genetically determined (Burmeister, 1999; Schuckit, 1998). During the past decade there has been a large multicenter effort to map the chromosomal regions involved in alcohol dependence through the Collaborative Studies on the Genetics of Alcoholism (COGA). To date, many studies have investigated the association between genomic loci and endophenotypes for alcoholism. These studies include behavioral (*e.g.*, low level of response to alcohol; Schuckit et al., 2001) and biological phenotypes (*e.g.*, P300 amplitude; Hesselbrock et al., 2001), which are thought to be genetically influenced. However, the genes that predispose humans to alcoholism remain to be elucidated. The identification of these genes in the human population is a daunting task due to genetic heterogeneity and the lack of marker genes known to be associated with alcohol dependence (Schuckit, 1994).

I.B. Alcohol Withdrawal

All species tested, including humans and mice, display a withdrawal syndrome following the removal of alcohol (see Metten and Crabbe, 1999b for

review). Depending on where in the time course of withdrawal (e.g., hours or days), symptoms present can include increased anxiety, psychomotor agitation, peripheral nervous system disturbances (e.g., tachycardia), and CNS hyperexcitability including milder symptoms like tremor to life-threatening convulsions. These CNS symptoms occur usually once ethanol is metabolized but do tend to wax and wane in intensity. Moreover, not all alcoholics experience every alcohol withdrawal sign.

I.C. Genetics and Alcohol Withdrawal

Clinical studies have shown that acute alcohol withdrawal syndromes may predict potential risk for future onset of alcoholism. In a survey study, sons of alcoholics reported greater hangover symptoms compared to sons of non-alcoholics. These symptoms are thought to represent an acute withdrawal syndrome. However, sons of alcoholics and non-alcoholics do not differ in amount or frequency of alcohol consumption (Newlin and Pretorius, 1990). Span and Earleywine (1999) reported that sons of alcoholics experienced greater acute withdrawal symptoms, including tremor, alcohol craving, and irritability, after consuming 0.5 g/kg of ethanol compared to sons of non-alcoholics. After ingesting 1.0 g/kg ethanol, college aged males who are family-history positive for alcoholism reported greater subjective feelings of "shakiness" compared to family-history negative males (McCaul et al., 1991). These studies indicate that acute ethanol withdrawal may be a predictor of future alcoholism, in that individuals with a history of alcoholism within their family may drink to alleviate the negative symptoms associated with acute ethanol withdrawal (Span and Earleywine, 1999). Although the above studies indicate that acute ethanol

withdrawal can predict risk for alcoholism, they do not begin to identify the genes that contribute to this trait.

I.D. Acute Ethanol Withdrawal Phenotype in Mice

Animal models offer an alternative approach for identifying genes that influence susceptibility to alcohol dependence because mice and humans share extensively conserved chromosomal regions. That is, if we know where a gene is located in the mouse genome we can predict what chromosome contains the human ortholog (gene with similar function). Mouse models cannot *per se* entirely mimic alcoholism but we are able to assess traits in mice that are thought to be related to alcoholism. As outlined above, acute ethanol withdrawal in sons of alcoholics (or family-positive males) is a trait in humans that may predict the development of alcohol dependence. There are behavioral assays that may model this phenotype in mice. In particular, ethanol withdrawal convulsions are a useful index of withdrawal severity because they are displayed in all species tested including humans (Friedman, 1980). In 1971, Goldstein and Pal demonstrated that ethanol withdrawal severity could be measured in mice following chronic ethanol vapor inhalation using what is now known as the handling-induced convulsion (HIC). Over the years, this behavioral assay has been described more thoroughly: the original scale ranged from 0 – 4, which only measured tonic and clonic seizures (Goldstein and Pal, 1971). Today, HIC is a 0-7 point scale with a more graded index that measures facial grimaces, tonic-clonic seizures either when lifted by the tail or following a spin and spontaneous or environmentally-induced convulsions (Crabbe et al., 1991).

Although most studies focused on HIC used chronic ethanol exposure, withdrawal-induced convulsions are observed following a single hypnotic dose of ethanol. The first known report that a single dose of ethanol could exacerbate convulsions in mice was published by McQuarrie and Fingl in 1958. This study tested the protective effect of acute and chronic ethanol exposure on pentylenetetrazol-induced (PTZ) seizures in mice. A large oral dose of ethanol (4 g/kg) increased PTZ seizure threshold 4 hours post-ethanol administration. However, McQuarrie and Fingl detected increased susceptibility to PTZ-induced convulsions 8 hours following the acute dose of ethanol. Goldstein (1972) reported that following 5 g/kg of ethanol, mice displayed convulsions upon handling. More recently, Kosobud and Crabbe (1986) demonstrated that Withdrawal Seizure-Prone mice (WSP), selectively bred for severe HIC following three days of ethanol vapor administration, also display moderate HIC following 4g/kg dose of ethanol (ip).

I.E. Mouse Strains Used to Study Acute Ethanol Withdrawal

I.E.1. DBA/2J and C57BL/6J Inbred Strains

Inbred mouse strains are ideal preclinical models to examine ethanol-related traits because their responses to ethanol remain stable over generations. For example, it was first demonstrated by McClearn and Rodgers in 1959 that C57BL/6 mice consume large amounts of ethanol compared to other strains including DBA/2. This drinking behavior has been reported consistently in the literature (see Belknap and Atkins, 2001). Moreover, DBA/2J (D2) and C57BL/6J (B6) strains are commonly used in ethanol research because they differ in many responses to ethanol besides ethanol drinking (reviewed by Crabbe et al., 1999).

D2 mice are a well-characterized strain that has severe acute ethanol withdrawal reactions, whereas B6 mice have mild acute ethanol withdrawal reactions (Belknap et al., 1993; Buck et al., 1997; Metten and Crabbe, 1994). Therefore, D2 and B6 mice and populations derived from them provide a unique opportunity to investigate the genetics of acute ethanol withdrawal in an attempt to identify genes that may be predictive of alcohol abuse or dependence in humans (See Section I.C).

Moreover, it was recently demonstrated that acute ethanol withdrawal severity is a heritable trait in mouse models derived from B6 and D2 progenitor strains (Buck et al. 1997). Acute ethanol withdrawal severity ranges in intensity in BXD recombinant inbred (RI) strains and B6D2 F2 intercross mice such that some strains (or individual mice) show little or no withdrawal, some show an intermediate phenotype whereas others show severe withdrawal reactions. Short-term selection (four generations) produced two lines of mice that differed for acute ethanol withdrawal liability (High acute Alcohol Withdrawal, HAW, and Low acute Alcohol Withdrawal, LAW lines) providing further support that this is a heritable trait in mice. The above populations of mice were subsequently used to identify specific regions in the mouse genome that contribute to differential acute ethanol withdrawal severity (see Section I.F; Buck et al., 1997).

I.E.2. DBA/2J and DBA/1J inbred mouse strains

The DBA inbred mouse strains date to 1909 when Charles C. Little began brother sister mating mice for coat color. Indeed, DBA stands for dilute, d, brown, b, and non-agouti, a (Russell, 1978) three classic coat color genes. Between 1929-1930 the inbred strains now known as DBA/2 (D2) and DBA/1 (D1) were reproductively isolated in order to develop separate DBA substrains

(Festing, 1990). D2 and D1 strains differ at several genetic loci including *Car2, Ce2, Hc, H2, If1, Lsh, Tla,* and *Qa3* suggesting that some heterozygosity remained in the DBA progenitor strains used to establish these inbred strains (Festing, 1994). Although some heterozygosity may have been present when these two strains originated and given that genetic drift will have occurred, D2 and D1 are extremely related. This is supported by molecular data currently available. For example, Atchley and Fitch (1993) examined 119 genetic markers across the genome between D2 and D1 mice and showed that these strains differ at only 11.8% of these loci. According to data collected by the Center of Inherited Disease Research, 51 out of 314 markers tested from the Massachusetts Institute of Technology (MIT) series differ between D2 and D1 (16.2% difference; http://www.cidr.org/mouse/mouse.html). In comparison, 217 markers out of this set are polymorphic between D2 and B6 mice – nearly 70% (Witmer et al., 2003). Beyond these studies, little genotypic information is known about D1 mice (although the Genomics Institute of the Novartis Research Foundation is in the process of identifying SNPs across numerous inbred strains including DBA/1J).

Phenotypically, the D1 strain is extensively used to investigate collagen-induced arthritis, a mouse model of rheumatoid arthritis, an autoimmune disease (for review see Holmdahl et al., 2002; Joe et al., 1999). This strain, however, has not been as well characterized behaviorally as D2 mice. Recent studies have shown that D1 mice rapidly develop fear-potentiated startle and that $GABA_A$ and $5HT_{1A}$ agonists attenuate this learned response, (Risbrough et al., 2003). According to unpublished data from these authors, B6 mice do not readily learn

the fear-potentiated startle task. D1 mice also show lower open field activity and spend less time in the center of the open field compared to B6 mice, suggesting higher levels of anxiety (Powell et al., 2003).

D2 mice have been extensively characterized for severe ethanol withdrawal reactions following both chronic (Crabbe, 1998; Buck et al., 2002) and acute administration (Belknap et al., 1993; Metten and Crabbe, 1994; Buck et al., 1997). D1 mice also have a more severe chronic and acute ethanol withdrawal reaction compared to several other standard inbred mouse strains, although it is less severe than the D2 strain (Metten and Crabbe, 1994; Crabbe et al., 1983b). D1 mice display a more severe withdrawal reaction following precipitated diazepam withdrawal (Metten and Crabbe, 1999a) compared to B6 mice, yet display a moderate reaction following acute pentobarbital (60 mg; ip) compared to D2 mice (Metten and Crabbe, 1994). Both D1 and D2 inbred strains show lower electroconvulsive shock thresholds (i.e., are more sensitive) in contrast to B6 mice (Frankel et al., 2001). However, both strains have low susceptibility to nicotine-induced seizures in a panel of 19 inbred strains (Marks et al., 1989), suggesting that D2 and D1 mice are not seizure-prone to all convulsants.

Overall D1 mice appear to be sensitive to ethanol. For example, D1 mice do not develop tolerance to ethanol-induced hypothermia following eight daily exposures to 3 g/kg of ethanol compared to other strains that show tolerance development such as C57BL/6N, 129/J and AKR/J (Crabbe et al., 1982), they experience an extended duration of loss of righting reflex following 4 g/kg ethanol (ip) compared to DBA/2N and C58/J and show increased motor incoordination in the dowel test after 3 g/kg ethanol compared to several other inbred strains

including A/HeN, 129/J and DBA/2N mice (Crabbe, 1983). Therefore, considering the genetic relatedness between the DBA strains and that D1 mice also show severe acute ethanol withdrawal symptoms (albeit less severe than D2), these strains and crosses derived from them make a useful tool for dissecting the genetics of acute ethanol withdrawal severity. Indeed, a population of F2 intercross mice derived from D1 and D2 progenitor strains (n = 160) were tested for acute zolpidem and acute ethanol withdrawal reactions. Preliminary results suggest that D1 allelic frequency on mouse Chromosome 11 may be associated with both acute zolpidem and ethanol withdrawal severity (Pamela Metten and John Crabbe, unpublished data).

I.F. Genetic Mapping of Acute Ethanol Withdrawal Loci

Quantitative trait locus (QTL) analysis, a form of genetic mapping, asks whether a continuously distributed trait (*e.g.,* acute ethanol withdrawal severity) is associated with genetic (allelic marker) variation (Belknap et al., 1996; Belknap et. al., 1997; Crabbe et al., 1999; Complex Trait Consortium, 2003). A QTL is a chromosome locus that is associated with phenotypic variability. A recent QTL analysis, using 21 BXD RI strains, B6D2 F2 intercross mice, and short-term selected lines of mice derived from B6 and D2 progenitor strains (see Section I.E.1), identified three significant loci in the mouse genome that influence acute ethanol withdrawal severity (Buck et al., 1997). These loci map to distal mouse Chromosome 1 (*Alcw1*), mid Chromosome 4 (*Alcw2*) and proximal Chromosome 11 (*Alcw3*; see Figure 1). The Chromosome 11 QTL accounts for 12% of the genetic variance. Interestingly, it is D2 alleles on Chromosome 11 that confer protection against severe acute ethanol withdrawal compared to mice that

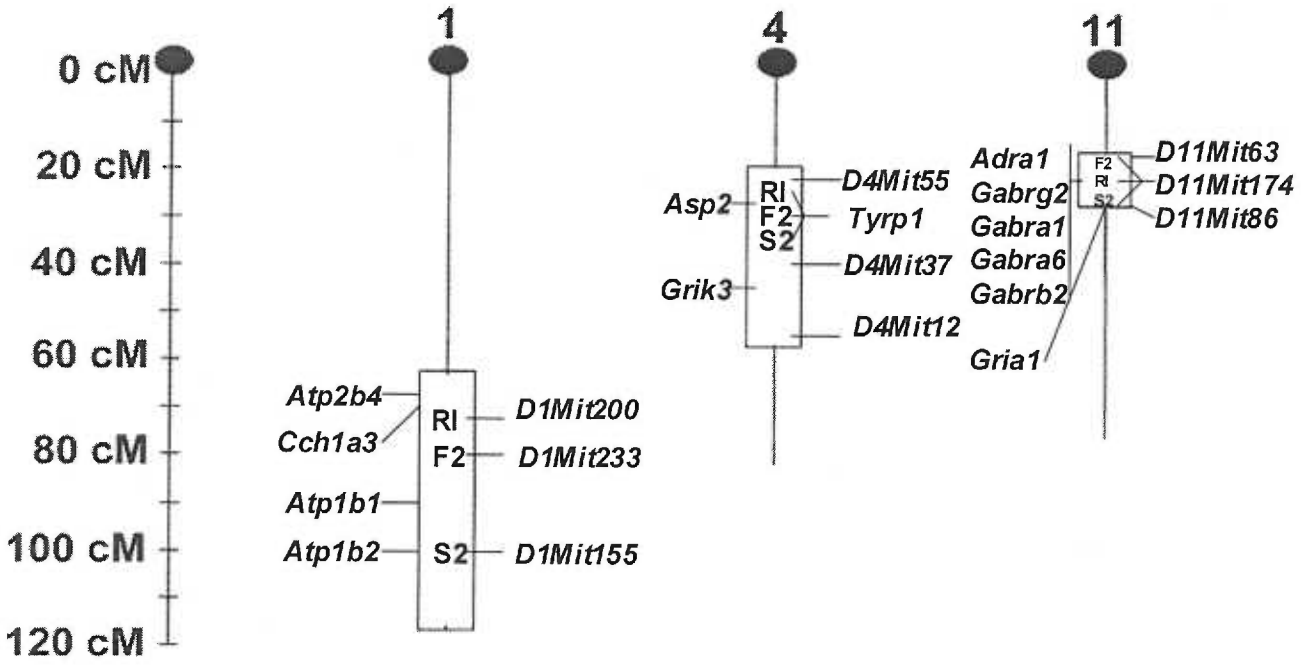

Figure 1. Acute ethanol withdrawal QTL detected in mice derived from D2 and B6 inbred strains. Three significant QTL were identified for acute ethanol withdrawal severity in mice on distal Chromosome 1 (*Alcw1*), mid Chromosome 4 (*Alcw2*) and proximal Chromosome 11 (*Alcw3*). As illustrated above, *Alcw3* maps near a cluster of $GABA_A$-R subunit genes, including *Gabrg2*. Also represented are other genes and molecular markers that are linked or near by the identified QTL. Boxes represent the 1-LOD confidence intervals for the identified QTL. Recombinant Inbred (RI) strains, B6D2 F2 intercross mice, and short-term selected (S2) lines were used to map these QTL. The marker with the strongest association with acute ethanol withdrawal severity for each population is shown to the right. (Figure adapted from Buck et al., 1997).

possess B6 alleles in all three populations tested. *Alcw3* maps to 20 cM, which is in proximity to a cluster of $GABA_A$ receptor subunit genes that contains γ_2 (*Gabrg2*), α_1 (*Gabra1*), α_6 (*Gabra6*) and the β_2 subunits, *Gabrb2* (Garrett et al., 1997).

I.G. The $GABA_A$ Receptor

γ-aminobutryic acid (GABA) is the main inhibitory neurotransmitter within the mammalian central nervous system (CNS). To date, three different GABA receptor subtypes have been identified: $GABA_A$, $GABA_B$, and $GABA_C$. $GABA_B$ receptors are metabotropic (see Bowery et al., 2002 for review) whereas $GABA_A$ and $GABA_C$ are ligand-gated ion channels. $GABA_C$ receptors, which contain ρ1-3 subunits, are located for the most part within the retina (reviewed by Bormann, 2000). Both $GABA_A$ (Fritschy and Mohler, 1995; Wisden et al., 1992) and $GABA_B$ (Fritschy et al., 1999) receptors are ubiquitously distributed throughout the CNS.

$GABA_A$ receptors ($GABA_A$-R) are a part of a ligand-gated ion channel superfamily that includes the nicotinic acetylcholine, glycine, and serotonin 3 (5-HT3) receptors. $GABA_A$-R are believed to be heteromeric pentamers coupled to an integral chloride channel (see Figure 2). This receptor binds GABA leading to influx of chloride anions and a hyperpolarization of neuronal membranes. Molecular biology thus far has identified 16 $GABA_A$-R subunits in the mammalian CNS: α1-6, β1-3, γ1-3, δ, ε, π, and θ subunits (for review see Mehta and Ticku, 1999; Macdonald and Olsen, 1994; Davies et al., 1997; Hedblom and Kirkness, 1997; Sieghart and Sperk, 2002). In addition, the genes that code for these

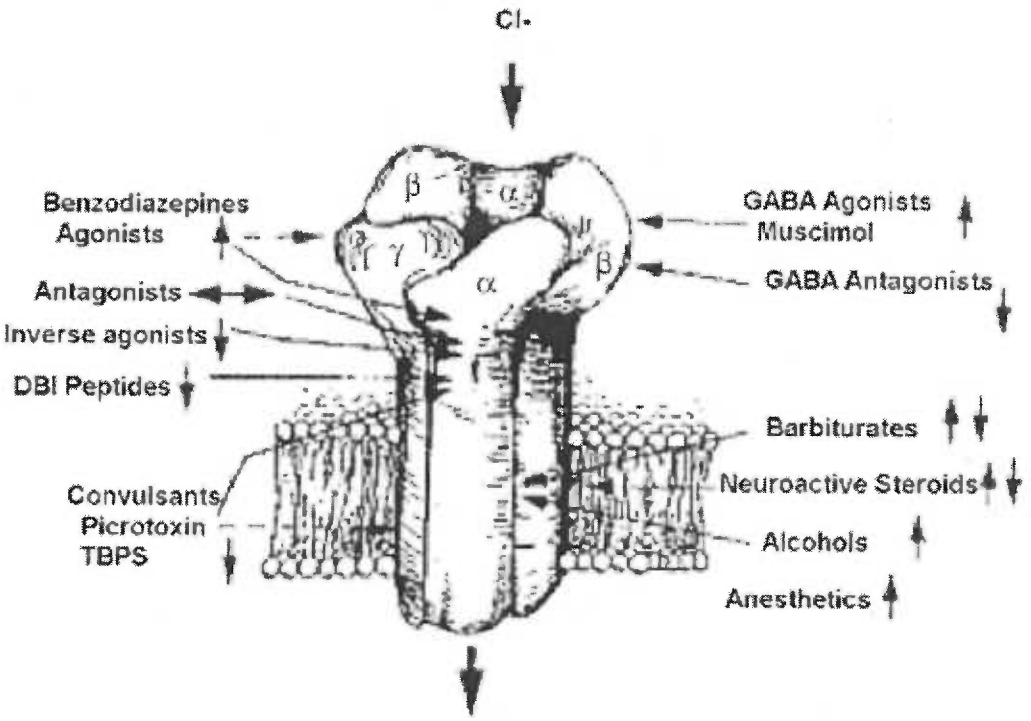

Figure 2. Predicted topography of the $GABA_A$ receptor. The $GABA_A$-R is predicted to be an oligomeric polypeptide composed of five subunits that are coupled to an integral chloride channel. When GABA binds to this receptor, it changes the confirmation of the subunits allowing chloride ions to flow into the channel leading to hyperpolarization of the neuron. To date, 16 different $GABA_A$-R subunits from multiple families have been identified (see Section I.G). The most prevalent combination of subunits in the mammalian brain is 2α1 2β2 and γ2 as depicted above (McKernan and Whiting, 1996). GABA binds at the interface of the α and β subunits, whereas, benzodiazepines bind at the interface of the α and γ subunits. Other ligands that bind to the $GABA_A$-R have either positive (e.g., pentobarbital) or negative (e.g., benzodiazepine inverse agonists) modulatory effects.

subunits are mostly found in clusters in both the mouse and the human genomes (Russek, 1999). *Gabrg1*, *Gabra2* and *Gabra4*, and *Gabrb1* are found on mouse Chromosome 5 (Human Chromosome 4p12); *Gabrg2*, *Gabra1*, *Gabra6*, and *Gabrb2* and *Gabrp* (which may or may not be part of this cluster; see Section III.E) are found on mouse Chromosome 11 (Human Chromosome 5q31-34); and *Gabrg3*, *Gabra5*, and *Gabrb3* are located on Chromosome 7 (Human Chromosome 15q11-13).

$GABA_A$-R subunits are predicted to possess a large extracellular amino-terminus, four transmembrane domains (TM) with a large intracellular loop between the third and fourth TM and a short carboxy-terminus (see Smith and Olsen, 1995). The most common subunit stoichiometry appears to be two α, two β and one γ (Mossier et al., 1994; Tretter et al., 1997; see Figure 2). Immunoprecipitation studies indicate that $\alpha1\beta2\gamma2$ receptors are the most preferred combination within the CNS (MacDonald and Olsen, 1994; Benke et al., 1994). The $\alpha1\beta2\gamma2$ combination is pharmacologically characterized as having high affinity for GABA and type I benzodiazepine receptor agonists (e.g. zolpidem; for review see Costa, 1998; Mehta and Ticku, 1999). The messenger RNA (mRNA) and polypeptide for the $GABA_A$-R $\alpha1$, $\beta2$ and $\gamma2$ subunits are the most abundant and ubiquitously distributed of their respective subunit family as shown from *in situ* and immunocytochemical studies performed using rat brain (Laurie et al., 1992a; Laurie et al., 1992b; Wisden et al. 1992; Pirker et al., 2000).

Multiple ligands and drugs modulate $GABA_A$-R. GABA binding occurs at the interface of the N-terminals of the α and β subunits whereas benzodiazepine binding occurs at the interface of the α and γ subunits (Smith and Olsen, 1995).

Neurosteroids are potent positive or negative modulators, whereas picrotoxin and bicuculline inhibit chloride influx by blocking the channel pore (for review see Mehta and Ticku, 1999). Other compounds that affect the $GABA_A$-R include inhaled anesthetics (Franks and Lieb, 1994) and barbiturates (Saunders and Ho, 1990). Lastly, both *in vivo* and *in vitro* (reviewed by Grobin et al., 1998; Mihic, 1999) studies have shown that ethanol affects $GABA_A$-R function and subunit expression (Reilly et al., 2001).

I.G.1. $GABA_A$-R and ethanol withdrawal

Numerous electrophysiological, biochemical and behavioral studies have implicated the $GABA_A$-R as a site for ethanol action (for review see Buck 1996; Grobin et al., 1998; Mehta and Ticku, 1999; Mihic and Harris, 1996; Mihic, 1999). The $GABA_A$-R plays a role in the modulation of ethanol withdrawal, which is characterized by increased anxiety and convulsion susceptibility (Buck et al., 1991c; File et al., 1989; Lister and Karanian, 1987). Ethanol withdrawal-induced audiogenic convulsions in rats are suppressed by microinjection of muscimol, a $GABA_A$-R agonist, into the inferior colliculus (Frye et al., 1983), and into the substantia nigra (Gonzalez and Hettinger, 1984). A single dose of flumazenil, a $GABA_A$-R antagonist, attenuates ethanol withdrawal severity as measured by handling-induced convulsions in mice (Buck et al., 1991c). As well, flumazenil decreases anxiety induced by ethanol withdrawal in rats by increasing the time spent in the open-arms of an elevated plus maze (Moy et al., 1997) and in a social conflict task (File et al., 1989).

Although the $GABA_A$-R has been implicated, the specific $GABA_A$-R subunits that modulate ethanol withdrawal severity remain to be elucidated. Several studies have found altered subunit messenger RNA (mRNA) or protein content in

rodents following chronic ethanol administration paradigms (See Table 1; Reilly et al., 2001). There also appears to be a genetic influence for differential regulation of $GABA_A$-R subunits in mice selectively bred for severe chronic ethanol withdrawal (Withdrawal Seizure-Prone, or WSP) or mild withdrawal reactions (-Resistant, WSR). Following chronic ethanol ingestion, WSP mice have decreased $GABA_A$-R α_1 subunit mRNA content within whole brain, whereas there was no change in WSR mice. In addition, $GABA_A$-R α_6 subunit mRNA content was lower in whole brain in control (pair-fed) WSR mice but not in WSP mice (Buck et al., 1991a). This difference in mRNA content may be associated with greater sensitivity to benzodiazepine inverse agonists (*e.g.*, DMCM and Ro15-4513) in ethanol-dependent WSP mice compared to WSR mice (Buck et al., 1991b).

Many studies have examined the effects of chronic ethanol exposure or withdrawal on $GABA_A$-R regulation/function, however, there are relatively few studies investigating modulation of the $GABA_A$-R following acute ethanol administration. In a study performed by Buck and Harris (1990), cortical membrane vesicles obtained from ICR mice 30 minutes following ethanol administration (4 g/kg ip) showed enhanced inhibition of muscimol-induced ^{36}Cl-flux by DMCM. Cerebellar microsacs prepared from DBA/2J mice treated with 4 g/kg ethanol (ip) one hour prior, do not show ethanol enhancement of muscimol stimulated ^{36}Cl- uptake compared to saline treated control mice (Allan and Harris, 1987). There is decreased ^{3}H-GABA binding in the cerebellum from Sprague Dawley rats three hours following administration of 3 g/kg ethanol (gavage) compared to control rats, however, this difference was not detected in other brain

Table 1: Effect of ethanol treatment on $GABA_A$ receptor subunit expression

Subunit	Alteration	References
$\alpha 1$ mRNA and peptides [a-d]	decreased	Morrow et al., 1990, 1992 Montpied et al., 1991 Mhatre et al., 1992, 1993 Devaud et al., 1995, 1997 Charlton et al., 1997 Cagetti et al., 2003
$\alpha 2$ mRNA and peptides [a]	decreased	Montpied et al., 1991 Mhatre et al., 1992, 1993
$\alpha 3$ mRNA [a]	no change	Montpied et al., 1991 Mhatre et al., 1993
$\alpha 4$ mRNA and peptides [a,c]	increased	Cagetti et al., 2003 Devaud et al., 1995, 1997 Mahmoudi et al., 1997
	no change	Matthews et al., 1998 Petrie et al., 2001
$\alpha 5$ mRNA [a,c]	increased no change	Devaud et al., 1995 Charlton et al., 1997 Petrie et al., 2001
$\alpha 6$ mRNA and peptides [b]	increased	Mhatre et al., 1992 Morrow et al., 1992 Wu et al., 1995 Petrie et al., 2001
$\beta 1$ mRNA [a]	increased no change	Mhatre et al., 1994 Devaud et al., 1995
$\beta 2$ mRNA and peptides [a,c,d]	increased	Devaud et al., 1997 Mhatre and Ticku, 1994 Reilly and Buck, 2000 Morrow et al., 1992

Table 1 (continued)

β2 mRNA and peptides [a,c,d]	no change	Devaud et al., 1995 Reilly and Buck, 2000 Matthews et al., 1998
β3 mRNA and peptides [a,c]	increased	Devaud et al., 1997 Mhatre and Ticku., 1994
β3 mRNA and peptides [a,c]	no change	Devaud et al. 1995 Matthews et al., 1998
γ1 mRNA and peptides [a]	increased no change	Devaud et al., 1995, 1997 Petrie et al., 2001
γ2S mRNA [a]	increased	Devaud et al., 1995
γ2L mRNA [a]	no change	Devaud et al., 1995
γ2 mRNA	increased	Cagetti et al., 2003
γ2 peptides [a,c]	no change	Devaud et al., 1997 Petrie et al., 2001 Matthews et al., 1998
γ3 mRNA [a]	no change	Devaud et al., 1995
δ mRNA [a]	no change	Devaud et al., 1995
δ mRNA [c]	decreased	Cagetti et al., 2003

Table adapted from Reilly et al., 2000.

[a] cerebral cortex
[b] cerebellum
[c] hippocampus
[d] whole brain

region examined (*i.e.*, striatum, substantia nigra and cortex; Reggiani et al., 1980). $GABA_A$-R α1 and β3 subunit mRNA was increased 30 minutes following a single ip injection of 4g/kg of ethanol in the cerebellum of female B6 mice, whereas no changes were observed for $GABA_A$-R α6, β2, or γ2 subunit message (Wu et al., 1995). To date, we know of no published studies that have investigated the effects of acute ethanol withdrawal on $GABA_A$-R function or subunit mRNA content in D2 and B6 mice.

However, differential acute ethanol withdrawal severity between D2 and B6 mice could result from innate differences in basal $GABA_A$-R expression/function. Yu and colleagues (1986) report that whole brain membrane vesicles prepared from young D2 mice (20-22 days old) are less sensitive to GABA (1 μM-1 mM) as measured by $^{36}Cl^-$ uptake compared to age matched B6 mice. This difference, however, disappears when tested between days 40-42. These authors also report that GABA agonist binding (muscimol and GABA) is lower in young B6 mice compared to the D2 strain. Interestingly, Ticku (1980) showed that GABA binding density (B_{max}) was less for D2 mice compared B6 mice at ~4 months of age. This result is inconsistent with that of Yu and coworkers (1986) where they showed no difference in GABA binding in adult D2 and B6 mice. Robertson (1980) reported that maximum ^{3}H-flunitrazepam binding (B_{max}) in whole brain of young D2 mice (22-24 days old) was greater than age matched B6 mice, however, no differences were observed when the mice were 40 days of age. In female C57BL/Jlbg mice, GABA greatly enhanced ^{3}H-flunitrazepam binding compared to other lines and strains of mice including DBA/2lbg mice, although they did not differ concerning ^{3}H-flunitrazepam binding itself (Marley and Wehner,

1987). These studies show putative functional and/or expression differences indirectly measured by B_{max} for the $GABA_A$-R between D2 and B6 mice, however, the specific gene(s) that are responsible remain to be elucidated.

I.G.2. Gabrg2 is a candidate gene for acute ethanol withdrawal severity

The $GABA_A$-R γ2 subunit was cloned in 1989 by Peter Seeburg's group (Pritchett et al., 1989). In this study, they also demonstrated that the $GABA_A$-R γ2 subunit is involved in benzodiazepine binding. Subsequently, it has been shown that the $GABA_A$-R γ2 subunit is the most prevalent γ subunit within the rat central nervous system (Fritschy and Mohler, 1995; Wisden et al., 1992). This subunit is involved in $GABA_A$-R benzodiazepine binding and pharmacology, $GABA_A$-R desensitization and normal chloride channel conductance as demonstrated by recombinant $GABA_A$-R studies and by null mutation of γ2 in mice (Benke et al., 1996; Dominguez-Perrot et al., 1996; Günther et al., 1995; Rudolph et al., 1999). Autoradiographic studies show that deletion of the γ2 subunit abolishes benzodiazepine binding while leaving GABA binding intact (Günther et al., 1995). The γ2 subunit gene is also involved in $GABA_A$-R synaptic clustering as shown by targeted disruption of the γ2 subunit and of the scaffolding protein gephyrin (Essrich et al., 1998; Kneussel et al., 1999). This reduction in synaptic clustering is associated with increased anxiety and a bias for threat cues in mice hemizygous for targeted disruption of *Gabrg2* ($\gamma2^{+/-}$ mice; Crestani et al., 1999).

Additionally, the γ2 subunit is alternatively spliced and it was suggested that the long variant of γ2 was responsible for mediating ethanol's effects on the $GABA_A$-R at intoxicating concentrations (Wafford et al., 1991). However, a recent study shows that γ2L knockout mice do not differ from wildtype littermates

in withdrawal severity following chronic intermittent alcohol withdrawal severity suggesting that ethanol withdrawal are not mediated by γ2L (Homanics et al., 1999). Recently, we identified amino acid variation within the amino-terminus of the $GABA_A$-R γ2 subunit (both in long and short variants) between B6 and D2 inbred mice (Buck and Hood, 1998; see Section III.A). B6 mice possess an alanine at amino acid residue 11 of the mature peptide (A11), whereas D2 mice have a threonine at this residue (T11). This amino acid substitution (A/T11) alters the predicted secondary structure of the protein (Buck and Hood, 1998). We sequenced *Gabrg2* from the 26 BXD RI strains which were derived from B6D2 F2 intercross mice (Bailey, 1981) and found a genetic correlation between this polymorphism and acute ethanol withdrawal severity and other motivationally negative ethanol traits (ethanol conditioned taste aversion, ethanol-induced motor incoordination and hypothermia; Hood and Buck, 2000). BXD RI that have the B6 allele show greater acute ethanol withdrawal severity compare to RI strains that possess the D2 allele. The variation in γ2 peptide does not alter sensitivity of $\alpha1\beta2\gamma2_{A11}$ vs. $\alpha1\beta2\gamma2_{T11}$ receptors to 200 mM ethanol, an anesthetic concentration *in vivo* (Mihic and Buck, in press). It is also plausible that other subunit combinations expressed with γ2 (e.g., α6 and β3) will yield different results. Moreover, ethanol potentiation (10-100 mM) was not observed in oocytes expressing $GABA_A$-R (Mihic and Buck, in press) whereas these intoxicating concentrations are effective in brain tissue. Therefore, cautious interpretation of these ethanol studies is warranted because it is not known whether γ2 peptide variation will alter function in either mammalian and/or

neuronal cells. *Xenopus* oocytes may also lack important cellular components that are required for receptor function.

However, the direct effect of pentobarbital is dramatically increased in $\alpha1\beta2\gamma2_{A11}$ receptors (Mihic and Buck, in press). This result is interesting because $\gamma2$ subunits are not absolutely required to see the direct effect of pentobarbital (e.g., Uchida et al., 1997). This suggests that variation in the $\gamma2$ subunit may affect $\gamma2$ expression (or assembly), and may not directly alter $\gamma2$ function. Alternatively, this polymorphism may simply be a marker for polymorphism elsewhere in *Gabrg2* (*e.g.*, promoter or other regulatory sequence) that effect *Gabrg2* mRNA content, or for a functional polymorphism in a nearby gene.

I.G.3. $GABA_A$-R associated proteins

One possible mechanism by which $GABA_A$-R $\gamma2$ expression differences may affect alcohol withdrawal severity is reduced synaptic clustering of the $GABA_A$-R. The majority of $GABA_A$-R are clustered on dendrites or soma across from pre-synpatic terminals in symmetrical synapses which is necessary for efficient synaptic transmission (Peters and Palay, 1996). The $GABA_A$-R $\gamma2$ subunit and gephyrin, a tubulin-binding protein, have been shown to be critical for synaptic clustering of the $GABA_A$-R (for review see Sassoe-Pognetto and Fritschy, 2000). $GABA_A$-R $\gamma2$ null mutant mice do not show gephyrin clustering and antisense oligonucleotides directed at gephyrin dramatically reduce $GABA_A$-R clustering in cultured hippocampal neurons (Essrich et al., 1998). The decrease in $GABA_A$-R clustering in turn alters GABA-induced current (Crestani et al., 1999). $GABA_A$-R clusters are also reduced in gephyrin null mutant mice (Kneussel et al., 1999).

However, it appears that gephyrin does not affect subunit assembly or receptor trafficking (see Moss and Smart, 2001).

Although gephyrin has been shown to be involved in synaptic $GABA_A$-R clustering, a direct interaction between gephyrin and $GABA_A$-R has yet to be demonstrated through protein-protein binding studies (Meyer et al, 1995; Kannenberg et al., 1997). Another protein that interacts with the $GABA_A$-R $\gamma 2$ subunit – named $GABA_A$ receptor- associated protein (GABARAP) was identified in 1999 by Richard Olsen's group (Wang et al., 1999). GABARAP's protein sequence is similar to the light chain-3 of microtubule-associated protein and was shown to bind to tubulin and microtubules, which are part of the synaptic cytoskeleton (Wang et al., 1999; Wang and Olsen, 2000). Therefore, it has been suggested that GABARAP may be a linker protein between $GABA_A$-R $\gamma 2$ subunits and gephyrin. To date, it is unclear what the exact mechanism of GABARAP action is. For example, it was shown that GABARAP and gephyrin co-localize intracellularly but not at the synapse indicating that GABARAP may be apart of intracellular transport and targeting (Kneussel et al., 2000). However, Chen and colleagues (2000) demonstrated in a quail fibroblast system that GABARAP is involved in $GABA_A$-R clustering which also may be necessary to see normal channel kinetics.

I.G.4. Linked genes

Other $GABA_A$-R subunit genes (*Gabra1*, *Gabra6*, and *Gabrb2*) linked to *Gabrg2* are also candidates for modulating acute ethanol withdrawal (Buck et al., 1997). Several association studies examining the relationship between the $GABA_A$-R subunit gene cluster on human Chromosome 5 (homologous to the mouse Chromosome 11 cluster) and alcohol dependence phenotypes have been

performed. Dependent upon genetic population (e.g., Scottish or Japanese) and phenotype (alcohol dependence with Korsakoff psychosis), there is *sometimes* significant association between $GABA_A$-R subunit polymorphisms and alcoholism (reviewed by Loh and Ball, 2000). Longitudinal studies have shown that sons of alcoholics who display low level of response to ethanol as measured by body sway are four times more likely to develop alcoholism compared to control males (Schuckit, 1994). In a small pilot study, Schuckit and colleagues (1999) investigated 17 men who had extremely low level of response to alcohol. They found that 7 of these men whose low response was in the extreme possessed two allelic variants of *GABRA6* (Pro/SerA6), whereas men that were Pro/Pro for *GABRA6* were less likely to develop alcoholism. The authors suggest that the $GABA_A$-R α6 subunit may play a role in developing alcohol dependence. However, their results are preliminary at best due to several limitations of this study including small sample size, possible population stratification and *a priori* knowledge of the subjects' phenotype.

Other genetic evidence for the involvement of $GABA_A$-R in ethanol-related phenotypes includes both a point mutation and deletion in the α6 subunit from rats selected for alcohol-induced motor impairment, Alcohol Non-Tolerant rats (Korpi et al, 1993, 1994). The point mutation alters amino acid sequence at residue 100 (Ala → Glu). Normally, $GABA_A$-R containing an α6 subunit are diazepam insensitive as measured by RO15-4315 displacement assays (Kleingoor et al., 1991); however, the glutamine substitution now makes GABAA-R with this subunit responsive to diazepam. While investigating this point mutation, Korpi and colleagues (1994) discovered a 10 amino acid deletion or

short variant of the α6 subunit. Whereas the amino acid substitution changes the pharmacology of the $GABA_A$-R that possess the α6 subunit, the deletion appears to affect subunit assembly in that α6Sβ2γ2 receptors behave like cells expressing β2γ2 – there is relatively little GABA induced current (Korpi et al., 1994). However, it is unknown whether these alterations in the α6 subunit affect alcohol withdrawal. Although these α6 variations were found in the ANT rat line, the α6 coding sequence does not differ between B6 and D2 mice (Buck and Finn, 2001) and targeted disruption of this gene does not affect ethanol sensitivity (Homanics et al., 1997) or chronic intermittent ethanol withdrawal hyperexcitability (Homanics et al., 1998). The amino acid sequences for the $GABA_A$-R α1 and β2 subunits do not differ between B6 and D2 mice (Kamatchi et al., 1995; Wang et al., 1992). Following a chronic liquid ethanol diet, handling-induced convulsions did not differ between $GABA_A$-R β2 null mutant and wildtype mice nor was withdrawal altered in mice that had a targeted deletion of the $GABA_A$-R α1 subunit compared to their wildtype counterparts (Blednov et al., 2003). Similarly, mRNA content for $GABA_A$-R β2 subunit does not differ in the cerebellum or cortex between naïve B6 and D2 strains, but does differ after chronic ethanol treatment in a complex manner that is dependent on genotype, blood ethanol concentration and brain region (Reilly and Buck, 2000). The gene sequencing and null mutation studies therefore suggest that *Gabrg2* is the most compelling candidate out of this gene cluster on mouse Chromosome 11.

I.G.5. $GABA_A$-R γ2 subunit involvement in human seizure phenotypes

Recently a number of rare human mutations in *GABRG2* have been detected that have phenotypic affects on CNS hyperexcitability (epilepsy; see below for discussion). In general, idiopathic epilepsies have a complex pattern of

inheritance (reviewed by Kaneko et al., 2002). Because GABA is the main inhibitory neurotransmitter within the CNS, many investigators have shown that altered GABA transmission or $GABA_A$-R function modulates seizures in animal models of epilepsy (reviewed by Treiman, 2002). Until recently, there was little empirical evidence demonstrating the genetic role of the $GABA_A$-R in human seizure phenotypes. Baulac and colleagues (2001) found an amino acid substitution at residue 289 (lysine → methionine; K → M) of the $GABA_A$-R $\gamma 2$ polypeptide in a large French family that had a syndrome similar to generalized epilepsy with febrile seizures plus (GEFS+). This is a heterogeneous syndrome with incomplete penetrance. Other phenotypes related to GEFS+ are childhood absence epilepsy and febrile seizures (see Scheffer and Berkovic, 1997 for review). The K289M substitution occurs at the putative extracellular loop between the second and third transmembrane domains. This lysine is conserved across human $GABA_A$-R subunits $\alpha 1$-6, $\beta 1$-3 and $\gamma 1$-3, suggesting functional importance. Maximal GABA-evoked currents (I_{GABA}) were reduced by 90% in oocytes expressing the mutated $\gamma 2$ protein in combination with $\alpha 1$ and $\beta 2$ subunits compared to wildtype $\alpha 1\beta 2\gamma 2$ receptors. Diazepam was able to potentiate I_{GABA} for $\alpha 1\beta 2\gamma 2_{K289M}$ receptors to the same relative degree as wildtype receptors indicating that the mutant $\gamma 2$ subunit was incorporated into receptors.

Interestingly, these functional results were not completely replicated in a study by performed by Bianchi and associates (2002). Here, they expressed $\gamma 2_{K289M}$ in human embryonic kidney (HEK) cells with $\alpha 1$ and $\beta 3$ subunits. Whole-cell recordings indicated that receptors containing the mutant $\gamma 2$ subunit responded to increasing GABA concentrations in a manner similar to wildtype receptors. In

this system, $\alpha1\beta3\gamma2_{K289M}$ receptors were equally sensitive to 1 μM diazepam as $\alpha1\beta3\gamma2$ receptors. However, rapid kinetic experiments revealed that K289M currents deactivated faster than γ2 wildtype containing receptors. Additionally, the K289M mutation affected paired-pulse inhibition, a measure of desensitization, in that inhibition produced by GABA was reduced following each application. Overall, the studies by Bianchi et al. (2002) and Baulac et al. (2001) suggest that the K289M mutation in the $GABA_A$-R γ2 subunit may lead to decreased neuronal activation.

Wallace and associates (2001) identified another mutation in the $GABA_A$-R γ2 subunit polypeptide in a large Australian pedigree in which individuals were affected by childhood absence epilepsy and/or febrile seizures. In this study, there was an arginine to glutamine substitution at residue 43 of the mature polypeptide (R → Q), which occurs in a putative high affinity benzodiazepine binding moiety (Boileau et al., 1998; Kucken et al., 2000). Consequently, diazepam did not potentiate GABA-evoked currents when oocytes expressed $\alpha1\beta2\gamma2_{R43Q}$ were compared to wildtype receptors (Wallace et al., 2001; Bowser et al., 2002). Unlike the K289M mutation, GABA-evoked currents for R43Q resembled wildtype $\alpha1\beta2\gamma2$ receptors in an oocyte expression system but showed slower deactivation and increased desensitization compared to wildtype $\alpha1\beta2\gamma2$ receptors when expressed in HEK cells which could lead to reduced GABAergic inhibition and increased susceptibility to seizures (Bowser et al., 2002).

In a subsequent study, another novel γ2 mutation was discovered in a separate Australian pedigree with a history of seizures including GEFS+ and

febrile seizures (Harkin et al., 2002). Sequencing results revealed a single base substitution at nucleotide 1168 (C → T) that creates a premature stop codon at residue Q351 in the intracellular loop between TM3 and TM4. This mutation (Q351X) leads to a truncated γ2 polypeptide that appears to inhibit proper receptor assembly and cell surface expression when expressed with α1 and β2 subunits in HEK cells. Also, GABA-induced currents are completely abolished when the truncated γ2 subunit is expressed with α1 and β2 subunits in *Xenopus* oocytes.

A fourth mutation in the *GABRG2* gene was identified in a German family with single-strand conformation analysis (Kananura et al., 2002). This mutation segregated with occurrence of childhood absence epilepsy and febrile convulsions. Sequencing results of *GABRG2* indicated that this mutation occurs at splice-donor site in intron six which is predicted to lead to a nonfunctional $GABA_A$-R γ2 subunit. Further empirical evidence concerning this splice site mutation is lacking.

Genetic association studies across multiple ethnic populations have also investigated the role of $GABA_A$-R γ2 polymorphism and idiopathic epilepsies. Results from these studies do not consistently indicate a role for *GABRG2* modulating seizure phenotypes. Nakayama and others (2002) did not detect a significant association between a SNP in exon 5 (C588T) and childhood absense epilepsy or febrile seizures in a Japanese population. However, the C588T SNP was significantly associated with febrile seizures in a population of Taiwanese children (Chou et al., 2003). In a Chinese population, transmission disequilibrium testing for a SNP in exon 5 of *GABRG2* did not reveal any allelic frequency

differences in 68 triads that had childhood absense epilepsy (Lu et al., 2002). These conflicting results indicate that idiopathic epilepsy is a genetically heterogeneous phenotype that may or may not involve the $GABA_A$-R $\gamma 2$ subunit.

Overall, it appears that rare mutations in the $GABA_A$-R $\gamma 2$ subunit found in three different families contribute to seizure disorders. These mutations appear to affect receptor function and/or surface expression. Also, in two individuals of German heritage there is a mutation in a splice-donor site that is predicted to affected expression of $GABA_A$-R $\gamma 2$ subunit mRNA. Thus, decreased $GABA_A$-R $\gamma 2$ expression/function appears to be associated with idiopathic seizure disorders. To date, however, mutations in human $GABA_A$-R $\alpha 1$, $\beta 2$ or $\alpha 6$ subunits have not been found that are related to seizure disorders.

I.H. Brain Regions Affected by Alcohol Administration and Withdrawal

The specific brain regions that mediate ethanol withdrawal-induced seizures have yet to be identified. Investigators have inferred plausible regions from other seizure models or epilepsy-like syndromes. Many studies have focused on cortical regions that may be involved in thalamo-cortical circuitry (for example see Blumenfeld, 2002). Microarray studies have shown that gene expression is altered in post-mortem tissue from the frontal cortex of alcoholics compared to controls (Lewohl et al., 2000; Mayfield et al., 2002). Autoradiographic 2-[^{14}C]-deoxyglucose studies using Sprague Dawley rats have shown that acute administration of ethanol (2 g/kg; intragastric) increases local glucose use in the lateral prefrontal cortex and the lateral septum (Williams-Hemby and Porrino, 1997). Preliminary results from our laboratory indicate that entorhinal and

prelimbic cortices (substructures of the frontal cortex) are more activated in ethanol-withdrawn D2 mice compared to Chromosome 11 congenics as measured by c-FOS mapping (Buck et al., in preparation). As well, many animal studies have shown that both gene expression and $GABA_A$-R function is altered in the cortex following chronic ethanol exposure (for review see Reilly et al., 2001).

I.I. Genetic Mapping and Candidate Gene Analysis

The genomic region on mouse Chromosome 11 that influences acute ethanol withdrawal severity in mice was first identified by the genetic mapping technique called quantitative trait locus (QTL) mapping (see Section I.F). This assessment is usually considered the first phase of many in the identification of genes that modulate complex traits (see Glazier et al., 2002 for review). The genomic regions identified in this step are rather large (10 – 30 cM) and contain many genes. Because of the size of these genomic intervals, investigators normally try to map these regions to smaller segments. There are several different ways to reduce the QTL interval size, including advanced intercross lines (reviewed by Darvasi and Soller, 1995), interval-specific congenic mice (see Darvasi, 1997), and recombinant progeny testing. A series of studies (Fehr et al, 2002; Shirley et al., submitted) that used congenic mice and a modified variation of recombinant progeny testing reduced a ~ 35 cM interval that modulates acute ethanol withdrawal severity on mouse Chromosome 4 to less than 1 cM (Buck et al., 1997).

Another possible way to reduce the QTL interval further is to use haplotype mapping. A haplotype is a chromosomal segment or markers that are inherited together in a block in contrast to a single gene (see Figure 3). One way to perform haplotype mapping is to test the trait of interest in two or more F2 populations and assess whether the QTL maps to the same chromosomal region in all or some of the populations. This method is also referred to as Multiple Cross Mapping (Hitzemann et al., 2002). Then, by comparing haplotypes across the progenitor strains used to derived the F2 populations, one can greatly narrow the genomic region by determining regions of the genome that are shared or are different (polymorphic) between these strains. Polymorphic regions shown to be significantly associated phenotype most likely contain the gene or genes underlying the trait. For example, Park and colleagues (2003) used multiple cross mapping to reduce a genomic region on mouse Chromosome 19 involved in metastatic efficiency from one that contained over 400 genes to one that contains 23 genes. This type of mapping is possible given the structural nature of the mouse genome because the inbred strains commonly used today were most likely derived from only a few progenitors therefore reducing the amount of potential polymorphism (Wade et al., 2002). Others have used outbred mice to fine map QTLs (see Mott et al., 2000). For example, Demarest and colleagues (2001) phenotyped a population of heterogeneous stock mice that were derived from eight inbred strains for ethanol-induced stimulation. Because heterogeneous stock mice have more recombinations compared to F2 intercross mice, these investigators were able reduce a Chromosome 2 QTL for ethanol-induced locomotor activity from a 20 cM interval to a 2 cM region.

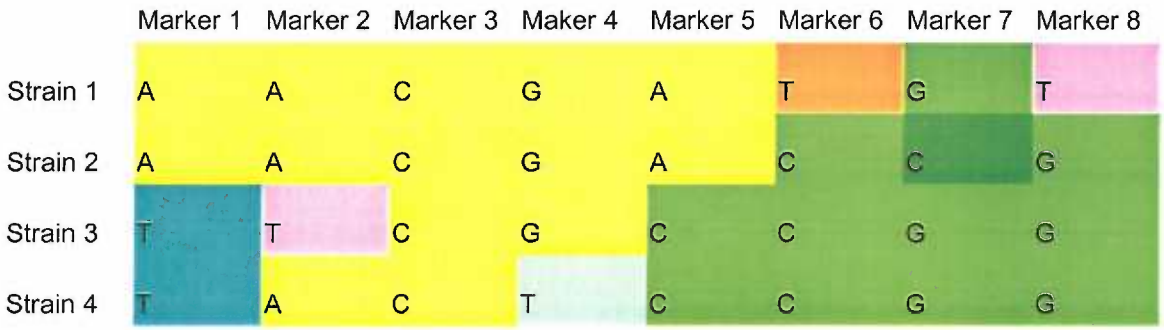

Figure 3. Haplotype illustration. This is a hypothetical example where four inbred mouse strains (strains 1-4) were assessed for several markers (or SNPs) across a chromosome (Markers 1-8) which are represented by polymorphic nucleotides (i.e., adenine – A, thymine-T, guanine – G, and cytosine – C). Each mouse in each strain is homozygous for the nucleotide shown for each marker. Haplotypes are defined where two or more strains share the polymorphism across several markers (or loci), which is represented by a block of the same color (e.g., Markers 1-5 show a haplotype shared by strains 1 and 2, shown in yellow). These similarities and differences can be used to dramatically decrease the size of QTL intervals by determining more precise polymorphic regions that may contain the gene of interest.

Recently it has been proposed that multiple methods should be used simultaneously to identify a quantitative trait gene or genes (QTG). For example, Hitzemann and colleagues (2003) have used Multiple Cross Mapping, gene expression arrays and DNA sequencing to identify *Kcnj9* as a strong candidate gene on mouse Chromosome 1 that modulates basal locomotor activity in mice. In addition, one can begin to dissect QTLs by using genotypic approaches, such as phenotyping transgenic mice that under- or overexpress the gene of interest (reviewed by Phillips et al., 2002). Together, multiple converging methods can reduce the number of plausible candidate genes to a manageable handful that can be functionally tested to ultimately identify the quantitative trait gene (Mackay, 2001) and perhaps the quantitative trait nucleotide (QTN) responsible for genetic difference that affects the phenotype of interest.

I.J. Overall Goal

The contribution of the GABAergic system to various aspects of alcohol reward, abuse, dependence and or withdrawal has been systematically studied over many decades (for review see Buck, 1996; Grobin et al., 1998; Mehta and Ticku, 1999; Mihic, 1999). However, it is unknown which specific subunits or combinations of subunits are responsible for phenotypic differences. This set of experiments was designed to fine map the Chromosome 11 QTL for acute ethanol withdrawal to determine whether the $GABA_A$-R subunit genes that map in proximity to *Alcw3* are viable candidate genes. Because we retained the $GABA_A$-R subunit cluster after reducing the QTL interval, we elected to focus on the $GABA_A$-R $\gamma 2$ subunit gene, *Gabrg2*, as a potential candidate gene for *Alcw3* using multiple techniques including sequence analysis, mRNA expression

studies assessing subunit abundance in mice that differ in withdrawal severity, and by behaviorally testing $\gamma 2$ null mutant mice. Ultimately, we show through our sequencing, behavior, expression and mapping data to date that *Gabrg2* remains the strongest candidate gene for *Alcw3* within the cluster of GABAergic genes.

II. General methods

II.A. Animals

II.A.1. Inbred Strains

The following inbred mouse strains used in these studies were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in either the Portland VAMC Veterinary Medical Unit or in the Hatfield Research Center at OHSU: DBA/2J (D2), C57BL/6J (B6), 129P3/J, A/HeJ, AKR/J, BALB/cJ, C3H/HeJ, C57BR/cdJ, C57L/J, CBA/J, CE/J, DBA/1J, PL/J, SJL/J, and SWR/J inbred strains and BXD recombinant inbred (RI) strains -1, -2, -5, -6, -8, -9, -11, -12, -13, -14, -15, -16, -18, -19, -21, -22, -23, -24, -25, -27, -28, -29, -30, -31, and –32. All strains were maintained on *ad libitum* food and water and a 12:12 light/dark cycle (lights on at 0600 hours). Mice were housed according to strain in filter top polycarbonate cages (28 x 17 x 11.5 cm), which were stored on Thoren racks (Thoren Caging Systems, Hazleton, PA). For all mice bred in house, offspring were weaned at approximately 21 days of age and housed according to strain and sex. All mice were either phenotyped or used in molecular studies between 60 and 100 days of age.

II.A.2. D2.B6-D11Mit174 Congenic Development

Dr. John K. Belknap at the Portland VA Medical Center developed Chromosome 11 congenic mice. Briefly, B6 mice were crossed with D2 mice to obtain B6D2 F1 hybrids. F1 mice were then backcrossed to D2 mice and their progeny were genotyped using the following markers: *D11Mit78* (2 cM), *D11Mit174* (20 cM) and *D11Mit179* (52 cM). Offspring that were heterozygous at all three markers were then backcrossed to the D2 strain. This procedure was repeated for ten backcross generations (N10; see Figure 4), which should

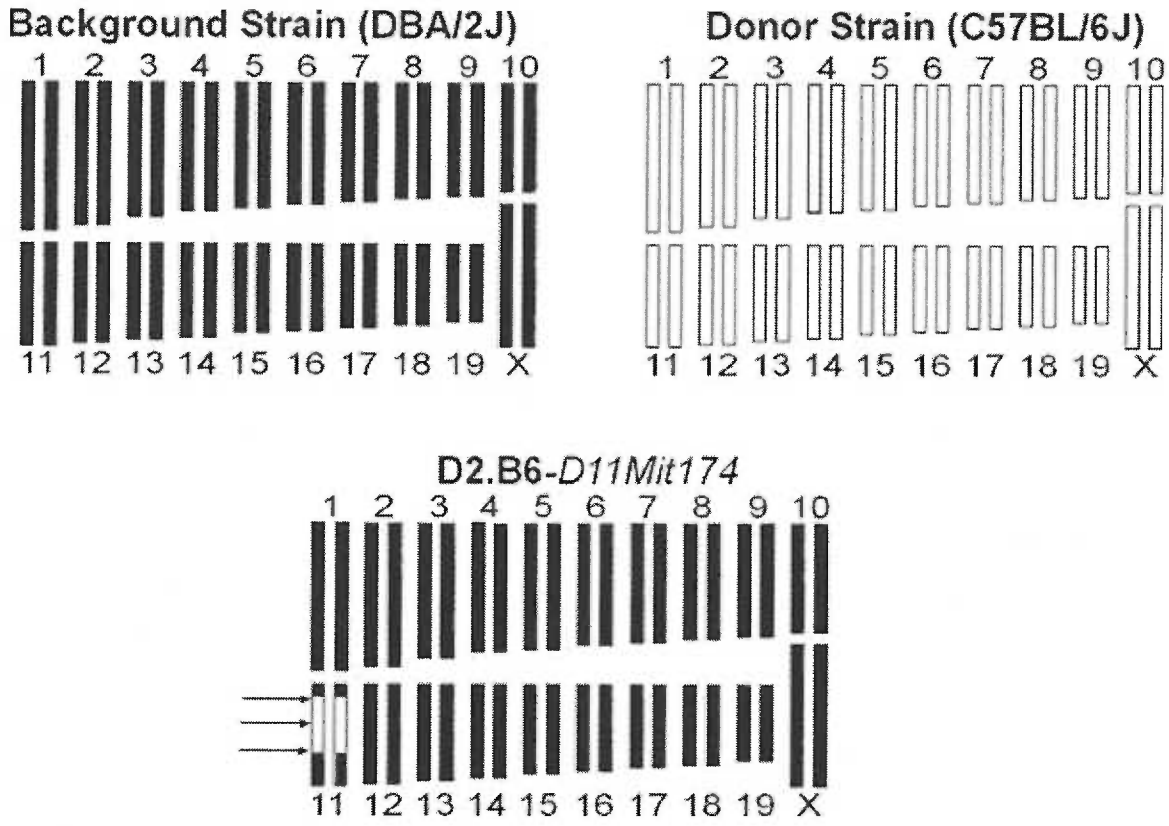

Figure 4. Development of the D2.B6-*D11Mit174* congenic strain.
Background DBA/2J mice were bred with the C57BL/6J donor strain to produce D2B6 F1 offspring. These F1 mice were then backcrossed to D2 mice. The resulting progeny were genotyped at three molecular markers on Chromosome 11: *D11Mit78* (2 cM), *D11Mit174* (20 cM) and *D11Mit179* (52 cM) as indicated by arrows in the lower panel. Mice heterozygous at these markers were backcrossed to the D2 strain for ten generations (N10). After the last backcross generation – mice heterozygous at all three markers were bred together to obtain congenic mice that were B6 homozygous in this 50 cM interval on Chromosome 11 as represented in the lower panel, but > 98% D2 genotype elsewhere.

achieve an inbreeding coefficient of ~0.99 (Falconer and MacKay, 1996). Mice that were heterozygous at all three markers at N10 were intercrossed to obtain B6 homozygosity from ~ 2 cM – 52 cM on Chromosome 11 (see Figure 4). The strain was named D2.B6-*D11Mit174* because this marker has the strongest association with the acute ethanol withdrawal phenotype in the original mapping population (Buck et al., 1997).

II.A.3. D1D2 F2 intercross mice

DBA/1J (D1) and DBA/2J (D2) inbred strains (both sexes) were obtained from the Jackson Laboratory. These inbred strains were reciprocally crossed to obtain D1D2 (female progenitor/male progenitor) and D2D1 F1 mice. These F1 progeny were then interbred to obtain D1D2 F2 mice.

II.A.4. $\gamma 2^{+/-}$ and $\gamma 2^{+/+}$ transgenic mice

We received three male heterozygous $GABA_A$-R $\gamma 2$ subunit null mutant mice ($\gamma 2^{+/-}$) from Dr. Hanns Mohler at the University of Zurich, Switzerland. Homozygous deletion of $\gamma 2$ is lethal (Günther et al., 1995). These mice were originally developed by Dr. Bernhard Lüscher (Günther et al., 1995) using E14 embryonic stem cells derived from strain 129/Ola and were originally on a mixed 129/SvJ and C57BL/6 genetic background. Subsequently, these mice were maintained on a C57BL/6 genetic background for over ten backcross generations (Dr. Lüscher, personal communication). In order to maintain this line of mice, we backcrossed male $\gamma 2^{+/-}$ to female B6 mice (from Jackson Laboratories) to obtain $\gamma 2^{+/-}$ and $\gamma 2^{+/+}$ offspring. Progeny were genotyped using either spleen or tail genomic DNA (see Sections II.G.1 and II.G.2 for extraction protocols) using primers (5'-CATCTCCATCGCTAAGAATGTTCGGGAAGT-3' and

5'-ATGCTCCAGACTGCCTTGGGAAAAGC-3') that span the null mutant allele. If the mice were heterozygous for the null mutant allele ($\gamma2^{+/-}$), PCR would have amplified a 1.4 Kb DNA fragment (Figure 5). If mice were wildtype ($\gamma2^{+/+}$), we would not detect this DNA segment. A control reaction was performed with primers that detect the wildtype *Gabrg2* allele with the following primers: 5'-GCTGACAAAATAATGCAGGGTGCCATACTC -3' and 5'- CATCTCCATCGCTAAGAATGTTCGGGAAGT-3'. This PCR amplifies a 2.0 Kb DNA fragment that should be present in both wildtype and hemizygous null mutant DNA samples (see Figure 5). PCR reactions were carried out using the following parameters: 95°C – 10 min, 35 cycles of 95°C – 30 sec, 56°C – 35 sec, 65°C – 2 min and a final extension at 72°C for 7 minutes. DNA fragments were visualized using a 1% agarose gel stained with ethidium bromide (see Figure 5).

II.B. DNA Sequence Analysis

II.B.1. Gabrg2 sequence analysis of D2, B6, and BXD recombinant inbred mice

II.B.1a. Sequencing

Two independent cDNA clones were sequenced for D2, B6 and each of the 26 BXD RI strains commonly used in ethanol-related phenotypic studies (Taylor, 1978; Crabbe et al., 1999), except the BXD-20 strain for which two independent genomic DNA clones were sequenced. Nine new BXD RI strains were recently developed (Taylor et al., 1999), but have not been behaviorally tested for alcohol response traits, and were not examined in the present study. Poly(A)$^+$ RNA was isolated from whole brain (minus cerebellum) using the FastTrack 2.0 mRNA Isolation Kit (Invitrogen; Carlsbad, CA). Briefly, tissue was homogenized in lysis

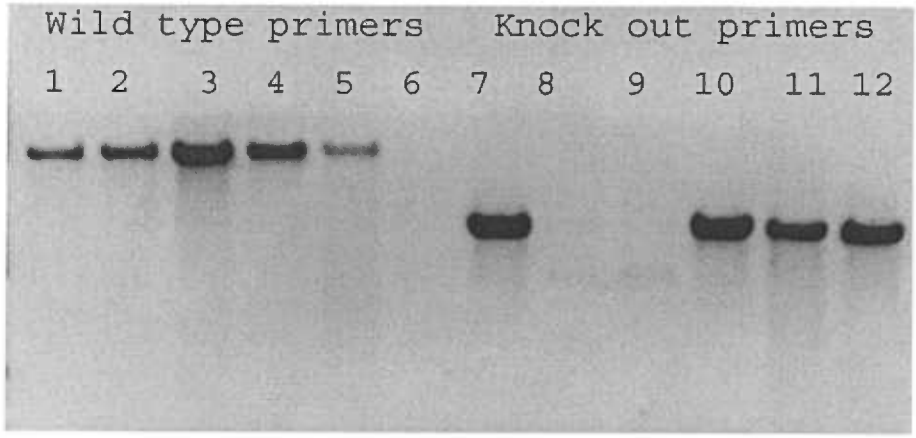

Figure 5. Agarose gel showing genotyping results for $GABA_A$-R $\gamma 2$ subunit heterozygous null mutant ($\gamma 2^{+/-}$) and wildtype ($\gamma 2^{+/+}$) mice using genomic DNA. In lanes 1 through 6, wildtype primers were used to amplify a 2.0 Kb DNA fragment. Primers that detect the null out allele (~1.4 Kb) were used in PCR (lanes 7 through 12). Mouse 1-1 is represented in lanes 1 and 7. Because it possessed both the wildtype allele and the knock out allele – it is $\gamma 2^{+/-}$, whereas mouse 1-2 (lanes 2 and 8) is $\gamma 2^{+/+}$ (no knock out allele). Lane 6 and 12 are control reactions carried out with cDNA from $\gamma 2^{-/-}$ mouse brain (provided by Dr. Bernhard Lüscher) – showing that the wildtype primers are specific to the natural allele.

buffer containing RNase and protein degraders. According to manufacturer's specification, messenger RNA (mRNA) was isolated with an oligo(dT) cellulose column, washed with low-salt buffer, and then eluted from the oligo(dT) columns. The isolated mRNA was ethanol-precipitated and quantified by optical density (A260/280 nm) ratios to determine concentration and quality. First-strand cDNA synthesis from mRNA was completed using Superscript II (GIBCO BRL; Carlsbad, CA) and a 1:1 mixture of oligo$(dT)_{12\text{-}18}$ (Pharmacia) and random hexamer primers (Invitrogen) at 42°C for 50 minutes. Two independent cDNA reactions were performed for each BXD strain, followed by PCR amplification of the protein-coding region. Two independent PCR amplifications were performed using DNA from each strain using the oligodeoxynucleotide primers 5'-TTGGGTACCTCTTCTGCAACCCAGAGGCGAG-3' (corresponding to nucleotides 133-154 of a published mouse BALB/c cDNA, GenBank accession number M62374, and a *Kpn* I site for cloning) and 5'-GTTGGATCCCACATTCGGTGACCACATAGG-3' (corresponding to antisense nucleotides 1665-1685 and a *Bam* HI site for cloning) and AmpliTaq Gold DNA polymerase (Perkin Elmer). PCR was carried out with 20 pmol of each primer for 35 cycles in 50 μl of polymerase buffer (95°C, 60 sec; 65°C, 60 sec; 72°C, 120 sec) and a final extension at 72°C for 7 minutes in a Perkin Elmer 9600 thermocycler. The PCR products were agarose gel purified and subcloned into pBluescript SKII- (Stratagene) for DNA sequence analysis. Before sequence analysis, a diagnostic digest was performed with *Kpn* I and *Bam* HI (GIBCO BRL) to insure that the *Gabrg2* fragment was subcloned. DNA sequence analysis was performed using fluorescent-labeled dye primers and Taq*ff* cycle sequencing kit

(Applied Biosystems) and was analyzed on an ABI model 373A Automated Sequencing System.

II.B.1b. Statistical Analysis

All ethanol-related behaviors were previously measured in other BXD RI studies (sources are given in Table 4 page 59; reviewed by Crabbe et al., 1999). In the present studies, the BXD RI strains we determined to be homozygous for the D2 allele for *Gabrg2* (with ACT coding for a threonine residue at position 11 of the γ2 peptide; T11) were assigned a genotypic score of 1, whereas BXD strains homozygous for the B6 allele (with GCT coding for an alanine at position 11; A11) were assigned a genotypic score of 0. Genetic correlations between γ2 genotype and alcohol phenotypes for which BXD strain means were available were determined by calculating the Pearson correlation coefficient (two-tailed) (Crabbe et al., 1990). Although *Gabrg2* sequence was determined for the 26 existing BXD RI strains (Taylor, 1978, but see Taylor et al. 1999), the number of strains represented in the genetic correlations ranges from 16 to 25 strains reflecting the number of BXD strains that were available in sufficient numbers when phenotypic testing was performed. Statistical comparisons between BXD RI strains that are homozygous for the B6 vs. D2 allele for *Gabrg2* were made using the Student's *t* test for each phenotype.

II.B.2. Standard inbred strain Gabrg2 sequence analysis

The *Gabrg2* sequencing results between the D2 and B6 inbred strains (see above) study identified three single nucleotide polymorphisms (SNPs) at coding sequence nucleotide residue 145, 225, and 744 (Buck and Hood, 1998; GenBank accession numbers AF233802 and AF233775). Here, we amplified and sequenced the coding sequence that contained these polymorphisms in

129P3/J, A/HeJ, AKR/J, BALB/cJ, C3H/HeJ, C57BR/cdJ, C57L/J, CBA/J, CE/J, DBA/1J, PL/J, SJL/J, and SWR/J inbred strains. Poly(A)$^+$ RNA was independently isolated from two whole brains (minus cerebellum) from each strain using the Micro FastTrack mRNA Isolation Kit (Invitrogen, Carlsbad, CA), according to manufacture's instructions. First-strand cDNA synthesis from mRNA was completed using Superscript II (GIBCO BRL) and a 1:1 mixture of oligo(dT)$_{12\text{-}18}$ (Pharmacia) and random hexamer primers (Invitrogen) at 42°C for 50 minutes. Two independent PCR reactions were performed for each inbred strain, followed by agarose gel purification (1.8% Seakem GTG). DNA sequence analysis was performed using fluorescent-labeled dye primers and Taq*ff* cycle sequencing kit (Applied Biosystems) and was analyzed on an ABI model 373A Automated Sequencing System. Results were aligned and compared to previously published D2 and B6 *Gabrg2* sequences using AssemblyLIGN (version1.0.9c; Oxford Molecular/GCG Inc., Madison, WI).

II.C. Phenotypic Measures

II.C.1 Acute Ethanol Withdrawal Phenotyping

Alcohol withdrawal symptoms include anxiety, autonomic hyperactivity, motoric dysfunction and convulsions, which are displayed in both humans and rodents. Handling-induced convulsions (HIC) are a particular sensitive index of acute ethanol withdrawal severity in mice (Metten and Crabbe, 1994; Buck et al., 1997, Fehr et al., 2002). The methods for HIC have been previously published (Crabbe et al., 1991; Terdal and Crabbe, 1994). Briefly, mice are lifted by the tail (and if no convulsion occurs, they are gently spun through an 180° arc) and scored 0-7 according to intensity (see Table 2 for complete description of

scores). Mice were rated twice, twenty minutes apart for baseline convulsions. Following baseline measurement, mice are injected with a hypnotic dose of ethanol (4 g/kg, 20% v/v in saline, 0.023 ml/kg). Withdrawal severity was assessed every hour starting 2 hours post-injection through hour 12.

II.C.2. Statistical Analysis

To assess alcohol withdrawal severity, scores were computed as the area under the curve (AUC) between 4 and 12 hr after alcohol administration in the experiment that compared the Chromosome 11 congenic strain, D2.B6-*D11Mit174* to its D2 genetic background strain and the D1D2F2 experiment. To calculate the AUC, HIC scores from hours 4 through 12 were summed. Hour 4 was selected because it was the first withdrawal time point that was greater than the average baseline score. Average baseline and AUC scores were analyzed using individual *t*-tests for the congenic and D2 comparison study. We tested for association between phenotype (average baseline and AUC) and genotype (*D11Mit174*) in the D1D2 F2 study by using Pearson's *r*.

Hours 5 through 12 were used for AUC measures in the $\gamma 2^{+/-}$ vs. $\gamma 2^{+/+}$ study because hour five was the first measure that exceeded average baseline. AUC takes into account both magnitude and duration of withdrawal severity. Both average baseline scores and AUC were analyzed using individual *t*-tests. Because the average baseline score was different between $\gamma 2^{+/-}$ and $\gamma 2^{+/+}$ mice, we used a corrected AUC score. This score was calculated by subtracting the average baseline score from each time point (i.e., from hour 5 through 12) followed by summation of these corrected values.

Table 2: Handling-Induced Convulsions: Seven-point scale used to index acute ethanol withdrawal severity

Briefly, mice are lifted by the tail (and if there is no convulsion they are gently spun through an 180° arc) and are scored for convulsion, according to the following scale.

0- no convulsion
1- facial grimace after spin
2- tonic convulsion elicited by spin
3- tonic-clonic convulsion following spin
4- tonic convulsion when lifted by the tail (no spin here after)
5- tonic-clonic convulsion when lifted by the tail, with delayed onset
6- severe tonic-clonic convulsion after being lifted by the tail, with quick onset, which often continues after the mouse is released
7- spontaneous or environmentally elicited severe tonic-clonic convulsion with quick onset and long duration

II.D. Quantitative RT-PCR (QRT-PCR) General Methods

II.D.1. Treatment or Experimental groups

We assessed the affects of acute ethanol withdrawal on $GABA_A$-R subunit gene expression in the frontal cortex of D2 and B6 mice. Briefly, mice were weighed and injected with either 4.0 g/kg ethanol (in physiological saline) or with saline and returned to their home cages where they remained undisturbed until time of tissue harvest. To control for cage or litter effects, every cage had at least one saline control animal. Seven hours following ethanol (or saline) administration, mice were killed by cervical dislocation and their frontal cortices were rapidly dissected and flash frozen in liquid nitrogen. Samples were stored at - 80°C until the time of processing. The 7-hour time point was chosen based on phenotypic data indicating where maximal acute ethanol withdrawal occurs for these strains.

We also tested whole brain expression in D2, B6, D1 and D2.B6-*D11Mit174* congenic. These mice were naïve at time of tissue harvest. Mice used in the expression studies were not tested for handling-induced convulsions.

II.D.2. Total RNA Isolation

Two frontal cortices were pooled from each treatment group (ethanol-withdrawn or saline control) and strain (D2 vs. B6) for a total of 12 independent RNA samples (N = 3/group). For whole brain expression studies, RNA was isolated from 3 individual mice per strain (D1, D2, B6, and D2.B6-*D11Mit174* strains) for a total of 3 independent RNA samples per strain. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), which is a modified one-step guanidine isothiocynate procedure first developed by Chomczynski and Sacchi (1987) as previously described (Daniels and Buck, 2002). Briefly, frozen

tissue was placed into Trizol reagent and homogenized using a Polytron homogenizer (Brinkmann, Westbury, NY) at setting three for ten seconds. Total RNA was retained in the aqueous phase following phenol:chloroform extraction at 12,000 g (9500 rpm) for 15 minutes. Isopropanol (300 μl) was added to the aqueous phase to precipitate the total RNA. The isopropanol/RNA sample was centrifuged as above for 10 minutes. The RNA pellet was washed in 75% ethanol (in DEPC-treated water), centrifuged and resuspended in 250 μl of RNase free water.

II.D.3. Poly(A)$^+$ RNA Isolation

In order to have more efficient QRT-PCR, we selected for poly(A)$^+$ RNA from our total RNA samples using oligo(dT) linked to polystyrene-latex particles (Oligotex Mini mRNA kit, Qiagen, Valencia, CA). Briefly, total RNA samples were incubated with 250 μl of binding buffer and 15 μl of oligo(dT) for 3 minutes at 70°C. Oligotex/poly(A)$^+$ RNA hybrids were collected on a spin column and washed twice. Poly(A)$^+$ RNA was eluted from the column with TE buffer (30 μl). mRNA concentration and quality was determined using spectrophotometry (260/280 nm ratios).

II.D.4. Primer Design and Evaluation for Specificity

Deoxyoligonucleotides used to amplify fragments of *Gabrg2*, *Gabra1*, *Gabra6*, and *Gabrb2* were designed using MacVector (version 6.5.3). Primers for the house keeping gene, *G6pd*, were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Forward and reverse primer sequences are listed for each gene in Table 3. Amplicon sizes ranged from 149-225 bp (see Table 3). These primers were designed to be specific for each gene and were assessed using BLAST

Table 3. Quantitative RT-PCR Primers

Primer Pair	Primer Sequence	PCR Amplicon Size (bp)
Gabra1 Forward	5' GGGTCTCTCTGACTATCTTTGGGGC	225
Gabra1 Reverse	5' GAAACTGGTCCGAAACTGGTGAC	
Gabra6 Forward	5' AGGCTGAAAGGCAGGCACAAAC	191
Gabra6 Reverse	5' TAAGATGGGCGTTCTACTGAGGGC	
Gabrb2 Forward	5' CCCTTCTGGAATACGCTTTGGTC	198
Gabrb2 Reverse	5' CCATTACTGCTTCGGATGTGGC	
Gabrg2 Forward	5' CGCAGTTCTGTTGAAGTGGGTG	180
Gabrg2 Reverse	5' GCAGGGAATGTAAGTCTGGATGG	
G6pd Forward	5' ATGGCCTTCTACCTGAAGATACC	149
G6pd Reverse	5' CATAGGAATTACGGGCAAAGA	

(http://www.ncbi.nlm.nih.gov/BLAST) to ensure that these primers would not amplify other genes.

Each primer pair was first tested on the LightCycler PCR System (Roche Applied Sciences, Indianapolis, IN) to check for proper amplification. This system measures real-time DNA amplification via fluorescence readings after each amplification cycle. For initial optimization, we ran a Mg^{++} gradient (4mM – 7mM) for each primer pair using poly(A)$^+$ RNA and a water negative control. The following parameters were used for each QRT-PCR experiment: reverse transcription at 55°C for 45 minutes, denaturation at 95°C for 30 sec followed by 45 amplification cycles of 95°C – 0 sec, 55°C – 10 sec, 72°C – 13 sec. A melting curve analysis was performed after each experiment to check for proper amplification (see Figure 6a-b). Briefly, each sample was slowly heated to 95°C (0.1°C per sec) and fluorescence was measured every second to determine the melting point of each product, which stays consistent across experiments unless a sample was contaminated. Each PCR product was run on an agarose gel to confirm the size of the amplified product and to confirm the absence of contamination.

II.D.5. PCR Efficiency

Whole brain poly(A)$^+$ RNA from eight B6 mice were pooled. QRT-PCR for each primer pair was performed across four different RNA amounts (43, 8.6, 4.3, 0.086 and 0.0086 ng) in quadruplicate. We used LightCyler Relative Quantification Software (Roche Molecular Biochemicals) to create coefficient files, which established the PCR efficiency of each primer pair for the above range of mRNA concentrations. This normalized for the variable PCR efficiencies of the target and reference genes.

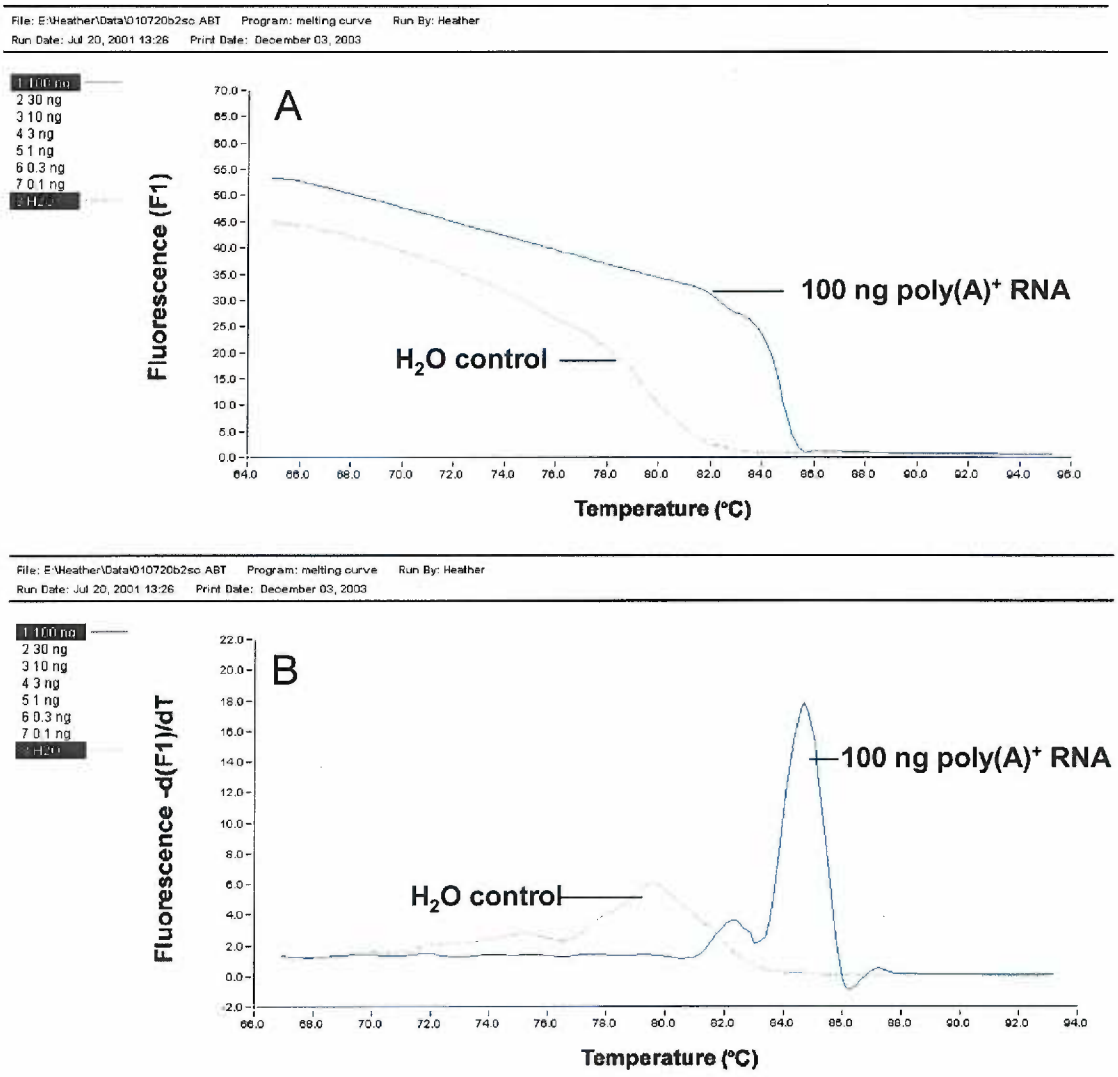

Figure 6a-b. Melting curve (a) and melting peak (b) for QRT-PCR primers. In the top panel, a 100 ng mRNA standard and a water control was amplified with *Gabrb2* specific primers. The amplicon obtained from the 100 ng mRNA sample melts later than the primer dimers observed in the water control. This is more clearly observed with the melting peak, where the 100 ng mRNA sample has a higher melting point of ~84° C compared to the primer dimer of the water control sample, which melts at ~79° C. If contamination were present, the negative control reaction (H_2O) would produce the same melting peak as the mRNA sample.

II.D.6. Quantification

Approximately 20 ng of poly(A)$^+$ RNA was used for each reaction. For each gene, duplicate amplifications were run per sample. Additionally, to normalize quantification, a house-keeping gene (*G6dh*) was assessed simultaneously for each experimental sample (in replicate). To determine relative mRNA amounts, triplicate reactions using known quantities of mRNA (calibrator) were run to compare to the external standard curve. The software uses the following normalization equation to determine the relative amount of starting RNA:

(Target $_{sample}$ /reference $_{sample}$) / (Target $_{calibrator}$/reference $_{calibrator}$).

For example, (*Gabrg2* $_{sample}$/*G6dh* $_{sample}$) / (*Gabrg2* $_{calibrator}$/*G6dh* $_{calibrator}$).

This equation corrects for pipetting errors, PCR efficiencies, RNA quality and run-to-run variability.

II.D.7. Experimental Design and Protocol

PCR reactions were carried out using the LightCycler RNA Amplification Kit SYBR Green 1 (Roche Molecular Biochemicals, Indianapolis, IN). The amount of poly(A)$^+$ RNA included in 15 µl reaction volume was approximately 20 ng based on the spectrophotometric analysis. The RT-PCR master mix contained reaction buffer, dNTPs with dUTP, SYBR Green 1 dye, $MgCl_2$ (final between 5 and 7 mM), forward and reverse primers (0.5 µM each), and RT-PCR enzyme mix. Samples and RT-PCR reagents were pipetted into glass capillary tubes, sealed, and centrifuged briefly to ensure that the reaction mix was in the tip of the capillary tube. Capillary tubes were transferred to the LightCycler and reverse transcription was carried out at 55°C for 45 min. RNA:cDNA hybrids were denatured at 95°C for 30 sec, and target DNA were amplified across 45 cycles (see Section II.D.4). In every experiment, the target gene (e.g., *Gabrg2*) and

house-keeping gene (i.e., *G6pd*) were amplified in replicate per mRNA sample. Included in every experiment were a negative control reaction containing no template RNA (H_2O blank) and three reactions (i.e., triplicate) that contained calibrator mRNA for both the target and house-keeping gene that were used to create the original coefficient file. These data were used to determine the ratios between the target gene and housekeeping gene that were subsequently analyzed.

II.D.8. Statistical Analysis

We used separate two-way ANOVAs to assess the effect of ethanol withdrawal and strain on *Gabrg2*, *Gabra1*, and *Gabrb2* gene expression in the frontal cortex. Individual *t*-tests were performed for each gene (including *Gabra6*) when we compared whole brain expression between D2 and B6 mice, D2.B6-*D11Mit174* congenic mice and D2 background strain, and D2 and D1 strains.

II.E. DNA Extraction

II.E.1. Spleen DNA extraction

We adapted a salting-out protocol from Miller and colleagues (1988). Briefly, frozen half spleens were thawed and placed into 5 ml of lysis buffer and then digested overnight with proteinase K at 55^{O}C (2.5 mg; Invitrogen). Samples were then incubated with RNase (10 μg; DNase free; company) for one hour at 37^{O}C. Saturated NaCl (5M; 1.65 ml) was added to each sample to precipitate the protein; these samples were centrifuged at 6000 g in a Beckman tabletop centrifuge for 20 minutes at 4^{O}C to pellet the protein. To remove the protein, the samples were strained through two layers of cheesecloth. Genomic DNA was

then ethanol precipitated (100% ethanol; 15 ml), collected on glass wands, washed with 75% ethanol, dried and rehydrated in 5 ml of TE buffer. Genomic DNA samples were stored at 4°C.

II.E.2. Tail DNA extraction

We used the Puragene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) per manufacturer's instructions. Briefly, mice were given morphine sulfate (40 mg/kg in physiological saline) prior to cutting a small piece of their tail (~ 4mm or less). Alternatively, tail tissue was collected post-mortem. This tissue sample was then placed into 300 μl of ice-cold cell lysis buffer. Proteinase K was added (1.5 μl; 20 mg/ml); samples were then incubated overnight at 55°C. Samples were incubated with RNase (6 μl; 500 μg/ml) for 60 minutes at 37°C. Protein precipitation solution was added to the cell lysate (100 μl); samples were vortexed and then centrifuged at 14,000 rpm for three minutes. Supernatant containing the genomic DNA was decanted to a clean microcentrifuge tube containing 300 ml of isopropanol. Samples were mixed by inverting 50 times. DNA was collected by centrifuging the sample at full speed in an Eppendorf minicentrifuge (Westbury, NY) for one minute. The supernatant was decanted and the DNA pellet was washed with 70% ethanol. DNA was rehydrated with 50 μl of TE buffer at stored at 4°C.

II.F. D1D2 F2 Genotyping

D1D2 F2 Mice were genotyped using microsatellite markers from the MIT series using protocols adapted from Dietrich et al. (1992) and Serikawa et al. (1992). Because *Alwc3* is linked to *D11Mit174* (Buck et al., 1997), we used this marker to assess whether Chromosome 11 genotype was associated with acute

ethanol withdrawal severity in D1D2 F2 mice. In other QTL studies, selective genotyping was used (Buck et al., 1997, 1999), where only the top and bottom quartiles of the phenotypic distribution were used. In this study we ascertained the genotypes for all mice behaviorally tested (N = 51). Mice that were D1D1, D1D2, or D2D2 were given the following genotypic scores: 0, 1 or 2, which represent the number of D2 alleles. The effect of allelic dosage on area under the withdrawal curve was assessed with correlation coefficients (Pearson's *r*).

II.G. Chromosome 11 Haplotype Mapping Using D1 and D2 Inbred Strains

Because acute ethanol withdrawal severity was significantly correlated with allelic dosage at *D11Mit174* (see Section III.I and Figure 12, page 60) we assessed the haplotype structure between D1 and D2 mice on Chromosome 11. Briefly, a haplotype is a segment of DNA (determined by SNPs and/or other molecular markers) that is inherited together in a block. We genotyped the entire length of Chromosome 11 between D1 and D2 mice with MIT series markers as described above (see Table 6 for markers used). This study was performed to narrow the genomic interval on mouse Chromosome 11 that contains the gene(s) that modulate acute ethanol withdrawal severity. We used 27 markers across Chromosome 11 with an average spacing of ~ 4 cM; the largest gap was 17 cM between *D11Mit199* (62 cM) and *D11Mit104* (79 cM).

III. Results

III.A. *Gabrg2* Sequence in the C57BL/6J and DBA/2J Inbred Strains

We isolated and sequenced a number of γ2 subunit cDNA clones from D2 and B6 mice and found three differences between these strains in their coding sequence. Two single nucleotide polymorphisms (SNPs) did not result in different polypeptide sequences: GGC and GGG both code for a glycine residue at position 37 of the immature polypeptide; and ACG and ACT both code for a threonine residue at position 210. These sequences were found in the D2 and B6 strains, respectively (see Figure 7). In contrast, one polymorphism at amino acid residue 11 did result in different predicted γ2 polypeptide sequences: ACT (codes for a threonine residue in the D2 strain; T11) versus GCT (codes for an alanine residue in the B6 strain; A11). We also found two amino acids in our D2 sequence that consistently differed from the original mouse γ2 sequence published by Kofuji and coworkers (1991) using the D2 strain (accession #M86572). We consistently found a glutamate residue at position 368 and an isoleucine residue at position 397, in contrast to Kofuji and colleagues (1991), who reported glutamine and methionine residues at these positions, respectively. Glutamine and methionine are found at the corresponding positions in the rat γ2 sequence (Shivers et al. 1989; accession #L08497). However, our D2 sequence is in agreement with our B6 mouse sequence and an unpublished sequence for the BALB/c mouse strain (accession #M62374), and suggests that the sequence published by Kofuji and associates (1991) is incorrect regarding amino acids at positions 368 and 397 (see Figure 8).

```
1    atgagttcgc caaatacatg gagcattgga agctcagtct actctcctgt attttcacag
61   aaaatgacgc tgtggattct gctcctgcta tcgctctacc caggcttcac aagccaaaag
121  tcagatgatg actatgaaga ttacActtct aataaaacat gggtgttgac tccaaaagtt
181  ccagagggtg atgtcactgt catcttaaac aacctgctgg aaggCtatga caacaaactt
241  cgacctgaca tcggagtgaa accaacatta attcatacag atatgtatgt gaacagcatt
301  ggtccagtga atgctatcaa tatggaatat acaattgata ttttttttgc ccaaacctgg
361  tatgacagac gtttgaaatt taacagtacc attaaagttc tccggttgaa tagcaatatg
421  gtggggaaaa tctggattcc agacactttc ttcaggaact ccaaaaaggc tgatgctcac
481  tggatcacca ctcccaacag gatgctgaga atttggaatg atggtcgagt tctctacacc
541  ttaaggctaa caattgatgc tgagtgccag ttgcaattac acaacttccc aatggatgaa
601  cactcctgcc ccctggagtt ctccagttat ggatatcctc gtgaagaaat tgtttatcaa
661  tggaagcgca gttctgttga agtgggtgac acaagatcat ggaggctgta tcaattttcc
721  tttgttggat tgaggaatac aacGgaagta gtgaagacaa cttctggtga ctatgtggtg
781  atgtctgtgt acttcgatct gagcagaaga atgggctact tcaccatcca gacttacatt
841  ccctgcacac tcatcgtggt cctgtcctgg gtgtccttct ggatcaataa ggatgctgtt
901  cctgccagaa catctttagg aatcactact gtcctgacca tgacaacttt aagcaccata
961  gccagaaaat ctctgcccaa ggtctcctat gtcacagcaa tggatctctt tgtatctgtt
1021 tgcttcatct ttgtgttttc tgctttggtg gagtatggca ccctgcatta ttttgtcagc
1081 aaccggaagc caagcaagga taaagacaaa aagaagaaaa accctcttct tcggatgttt
1141 tccttcaagg cccctaccat tgatattcgt cccagatcag caaccattca aatgaacaat
1201 gccacacacc ttcaagagag ggatgaagaa tatggctatg agtgtttgga tggcaaggac
1261 tgtgccagtt tcttctgctg ttttgaagat tgccgaacag gagcctggag acatgggagg
1321 atacatattc gcattgccaa aatggactcc tatgctcgga tcttcttccc taccgccttt
1381 tgcttgttca atcttgttta ctgggtctcc tatctttatc tgtaaagagg tatgggtttt
1441 attgataggg gtgttattcg ctgaatctta tggagacaaa atgcactttc taagtccaac
1501 gatataatcc cctatgtggt caccgaatgt g
```

Figure 7. Sequencing results for the coding region between D2 and B6 mice. Data are from GenBank accession # AF233802 (D2 coding sequence). Capitalized and bolded nucleotides indicate SNPs between D2 and B6 mice. Codon **A**C**T** starting at nucleotide 145 codes for a threonine (T11) in the D2 strain, whereas **G**C**T**, found in the B6 strain codes for alanine (A11). We also found two silent SNPs between D2 and B6 mice located at nucleotides 225 (GG**C** vs. GG**G**, which code for glycine) and 744 (AC**G** vs. AC**T**, coding for threonine).

```
1   MSSPNTWSIG SSVYSPVFSQ KMTLWILLLL SLYPGFTSQK SDDDYEDYTS nktWVLTPKV
23  PEGDVTVILN NLLEGYDNKL RPDIGVKPTL IHTDMYVNSI GPVNAINMEY TIDIFFAQTW
83  YDRRLKFNST IKVLRLNSNM VGKIWIPDTF FRNSKKADAH WITTPNRMLR IWNDGRVLYT
143 LRLTIDAECQ LQLHNFPMDE HSCPLEFSSY GYPREEIVYQ WKRSSVEVGD TRSWRLYQFS
203 FVGLRNTTEV VKTTSGDYVV MSVYFDLSRR MGYFTIQTYI PCTLIVVLSW VSFWINKDAV
263 PARTSLGITT VLTMTTLSTI ARKSLPKVSY VTAMDLFVSV CFIFVFSALV EYGTLHYFVS
323 NRKPSKDKDK KKKNPLLRMF SFKAPTIDIR PRSATIQMNN ATHLQERDEE YGYECLDGKD
386 CASFFCCFED CRTGAWRHGR IHIRIAKMDS YARIFFPTAF CLFNLVYWVS YLYL
```

Figure 8. Primary amino acid structure for the $GABA_A$-R γ2 subunit polypeptide found in the D2 strain. Italicized amino acids comprise the potential signal sequence of the immature polypeptide and are not numbered in this sequence. Numbering therefore reflects the mature peptide expressed at the cell surface. There is a nonconservative amino acid substitution at residue 11 of the mature polypeptide; D2 mice possess a threonine, (bold; T11) whereas the B6 derived polypeptide contains an alanine (A11; not shown). Two amino acids downstream from this variation there is a putative N-linked glycosylation motif (NKT; in lower case). The alanine at residue 11 found in the D2 strain is predicted to disrupt a helical motif of the γ2 polypeptide, which may theoretically affect glycosylation.

Following the methods of Garnier et al. (1978) for the prediction of the secondary structure of proteins, we found that in mice where the γ2 subunit is derived from the B6 strain, residue A11 lies in a region that corresponds to an alpha helix. In contrast, residue T11 in the D2 progenitor strain is predicted to disrupt this alpha helix region. It is therefore possible that this modification in the secondary structure of the γ2 subunit, owing to the single amino acid exchange, affects $GABA_A$-R function, considering that two amino acids downstream from this substitution there is a putative N-linked glycosylation site (NKT; see Figure 8).

III.B. Correlation Between *Gabrg2* Sequence Polymorphism and Acute Ethanol Withdrawal Severity in BXD RI Strains

The B6 and D2 progenitor strains differed in their $GABA_A$-R γ2 subunit cDNA coding sequences and their predicted γ2 peptide sequences (Buck and Hood, 1998). In these studies, we cloned and sequenced the *Gabrg2* gene from the 26 BXD RI strains (Taylor, 1978), which have been used by numerous investigators to map a number of ethanol response QTLs (reviewed by Crabbe et al., 1999). Figure 9 shows that 14 BXD RI strains were homozygous for the D2 allele for *Gabrg2* (with ACT coding for a threonine residue at position 11 of the γ2 peptide, T11) and 12 BXD strains were homozygous for the B6 allele (with GCT coding for an alanine at position 11, A11). The B6, D2, and BXD sequence data have been assigned consecutive GenBank accession numbers AF233775-AF233802. The BXD strain distribution for *D11Mit174*, a genetic marker linked to *Alcw3* (Buck et al., 1997) and that maps within the $GABA_A$-R cluster (http://www.ensembl.org/Mus_musculus/ and Celera Discovery System),

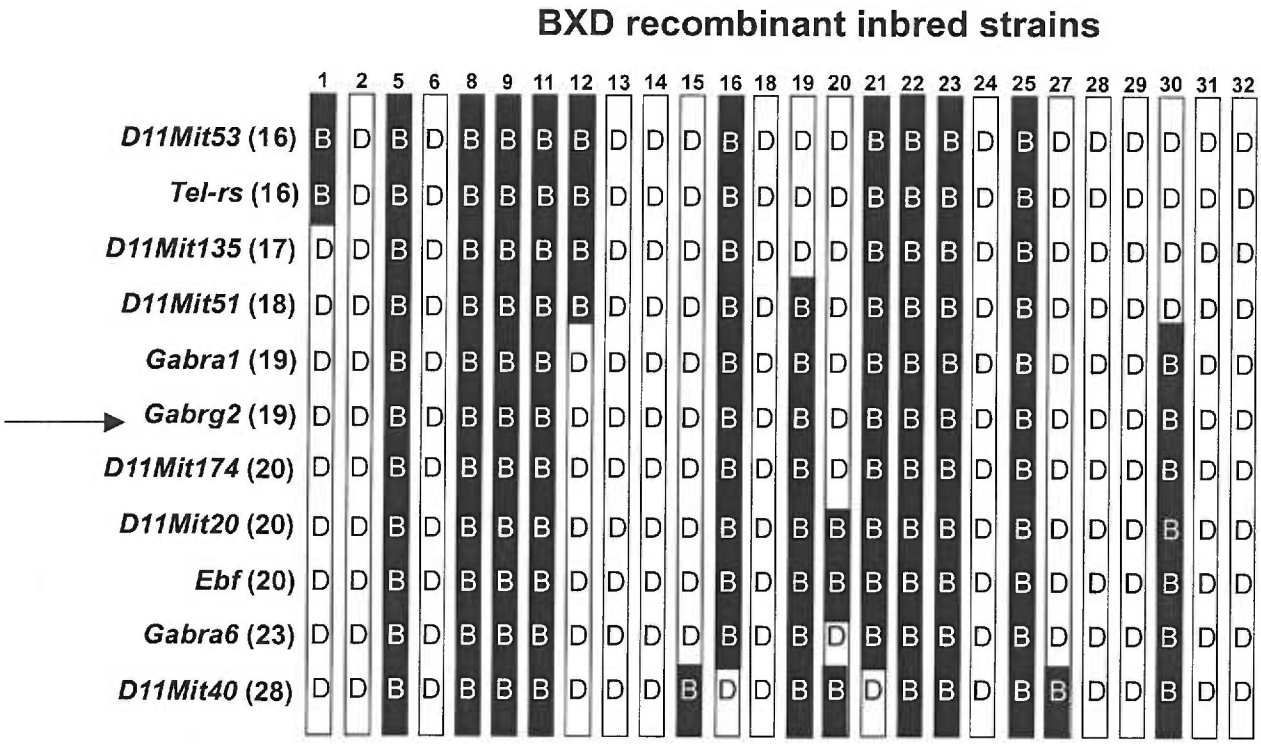

Figure 9. Allelic status for *Gabrg2* and nearby genes in BXD recombinant inbred mice. Allelic status for *Gabrg2* (see arrow) and ten nearby genes and markers on mouse Chromosome 11 (i.e., B for the B6 allele, D for the D2 allele) is indicated for each of the 26 BXD strains for which we sequenced *Gabrg2* (e.g., column 1 represents strain BXD-1). Gene and marker positions are also given in centiMorgan (cM) from the centromere. Genes: *Tel-rs1*, telomere related sequence 1; *Gabra1*, $GABA_A$-R, α1 subunit; *Gabrg2*, $GABA_A$-R, γ2 subunit *Ebf*, early B-cell factor; *Gabra6*, $GABA_A$-R, α6 subunit. *D11Mit 53*, *D11Mit135*, *D11Mit51, D11Mit174*, *D11Mit20*, *D11Mit40* are microsatellite markers. Strain distribution patterns and chromosome location are from Mouse Genome Informatics (MGI). The map predicted from Ensembl gives a slightly different physical order for the genes and molecular markers: *D11Mit53* (32.4 Mb), *Tel-rs* (not mapped), *D11Mit135* (34 Mb), *D11Mit51* (36.7 Mb), *Gabrg2* (42.2 Mb), *Gabra1* (42.6 Mb), *Gabra6* (42.8 Mb) *Gabrb2* (not shown in above figure; 42.9), *D11Mit 174* (43.1; maps within *Gabrb2*), *D11Mit20* (45.2 Mb), *Efb* (not mapped), and *D11Mit40* (80.0 Mb). Recently, 9 additional RI strains have been developed (Taylor et al., 1999), however, none possess recombinations that separate the $GABA_A$-R subunit cluster (data not shown).

was identical to that for *Gabrg2*. The BXD strain distribution for *Gabrg2* was also identical to that for restriction fragment length polymorphisms in the $GABA_A$-R α1 and α6 subunit genes, *Gabra1* and *Gabra6*, indicating no recombinations among these genes (Garrett et al., 1997; See Figure 9). Moreover, *D11Mit174* was located within *Gabrb2.*

The handling-induced convulsion was used to index alcohol withdrawal severity following acute administration of a high dose (4 g/kg, ip) of ethanol as previously described (Crabbe et al., 1991). To correct for BXD strain differences in baseline HICs, post-ethanol HIC scores were regressed on baseline (pre-ethanol) scores, and the regression residual scores were used to score alcohol withdrawal severity (Buck et al., 1997). This method yielded alcohol withdrawal scores that were normally distributed and guaranteed that the derived scores had a correlation of exactly 0 with baseline scores. We found that *Gabrg2* allelic variation was genetically correlated with acute alcohol withdrawal (r = -0.44, p = 0.04, see Figure 10 and Table 4). Figure 10 illustrates that BXD strains that possesd the D2 allele for *Gabrg2* showed significantly less severe acute alcohol withdrawal than BXD strains that expressed the B6 allele (-0.95 ± 0.67 vs. 1.61 ± 1.05, residual score ± SEM). This was consistent with the direction of effect predicted by a QTL involved in acute alcohol withdrawal (*Alcw3)* identified near *Gabrg2* (Buck et al., 1997).

Other ethanol related traits were tested for correlation with γ2 variation in BXD RI mice. As shown in Table 4, ethanol-conditioned taste aversion (2g/kg), ethanol- induced hypothermia (4 g/kg) and ethanol-induced motor-incoordination (screen test) were significantly correlated with γ2 polymorphism.

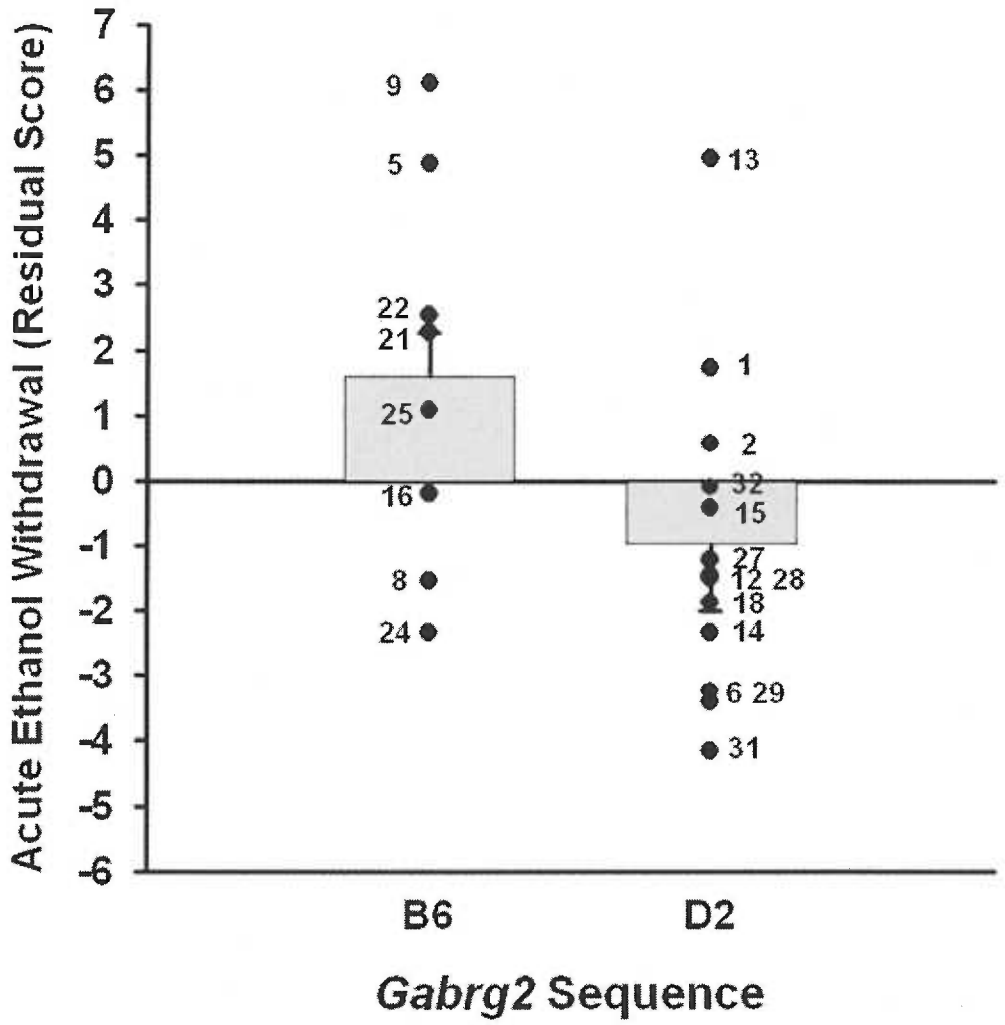

Figure 10. Allelic variation in the $GABA_A$-R $\gamma 2$ subunit is genetically correlated with acute alcohol withdrawal in BXD mice. Each data point identifies the strain mean for a BXD strain (e.g., 9 represents strain BXD-9). *Gabrg2* is associated with acute alcohol withdrawal severity calculated as residual area under the curve (*p = 0.04, withdrawal scores are from Buck et al., 1997). The bars show the mean withdrawal residual score ± SEM for BXD strains with the B6 allele (N = 8) vs. the D2 allele (N = 13). This is consistent with the direction of effect predicted by *Alcw3*, a significant QTL for acute alcohol withdrawal on Chromosome 11 where the D2 allele is associated with lower acute ethanol withdrawal severity while the B6 allele is associated with more severe withdrawal (Buck et al., 1997).

Table 4. Genetic correlations between $GABA_A$ receptor γ2 subunit sequence (A/T11) and strain means for selected behavioral responses to ethanol measured in other BXD RI studies.

Response to Ethanol	*r*	*p*	n	Reference
Acute withdrawal	-.44*	.04	21	Buck et al., 1997
Chronic withdrawal (AREA 25)	-.34	.11	24	Crabbe, 1998
Chronic withdrawal (DELAREA 25)	-.22	.28	24	Crabbe, 1998
Conditioned taste aversion (2 g/kg, consumption)	.55*	.01	20	Risinger & Cunningham, 1998
Conditioned taste aversion (2 g/kg, residual)	.52*	.02	20	Risinger & Cunningham, 1998
Conditioned taste aversion (4 g/kg, consumption)	.32	.16	20	Risinger & Cunningham, 1998
Conditioned taste aversion (4 g/kg, residual)	.25	.29	20	Risinger & Cunningham, 1998.
Conditioned Place Preference	-.10	.67	20	Cunningham, 1995
Motor incoordination (screen test)	.55*	.004	25	Browman & Crabbe, 2000
Ataxia (onset threshold, rotarod test)	-.01	.95	25	Gallaher et al., 1996
Ataxia (maximal threshold, rotarod test)	-.32	.12	25	Gallaher et al., 1996
Tolerance to ethanol-induced ataxia (rotarod test)	-.25	.23	25	Gallaher et al., 1996
Ataxia (grid test error)	.40	.06	24	Phillips et al., 1996
Tolerance to ethanol-induced ataxia (grid test)	.32	.12	24	Phillips et al., 1996
Loss of righting reflex (duration)	.36	.09	24	Browman & Crabbe, 2000
Loss of righting reflex (BEC at recovery)	-.02	.94	24	Browman & Crabbe, 2000
Hypothermia (2 g/kg)	.03	.90	23	Crabbe et al., 1996
Hypothermia (3 g/kg)	-.19	.38	23	Crabbe et al., 1996
Hypothermia (4 g/kg)	-.46	.02	23	Crabbe et al., 1996
Tolerance to ethanol-induced hypothermia (2 g/kg)	-.36	.09	23	Crabbe et al., 1996
Tolerance to ethanol-induced hypothermia (3 g/kg)	-.37	.08	23	Crabbe et al., 1996
Tolerance to ethanol-induced hypothermia (4 g/kg)	-.22	.32	23	Crabbe et al., 1996
3% Ethanol intake (g/kg/day)	.17	.54	16	Phillips et al., 1994
3% Ethanol preference (vs. water)	.04	.89	16	Phillips et al., 1994
10% Ethanol intake (g/kg/day)	.17	.54	16	Phillips et al., 1994
10% Ethanol preference (vs. water)	.19	.43	19	Phillips et al., 1994

BXD strain means are from the references indicated.

* $p < 0.05$

For both ethanol-conditioned taste aversion and ethanol-induced motor-incoordination, there were suggestive QTLs mapped to Chromosome 11 using BXD RI strains (Risinger and Cunningham, 1998; Browman and Crabbe, 2000), suggesting that *Gabrg2* has possible pleiotropic effects on the aversive effects of ethanol.

III.C. *Gabrg2* Sequence Analysis Among a Panel of Inbred Mouse Strains

We sequenced *Gabrg2* in a panel of inbred mice previously tested for acute ethanol withdrawal severity (Metten and Crabbe, 1994; 129P3/J, A/HeJ, AKR/J, BALB/cJ, C3H/HeJ, C57BR/cdJ, C57L/J, CBA/J, CE/J, DBA/1J, PL/J, SJL/J, and SWR/J inbred strains). Results showed that all the strains sequenced here except for the D2 strain had the same nucleotides at residues 145, 225, and 744. DBA/1J (D1) and D2 mice are closely related genetically (Atchley and Fitch, 1993; Taylor, 1978), but did not possess the same $GABA_A$-R γ2 variant. Furthermore, D2 mice displayed 2-fold greater ethanol withdrawal severity compared to D1 mice (behavioral data from Metten and Crabbe 1994, see Figure 11). Therefore, we hypothesized that crosses derived from D1 and D2 inbred strain mice may be useful in finer mapping of the Chromosome 11 QTL.

III.D. A Chromosome 11 QTL Regulates Acute Ethanol Withdrawal Severity in D1D2 F2 Mice

Because the D2 and D1 inbred strains are closely genetically related, yet possessed different $GABA_A$-R γ2 variants, we developed a reciprocal D1D2 F2 intercross to test the hypothesis that *Gabrg2*, or a linked gene, modulates acute

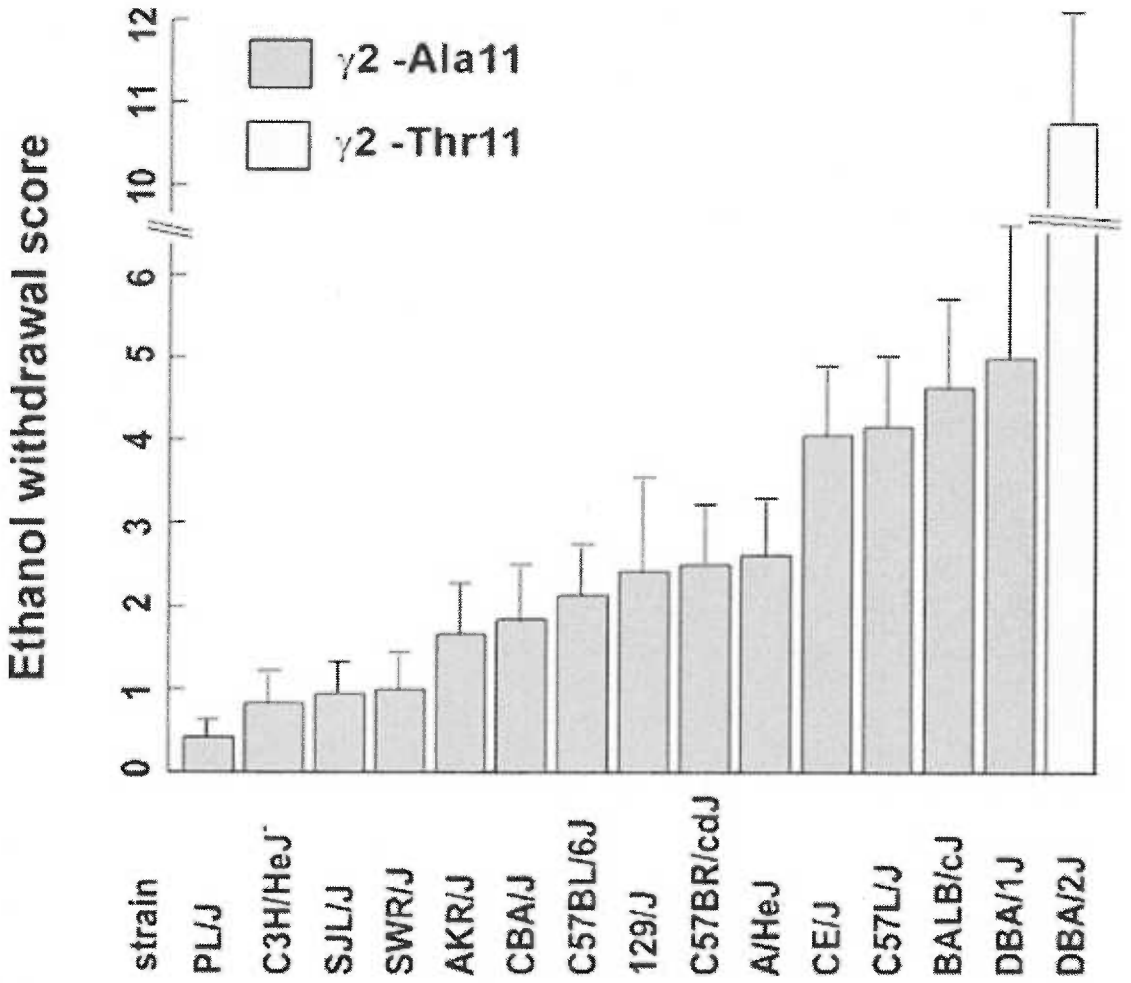

Figure 11. D2 mice possess a unique γ2 variant and display the most severe acute ethanol withdrawal compared to 14 other standard inbred strains. We sequenced *Gabrg2* across a panel of inbred mouse strains previously tested for acute ethanol withdrawal severity (Metten and Crabbe, 1994). D2 mice possess a threonine at residue 11 (T11) of the mature γ2 polypeptide, whereas the other 14 strains, including the genetically similar DBA/1J strain, have an alanine (A11) at this position. Moreover, D2 mice show at least a 2-fold greater acute ethanol withdrawal reaction compared to all other strains tested.

ethanol withdrawal severity. *D11Mit174* allelic status was correlated with acute ethanol withdrawal severity in this population of mice (r = -0.36; $p < 0.008$, two-tailed; Figure 12, inset). Additionally, this locus accounted for 13% of the phenotypic variance (r^2; Belknap et al., 1996). F2 mice that were D1D1 homozygous at *D11Mit174* have more severe acute ethanol withdrawal than D2D2 homozygotes. Mice that were D1D2 at *D11Mit174* show less severe acute ethanol withdrawal, similar to F2 mice that were D2D2 at this locus. These results suggest that the D2 allele at the relevant gene may act dominantly in this genetic population. D1D1 mice also had a more severe ethanol withdrawal reaction as shown by time course analysis of data for hours 4 through 12 ($F_{[8,216]}$ = 8.4, $p < 0.001$, Figure 12). Baseline handling induced convulsions did not differ among genotypes ($F_{[2,48]}$ = 0.45, p = 0.65; two-tailed). These results indicated that the D1 allele was associated with increased acute ethanol withdrawal severity. Similarly, in B6D2 F2 populations, the B6 derived *D11Mit174* allele was associated with increased acute ethanol and pentobarbital withdrawal severity (Buck et al., 1997, 1999). The D1 inbred strain had less severe acute ethanol withdrawal than D2 mice; however the D1 *D11Mit174* allele was associated with more intense withdrawal suggesting that there were other QTL regulating this phenotype in D1D2 F2 mice. This is highly plausible because another QTL study that used D1D2 F2 mice to assess the genetic contribution to acute zolpidem withdrawal detected the Chromosome 11 QTL along with a genomic region on Chromosome 2 that modulated this phenotype. In contrast, the D2 allele on Chromosome 2 was

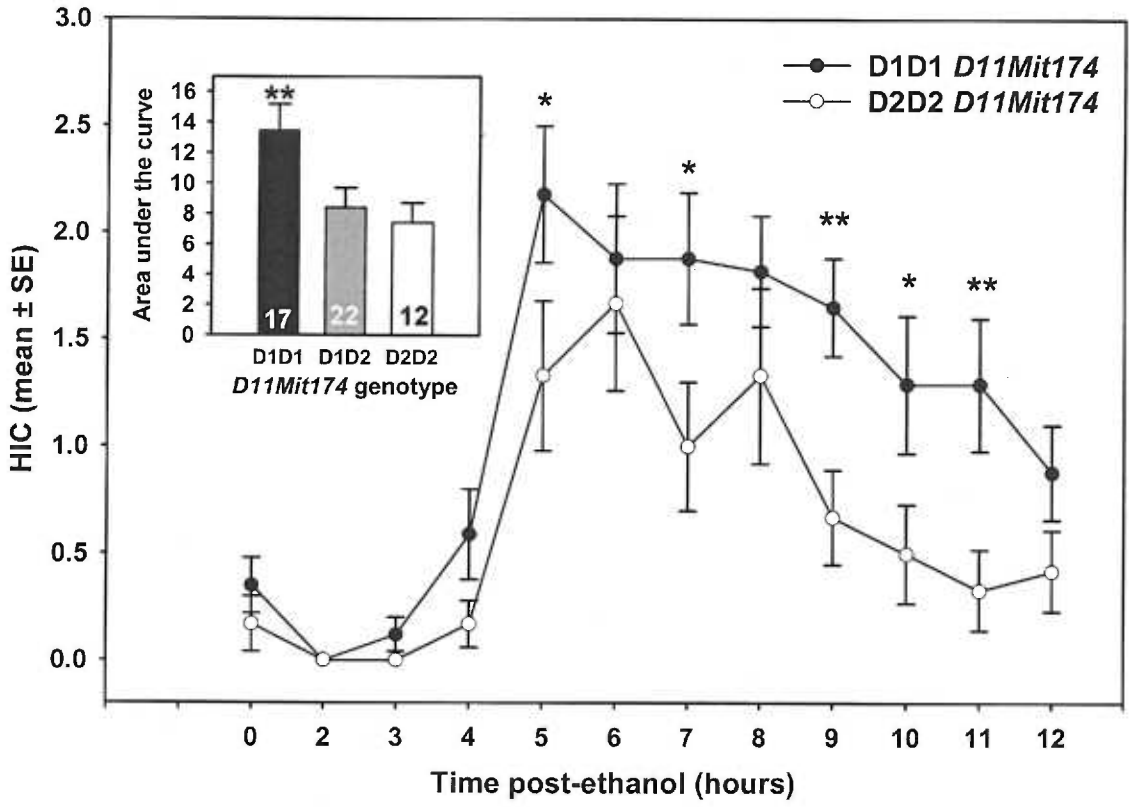

Figure 12. Mice that are D1D1 at the molecular marker *D11Mit174* have more severe acute ethanol withdrawal compared to mice that are D2D2 homozygotes. (Inset) Acute ethanol withdrawal severity was correlated with *D11Mit174* status as measured by AUC (r = -0.36; p = 0.008, two-tailed). Mice that were D1D1 homozygous at the marker displayed more severe withdrawal reactions. Our results suggested that the D2 allele acted dominantly because mice that are heterozygous (D1D2) have a similar degree of ethanol withdrawal severity as the D2D2 homozygotes. Ethanol withdrawal time course illustrated that mice D1D1 at *D11Mit174* had a higher withdrawal reaction compared to D2D2 homozygotes.

*** $p < 0.05$; ** $p < 0.01$.**

associated with greater zolpidem withdrawal severity (Metten and Crabbe, unpublished data).

We attempted to perform interval mapping for Chromosome 11; however, we were unable to find markers that were polymorphic between D1 and D2 mice either upstream or downstream from *D11Mit174* (at least 10 cM away). This result is not particularly surprising because D1 and D2 mice are genetically related. The Center for Inherited Disease Research has performed a genome-wide scan using ~300 markers from the MIT series for 54 inbred strains including D1 and D2. They report 51 markers (none within the narrowed QTL interval), across the genome, as being polymorphic between D1 and D2 mice (~17% difference; http://www.cidr.jhmi.edu/mouse/mouse_dif.html). In a study by Atchley and Fitch (1993), 119 markers were assessed across the genome between D1 and D2 mice revealing only 11.8% difference between them or ~ 14 polymorphic markers.

III. E. Chromosome 11 Haplotype Mapping between DBA/2J and DBA/1J Inbred Strains

In order to obtain a finer resolution map of the Chromosome 11 QTL interval, we exploited the genetic relatedness between the DBA/1J and DBA/2J inbred strains. We genotyped microsatellite markers across mouse Chromosome 11 to determine the extent of allelic differences between these two strains. Our results showed that there was an genomic interval between 17 and 20 cM that was polymorphic between D1 and D2 mice, however, the other markers tested either proximal or distal to this region were not different between these strains (see

Marker	Cm (MGI)	Mb (Ensembl)	Mb (Celera)	polymorphic? D1 vs D2
D11Mit226	1.5	8.9	6.3	no
D11Mit63	2	17.18	14.4	no
D11Mit78	2	18	17.1	no
D11Mit148	3.3	14.8	12.2	no
D11Mit79	10.9	19.8	18.8	no
D11Mit163	16	27.8	26.9	no
D11Mit53	16	32.4	31.69	no
D11Mit173	17	34.3	33.37	no
				unknown
D11Mit231	17	35.9	36.4	yes[1]
D11Mit108	18	36.3	UN	yes[2]
D11Mit217	19	37.8	38.4	yes[2]
D11Mit174	20	43.1	43.4	yes
D11Mit296	20	43.4	43.9	yes[2]
D11Mit21	20	44.7	UN	yes
				unknown
D11Mit206	20	45.1	46.2	no
D11Mit188	20	45.8	46.9	no
D11Mit140	28	54.8	55.7	no
D11Mit349	32	56.3	57.5	no
D11Mit208	33	59.1	60.3	no
D11Mit261	34	62.8	UN	no
D11Mit177	36	65.8	69.1	no
D11Mit115	37	68.2	71.5	no
D11Mit90	42	71.1	75	no
D11Mit195	47	85.6	91.4	no
D11Mit179	52	90.6	96.4	no
D11Mit199	62	102.7	110.5	no
D11Mit104	79	120.1	128	no

1: Center of Inherited Disease Research
http://www.cidr.jhmi.edu/cgi-bin/markers2002.cgi
2: Hood et al., in preparation

Table 5. Haplotype mapping between DBA/2J and DBA/1J mice. We genotyped D1 and D2 inbred strains using 27 molecular markers distributed across the length of Chromosome 11. Distance (in centiMorgan; cM) was obtained from Mouse Genome Informatics (MGI), and physical location (megabase; Mb) were obtained from public (Ensembl) and private (Celera) databases. The region highlighted in yellow indicates the region of Chromosome 11 that contains several polymorphic markers between D2 and D1 mice. The gene(s) that underlie *Alcw3* most likely is contained in this section.

Table 5). This region, according the Mouse Genome Sequencing Consortium and to the privately funded mouse genome sequencing initiative spanned an interval of approximately ~ 8.8 megabases (Mb). Database queries of both Celera and Ensembl showed that there are 35 known (including RIKEN clones) and 11 predicted (novel) genes within this interval (see Table 6), including *Gabrg2*, *Gabra1*, *Gabra6*, and *Gabrb2*. In addition to these $GABA_A$-R subunits, *Gabrp*, the gene that encodes the π subunit also maps to mouse Chromosome 11. This subunit may be expressed in human hippocampal and cortical tissue (Hedblom and Kirkness, 1997). As well, the π subunit may attenuate $GABA_A$-R sensitivity to pregnanolone, an endogenous neurosteroid (Hedblom and Kirkness, 1997). However, based on our haplotype analysis, *Gabrp* mapped outside of the polymorphic interval between D1 and D2 mice (Table 5). Based upon the distance between *Gabrp* and *Gabrg2* (8.5 Mb) and the fact that there were other intervening genes, the π subunit is probably not part of the $GABA_A$-R subunit cluster. Other genes of interest contained within this interval include Cyclin G, which is part of an apoptosis pathway and the α1B- adrenoreceptor. Some of these genes have been shown to be expressed within the mouse CNS mainly through cDNA amplification (UniGene; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). However, it remains to be determined if these genes are differentially expressed between D1 and D2 or between B6 and D2 mice.

III.F. Whole Brain $GABA_A$-R Subunit Gene Expression Between Ethanol-naïve D2 and B6 Mice

Gabrg2, *Gabra1*, *Gabra6*, and *Gabrb2* expression levels between naïve D2 and B6 mice were measured using QRT-PCR. Our initial expression studies

(Mb)	Symbol	Description	Function	Brain?
33.9	*Gabrp*	$GABA_A$-R π subunit	steroid modulation	?
34.3	*Kcnmb1*	Ca^{2+} -activated K^+ channel β1 subunit	vascular tone	-
34.4	*Lcp2*	Lymphocyte cytosolic protein 2	protein adaptor	-
34.5	*Foxi1*	Forkhead box I1	inner ear development	-
34.5	*Dock2*	Dedicator of cyto-kinase 2	cell migration	-
36	*Pank3*	Pantothenate kinase 3	co-A synthesis	+
36.2	*Odz2*	Odd OZ/Ten-m homolog 2 (drosophila)	CNS development?	+
41.1	*Akrb3*	Aldose reductase	oxidoreductase activity	+
41.1	*Hmmr*	Hyanluronan mediated motility receptor	cell adhesion molecule	+
41.1	*Ccng1*	Cyclin G	Apoptosis	+
42.4	*Gabrg2*	$GABA_A$-R γ2 subunit	neural inhibition	+
42.6	*Gabra1*	$GABA_A$-R α1 subunit	neural inhibition	+
42.8	*Gabra6*	$GABA_A$-R α6 subunit	neural inhibition	+
42.9	*Gabrb2*	$GABA_A$-R β2 subunit	neural inhibition	+
43.9	*Pttg1*	Pituitary tumor transforming gene	Mitosis	+
43.9	*Slu7-pending*	Step II splicing factor	pre-mRNA splicing	+
44.1	*Fabp6*	Gastropin	bile salt binding	-
44.3	*Adra1b*	α1B adrenergic receptor	G-protein coupled R	+
45.5	*Ebf1*	Transcription factor early β cell	neural development	+
49.8	*Zfp62*	Zinc finger protein 66	nucleic acid binding	+

Table 6. Known genes contained within a non-shared genomic block between D1 and D2 mice. Physical location, obtained from Ensembl (version 17.30.1, updated May 6, 2003), is shown in megabases (Mb). Genes within non-polymorphic regions are highlighted in blue. As shown above, the gene the codes for the $GABA_A$-R π subunit, *Gabrp*, is within a region predicted to be shared between these two strains. However, *Gabrg2*, *Gabra1*, *Gabra6*, and *Gabrb2* are within a polymorphic section between D2 and D1 mice (represented in yellow). The gray region represents recombination boundaries; the proximal boundary contains known genes; however, all evidence to date suggests that these genes are not centrally expressed.

used whole brain in order to capture all brain regions regulating acute ethanol withdrawal. We did not observe innate expression differences in whole brain mRNA between D2 and B6 mice for *Gabrg2* ($t_{[1,4]}$ = 0.75; p = 0.50), *Gabra1* ($t_{[1,4]}$ = 0.83; p = 0.45) or *Gabrb2* ($t_{[1,4]}$ = -0.81; p = 0.46; Figure 13). Interestingly, these results did not confirm Affymetrix microarray expression data that suggested differential whole brain *Gabra1* expression between naïve B6 and D2 mice (B6/D2 ratio = 1.92; R. Hitzemann, unpublished data). On the other hand, *Gabra6* expression was significantly different between D2 and B6 mice for both QRT-PCR and microarray experiments. As shown in Figure 13c, D2 *Gabra6* content was greater compared to B6 mice ($t_{[1,4]}$ = -6.67; p = 0.003). These results are consistent with the direction of effect (D2>B6) observed in the microarray data. However, congenic and background strain mice did not differ in *Gabra6* expression, which did not support a role for *Gabra6* in acute ethanol withdrawal severity (see Section III.E).

III.G. $GABA_A$-R subunit mRNA Expression between Ethanol-naïve and Withdrawn D2 and B6 Mice

c-FOS mapping indicated that the frontal cortex of D2 and B6 mouse strains was differentially activated during acute ethanol withdrawal and therefore may be part of a neural circuit that modulates acute ethanol withdrawal severity (Buck et al., in preparation). *Gabrg2*, *Gabra1*, and *Gabrb2* mRNA content was measured in frontal cortex of ethanol-withdrawn D2 and B6 mice (7 hours post-injection; 4 g/kg ethanol; 20% v/v in physiological saline) and compared to vehicle control mice using QRT-PCR.

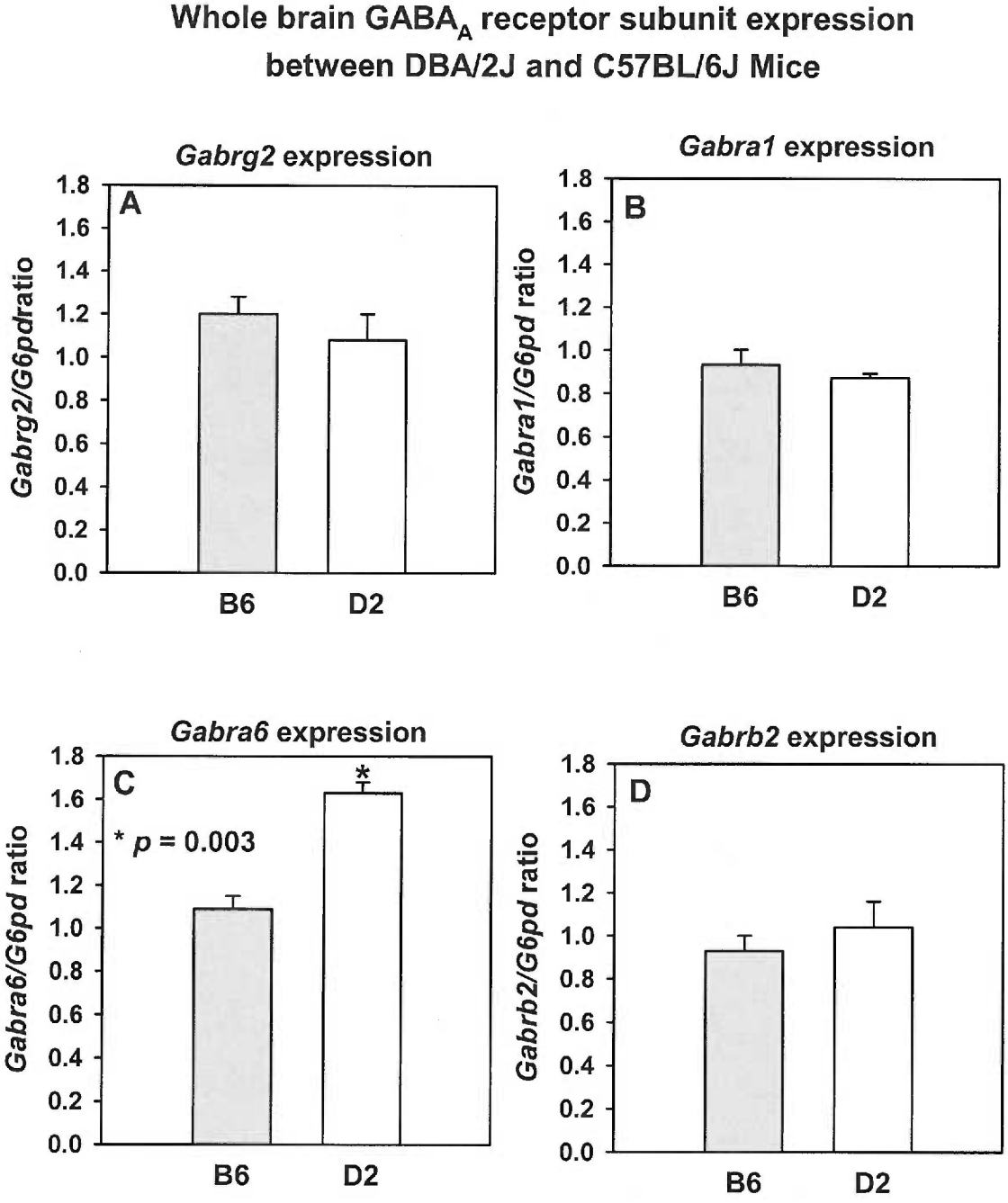

Figure 13. *Gabra6* is differentially expressed between D2 and B6 mice. (A) Whole brain *Gabrg2* expression did not differ between D2 and B6 mice ($t_{[1,4]}$ = 0.75; p = 0.49). **(B)** *Gabra1* was similarly expressed between D2 and B6 mice ($t_{[1,4]}$ = 0.83; p = 0.45). **(C)** D2 mice had greater levels of *Gabra6* compared to B6 mice ($t_{[1,4]}$ = -6.67; p = 0.003). **(D)** No differences for *Gabrb2* expression were detected between D2 and B6 mice ($t_{[1,4]}$ = -0.81; p = 0.46).

Our data showed that there was a significant effect of strain on *Gabrg2* expression. D2 mice had higher cortical levels of *Gabrg2* compared to B6 mice ($F_{[1,8]} = 18.89$; $p = 0.002$, two-tailed; Figure 14a). There was a trend for an effect of withdrawal ($F_{[1,8]} = 3.15$, $p = 0.11$, two-tailed) and a significant interaction between strain and treatment where ethanol-withdrawn D2 mice had *Gabrg2* expression levels similar to B6 withdrawn and saline-control mice ($F_{[1,8]} = 8.08$, $p = 0.02$, two-tailed). Tukey post-hoc tests indicated that *Gabrg2* expression in D2 saline control mice was significantly different compared to the three other groups tested (verses D2 ethanol-withdrawn mice, $p = 0.04$; verses B6 saline control mice, $p = 0.004$; verses B6 ethanol-withdrawn, $p = 0.01$).

Some studies have shown an association between differential *Gabra1* expression and genetic predisposition for ethanol withdrawal severity using WSP and WSR whole brain (Buck et al., 1991; Keir and Morrow, 1994). Following chronic ethanol diet, WSP mice showed decreased whole brain *Gabra1* expression compared to WSR mice (Buck et al., 1991). However, *Gabra1* expression was higher in the cerebellum of ethanol-naïve WSP mice compared to WSR selected line. Here we did not detect a difference between D2 and B6 cortical *Gabra1* baseline expression ($F_{[1,8]} = 1.35$, $p = 0.28$, two-tailed; Figure 14b). Ethanol withdrawal did not affect *Gabra1* expression in frontal cortex of B6 and D2 mice ($F_{[1,8]} = 1.35$, $p = 0.28$, two-tailed). No interaction between strain and treatment was detected for *Gabra1* expression ($F_{[1,8]} = 0.53$, $p = 0.49$, two-tailed).

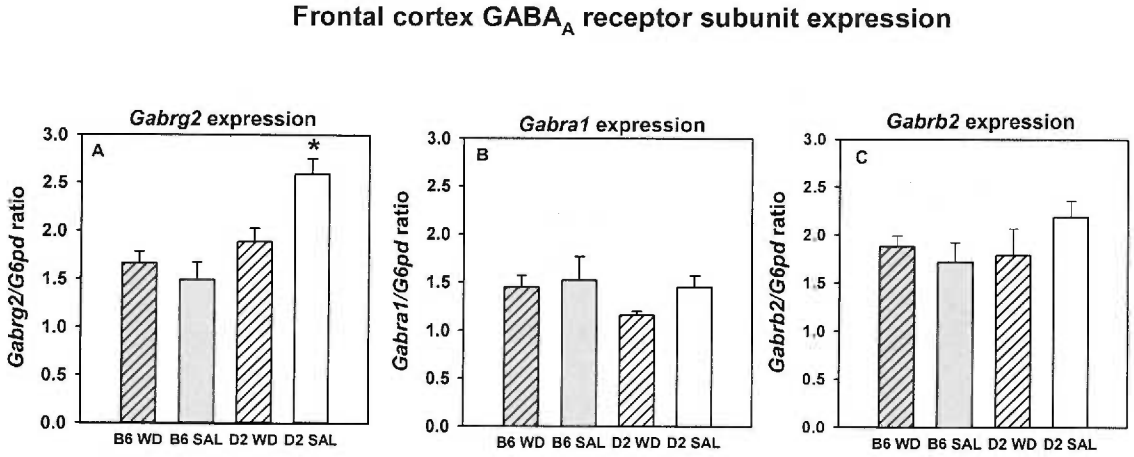

Figure 14a-c. *Gabrg2* expression is greater in frontal cortex of D2 inbred strain mice compared to the B6 strain. (A) *Gabrg2* frontal cortex expression in D2 and B6 mice cortex following acute ethanol withdrawal (WD) or saline controls (SAL). D2 mice expressed more *Gabrg2* compared to the B6 inbred strain in frontal cortex ($F_{[1,8]}$ = 18.89; p = 0.002, two-tailed, indicated with an asterisks). There was a trend for an effect of treatment ($F_{[1,8]}$ = 3.15, p = 0.11). The data indicated that there was a strain by treatment interaction in that acute ethanol withdrawal reduced D2 *Gabrg2* expression in frontal cortex ($F_{[1,8]}$ = 8.08, p = 0.02). **(B)** *Gabra1* levels were not different between D2 and B6 mice. We did not observe an effect of strain ($F_{[1,8]}$ = 1.35, p = 0.28), treatment ($F_{[1,8]}$ = 1.35, p = 0.28), nor a strain by treatment interaction ($F_{[1,8]}$ = 0.53, p = 0.49) for *Gabra1* cortical expression. **(C)** GABA$_A$-R β2 subunit gene expression was not different between D2 and B6 frontal cortex. We did not detect any expression differences between strains ($F_{[1,8]}$ = 0.87, p = .38, two-tailed), treatment ($F_{[1,8]}$ = 0.38, p = 0.55). We did not observe a strain by treatment interaction for *Gabrb2* expression ($F_{[1,8]}$ = 1.89; p = .76; two-tailed).

Lastly, we assessed *Gabrb2* expression in the frontal cortex of D2 and B6 mice. Previous studies from our lab have shown that this gene was complexly regulated by chronic ethanol exposure that is dependent upon strain, blood ethanol concentration and time point. However, no differences in baseline expression were observed in cortex or cerebellum (Reilly and Buck, 2000). Here, we measured *Gabrb2* mRNA content in the frontal cortex following acute ethanol withdrawal in D2 and B6 mice. Steady-state *Gabrb2* expression did not differ between D2 and B6 mice ($F_{[1,8]} = 0.87$, $p = 0.38$, two-tailed; Figure 14c). Ethanol withdrawal did not affect *Gabrb2* expression ($F_{[1,8]} = 0.38$, $p = 0.55$, two-tailed) nor did we observe a strain by treatment interaction $F_{[1,8]} = 1.87$, $p = 0.21$, two-tailed; Figure 12c). We did not measure *Gabra6* expression in this experiment because it is restricted to the granule cells of the cerebellum and cochlear nucleus (Wisden et al., 1992).

Our expression data suggested that cellular heterogeneity may dilute differences in gene expression. We were most likely able to detect differential *Gabra6* expression between D2 and B6 mice because this gene's expression is restricted to the cerebellum and thus "noise" from the brain regions is not a factor. The cellular composition of the cerebellum is estimated to be ~ 85% granule cells. However, *Gabrg2*, *Gabra1*, and *Gabrb2* are ubiquitously expressed throughout the brain and in numerous cell types (Fritschy and Mohler, 1995; Wisden et al., 1992, Pirker et al., 2000). When gene expression was measured in the frontal cortex, we were able to detect differential *Gabrg2* expression between D2 and B6 inbred strains. Moreover, our data showed that ethanol withdrawal regulates *Gabrg2* expression. These studies provide

supporting evidence for the $GABA_A$-R $\gamma 2$ subunit gene as a strong candidate for *Alcw3*. However, these experiments did not exclude the other Chromosome 11 $GABA_A$-R subunits because we only measured their mRNA content at one withdrawal and developmental time-point. For instance, there could be differential expression during embryonic development between D2 and B6 mice that affects synaptic development. However, tests using null mutants for $\gamma 2$, $\alpha 1$, $\beta 2$ and $\alpha 6$ support the former hypothesis (below).

III.H. Heterozygous Null mutant $\gamma 2$ Mice Have More Severe Acute Ethanol Withdrawal Than Wildtype Littermates

$\gamma 2^{+/-}$ null mutant mice were developed by homologous recombination in exon eight of *Gabrg2* (Günther et al., 1995). Homozygous deletion of $GABA_A$-R $\gamma 2$ subunit is lethal whereas heterozygous mice survive into adulthood and are visually indistinguishable from wildtype littermates. $GABA_A$-R $\gamma 2$ peptide was nearly 50% less in $\gamma 2^{+/-}$ mice compared to their wildtype littermates, $\gamma 2^{+/+}$ (Günther et al. 1995; Crestani et al., 1999). We therefore used $\gamma 2^{+/-}$ mice as an indirect method to test the hypothesis that lower $\gamma 2$ content is associated with a more severe alcohol withdrawal reaction. Acute ethanol withdrawal severity as measured by HIC was compared between $\gamma 2^{+/+}$ and $\gamma 2^{+/-}$ mutant mice. $\gamma 2^{+/-}$ mice showed increased withdrawal severity compared to their wildtype littermates as measured by AUC ($t_{[1,66]} = 2.54$, $p = 0.01$, two-tailed). Because average baseline score between the genotypes were also different in that $\gamma 2^{+/-}$ mice had higher baseline scores ($t_{[1,66]} = 2.14$, $p = 0.032$, two-tailed), withdrawal scores were corrected to remove the possible influence of baseline differences. We subtracted the product of the average baseline score multiplied by the number of

time points summed for AUC (i.e., 8 time points) for each mouse from its respective area under the curve score (see Metten and Crabbe, 1994). This corrected score between the two different genotypes (if limited to zero or positive numbers) was also statistically significant in that $\gamma2^{+/-}$ mice had more severe acute ethanol withdrawal compared to their wildtype littermates as shown in Figure 15 inset ($t_{[1,66]} = 2.52$, $p = 0.01$, two-tailed). AUC and this corrected AUC score, moreover, were highly correlated ($r = 0.98$; $p < 10^{-6}$). However, if the corrected area under the curve score was allowed to be negative for individual mice, baseline differences appeared to mask withdrawal severity in $\gamma2^{+/-}$ mice ($t_{[1,66]} = 0.45$; $p = 0.65$, two-tailed). Because of the subtlety of the withdrawal reaction, which was clearly observable in the withdrawal time course (Figure 15), we also analyzed regression residuals, which removed the influence of baseline measures from AUC and peak withdrawal severity (a measure of magnitude). Residual scores, which had a correlation of exactly zero with baseline convulsions, showed a trend for higher withdrawal for $\gamma2$ heterozygous null mutant mice ($t_{[1,66]} = 1.68$, $p = 0.1$, two-tailed; Figure 16a). For each individual mouse, we determined the highest HIC score and then averaged that time point with the one point that preceded and followed it to obtain a peak ethanol withdrawal measure. As demonstrated in Figure 16b, $\gamma2^{+/-}$ mice had a trend toward greater peak withdrawal score compared to wildtype littermates ($t_{[1,66]} = 2.1$, $p = 0.08$, two-tailed).

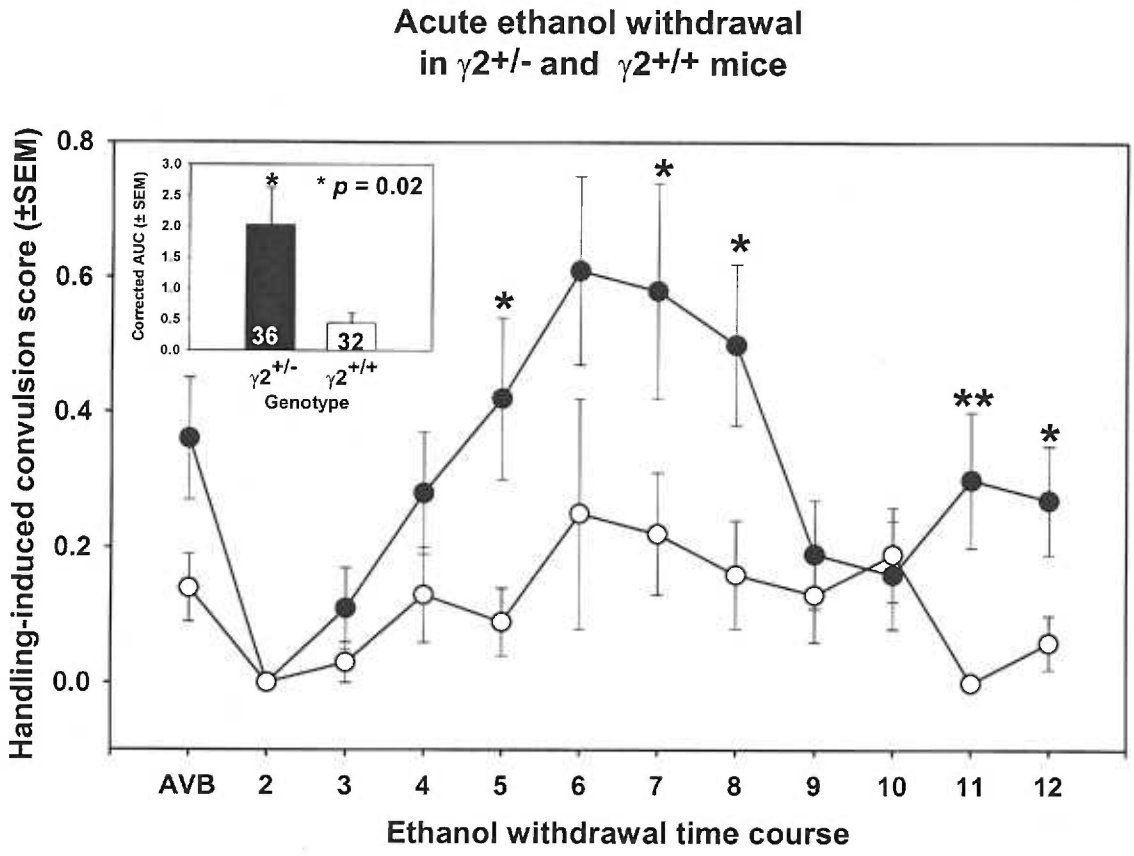

Figure 15. $\gamma 2^{+/-}$ mice have greater acute ethanol withdrawal severity compared to their wildtype littermates. Although $\gamma 2^{+/-}$ and $\gamma 2^{+/+}$ mice are on C57BL/6 genetic background, we were able to observe altered acute ethanol withdrawal severity. Mice that possess one functional γ2 allele (solid symbols) displayed more severe withdrawal reactions compared to mice that are homozygous for the wildtype allele (open symbols). As shown in the inset, $\gamma 2^{+/-}$ mice had a greater duration and magnitude of acute ethanol withdrawal as measured by corrected AUC compared to their wildtype littermates ($t_{[1,66]} = 2.52$, $p = 0.02$, two-tailed).

*** $p < 0.05$; ** $p < 0.01$**

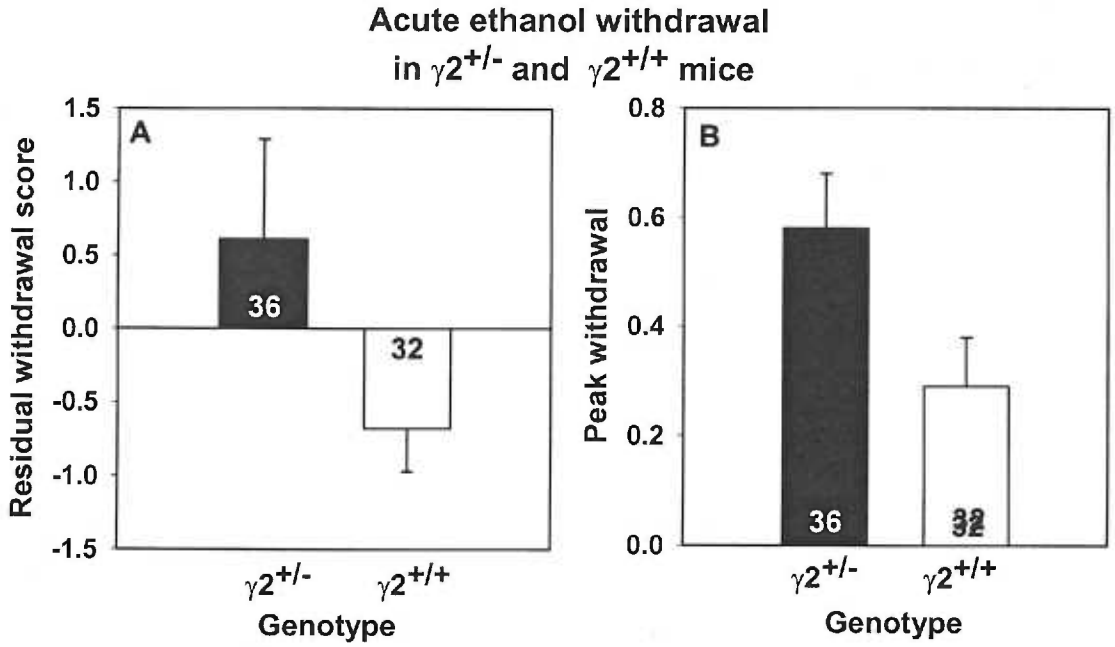

Figure 16a-b. $\gamma 2^{+/-}$ mutant mice display greater acute ethanol withdrawal severity compared to their wildtype littermates. (A) The residual withdrawal score was derived by regressing AUC onto average baseline score for each individual mouse. This measure ensured that withdrawal severity was not statistically correlated with baseline observations. $\gamma 2^{+/-}$ mice had a trend toward greater regression residual scores, which indicates higher acute ethanol withdrawal severity compared to their wildtype littermates. **(B)** Peak scores assessed the magnitude of acute ethanol withdrawal severity. In concordance with the residual scores, $\gamma 2^{+/-}$ mice had a trend toward a higher peak withdrawal than $\gamma 2^{+/+}$ mice.

Overall, with the varied metrics of assessing acute ethanol withdrawal severity, it appeared that decreased $\gamma 2$ peptide content was associated with increased withdrawal severity. An alternative explanation for increased acute ethanol withdrawal severity in $\gamma 2^{+/-}$ mice is that these mice carry strain 129 linked genes including the other $GABA_A$-R subunits. Although these mice have been backcrossed for over 10 generations to a C57BL/6 strain, it is estimated that they will still posses ~ 16 cM of linked 129 genome from the E14 ES cells (129/Ola; Günther et al., 1995) used for homologous recombination (Gerlai, 1996). Because there was not a deliberate attempt to select against these linked genes during backcrossing, we genotyped two mice that were $\gamma 2^{+/-}$ and two mice that were $\gamma 2^{+/+}$ (both sets were littermates but from different male $\gamma 2^{+/-}$ breeders; Table 7) with numerous microsatellite markers. These markers were predicted to be polymorphic between 129/Ola and B6 mice either from the CIDR database or from personal communication with Drs. Alexander Bachmanov (Monell Chemical Senses Center, Philadelphia, PA) and Margriet Snoek (The Netherlands Cancer Institute). Although we genotyped many markers, we were not able to find ones that could distinguish between wildtype and heterozygous null mutant mice (see Table 7). Thus, we could not determine the extent of 129 linkage in these mice to rule out other potentially linked genes. However, ethanol withdrawal studies using $\alpha 1$, $\alpha 6$, or $\beta 2$ null mutants showed that these null mutants do not display differential withdrawal severity compared to wildtype littermates (Blednov et al., 2003; Homanics et al., 1998), indicating that linked strain 129 genes most likely did not regulate ethanol withdrawal severity.

Table 7. Polymorphisms between $\gamma 2^{+/-}$ and $\gamma 2^{+/+}$ mice

Symbol	cM (MGI)	Mb Ensembl	Polymorphism detected?
D11Mit62	1.5	9.1	no
D11Mit226	1.55	8.9	no
D11Mit77	2	17.9	no
D11Mit78	2	18	no
D11Mit79	10.9	19.8	no
D11Mit185	12	28	no
D11Mit151	13	25	no
D11Mit82	14	26.1	no
D11Mit163	16	27.8	no
D11Mit174	20	43.1	no
D11Mit20	20	45.2	no
D11Mit21	20	44.7	no
D11Mit296	20	43.4	no
D11Mit310	24	54.7	no
D11Mit154	27.5	52.6	no
D11Mit23	28	54.8	no
D11Mit140	28	54.8	no
D11Mit349	32	54.3	no
D11Mit164	32	57.5	no
D11Mit157	34.25	62.6	no
D11Mit177	36	65.8	no
D11Mit4	37	69.2	no
D11Mit5	37	67.8	no
D11Mit90	42	71.1	no
D11Mit320	43	71.6	no
D11Mit7	44.3	76.8	no

III.I. D2.B6-*D11Mit174* Congenic Mice Capture *Alcw3*.

The conventional congenic strain, D2.B6-*D11Mit174,* was developed by Dr. John K. Belknap. Briefly, B6D2 F2 intercross mice were repeatedly backcrossed to D2 mice for 10 generations. Progeny were identified by genotyping markers on Chromosome 11. After the tenth backcross generation, individual congenic mice were bred together to create a congenic strain that is considered fully inbred (Bailey, 1981). The D2.B6-*D11Mit174* strain is B6 homozygous from ~ 2-52 cM on Chromosome 11, whereas the remaining ~98% of the genome is D2 derived (see Figure 4, page 31). Our previous studies predicted that the B6 derived genomic region on Chromosome 11 of the D2.B6-*D11Mit174* strain should be associated with a more severe withdrawal reaction compared to the D2 genetic background strain (Buck et al., 1997; Hood and Buck, 2000).

During the development of the D2.B6-*D11Mit174* strain preliminary data were gathered at backcross generations N5 and N6 to ensure that we were capturing the phenotype. Data were pooled between these backcross generations because genetic background differences would be random and therefore would not bias our statistical analysis. In Figure 17, mice that were heterozygous at *D11Mit174* showed a trend for increased acute ethanol withdrawal as measured by AUC compared to mice that were D2 homozygous ($t_{[1,47]} = -1.77$, $p = 0.08$, two-tailed). Average baseline scores between groups did not differ ($t_{[1,47]} = -0.14$, $p = 0.89$).

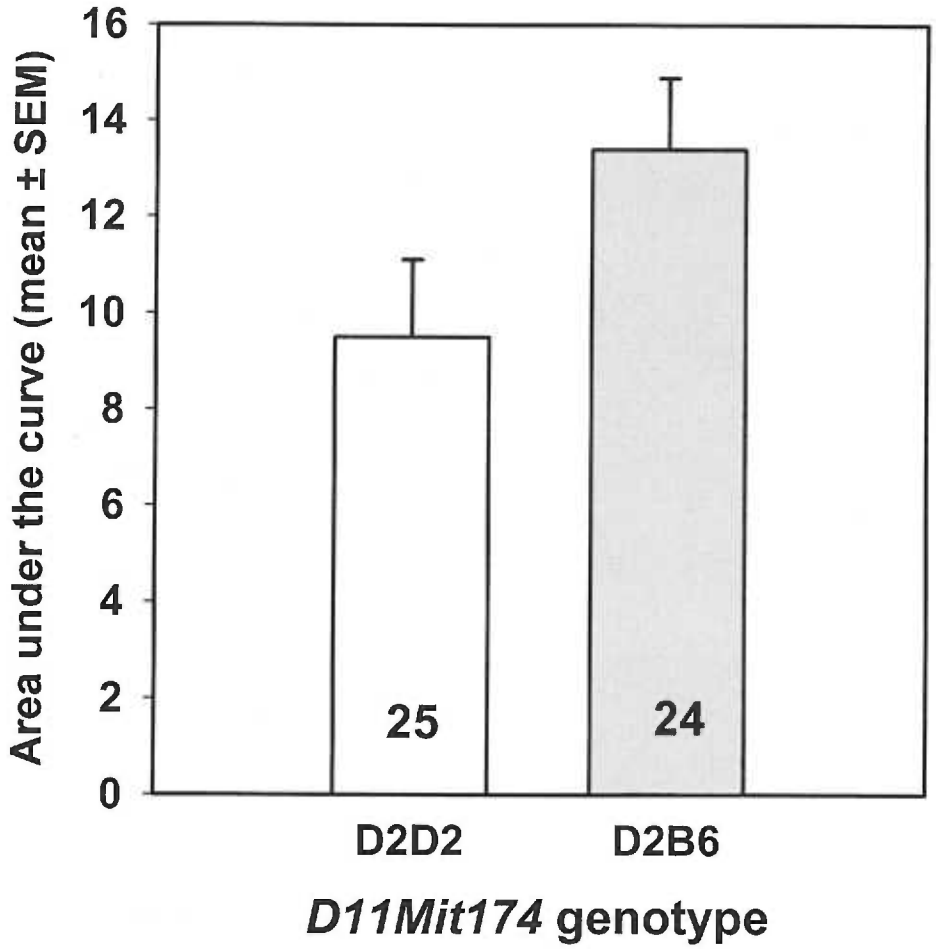

Figure 17. Test for capture of *Alcw3* in backcross generations N5 and N6. We assessed two different backcross generations in order to monitor the phenotype of the D2.B6-*D11Mit174* congenic strain while under development. Data were pooled between N5 and N6 backcross generations. As shown above, mice that are heterozygous at *D11Mit174* display a trend towards more severe acute ethanol withdrawal reactions compared to mice that are D2 homozygous.

More recently, we phenotyped the full congenic strain for confirmation. As shown in Figure 18a, D2.B6-*D11Mit174* mice showed a higher residual withdrawal score compared to the D2 background strain ($t_{[1,60]} = 2.60$, $p = 0.012$, two-tailed; Figure 18a). In addition, D2.B6-*D11Mit174* mice had significantly greater AUC scores, which measured both the magnitude and duration of acute ethanol withdrawal, compared to D2 mice ($t_{[1,60]} = 2.31$, $p = 0.02$, two- tailed). Residual and AUC scores were highly correlated ($r = 0.96$ $p < 10^{-6}$). Withdrawal time course analysis (Figure 18b) showed that these strains do not differ in average baseline measures ($t_{[1,60]} = -0.62$, $p = 0.54$, two-tailed). Additionally, repeated measures ANOVA (hours 4 through 12) detected a difference in strain ($F_{[1,60]} = 5.34$, $p = 0.02$, two-tailed) but not an hour by strain interaction ($F_{[8,480]} = 0.24$, $p = 0.98$, two-tailed).

These data show that the D2.B6-*D11Mit174* congenic strain captures *Alcw3*. These mice will be useful for higher-resolution mapping and delineation of the neural circuitry involved in acute ethanol withdrawal. Moreover, these mice may provide a less noisy genetic background than their progenitor strains to study mechanisms (e.g., gene expression) that underlie this acute ethanol withdrawal QTL.

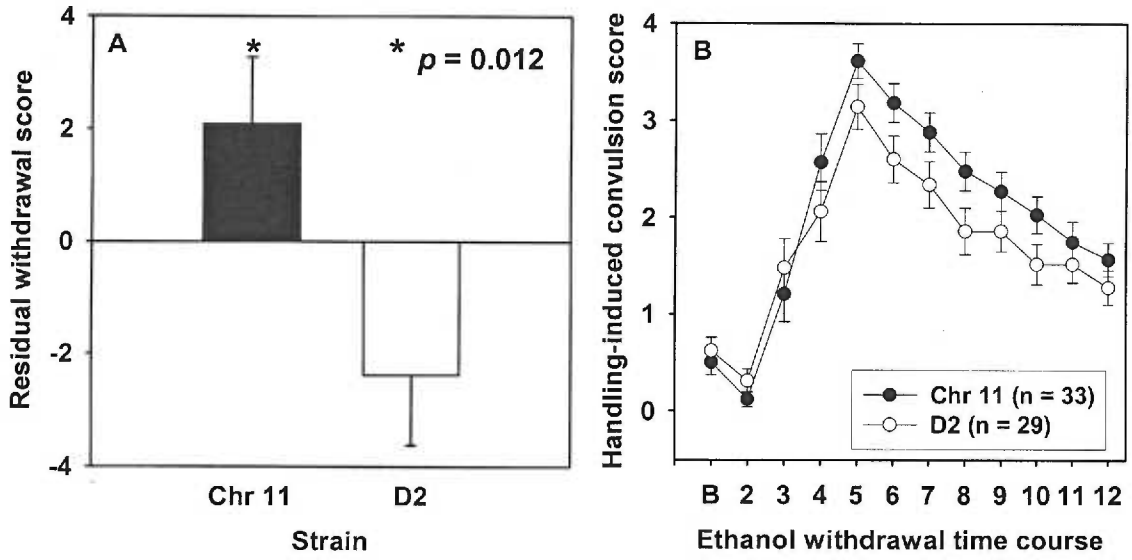

Figure 18a-b. D2.B6-*D11Mit174* congenic strain (Chr 11) mice show greater acute ethanol withdrawal severity compared to the D2 background strain mice. (A) Residual withdrawal scores are significantly lower for the D2 strain in comparison to Chromosome 11 congenic mice. This result is consistent with previous observations that Chromosome 11 B6 alleles linked to *D11Mit174* are associated with increased acute ethanol withdrawal severity (Buck et al., 1997; Hood and Buck, 2000) **(B)** Handling-induced convulsion time course also shows that congenic mice have a greater magnitude of withdrawal as measured by HIC.

III.J. $GABA_A$-R subunit Expression between D2.B6-*D11Mit174* Congenic and D2 Inbred Strains

Because D2.B6-*D11Mit174* mice captured the phenotypic effect of *Alcw3*, we compared whole brain $GABA_A$-R subunit mRNA expression between ethanol-naïve congenic and D2 inbred mice. Our results did not detect differential gene expression for *Gabrg2*, *Gabra1*, *Gabra6*, or *Gabrb2* (Figure 19a-d). This was in contrast to our D2 and B6 whole brain comparison that showed more D2 *Gabra6* expression. Moreover, BXD RI strain whole brain *Gabra6* expression was not correlated with acute ethanol withdrawal (r = -0.13, p = 0.57; expression data from WebQTL, http://www.webqtl.org/; Figure 20) or allelic status at Chromosome 11 (r = 0.20, p = 0.38). In addition, using BXD RI *Gabra6* expression as the phenotype, interval mapping as performed using WebQTL statistical options, showed that this *trans*-regulation comes from genes on Chromosome 13 and 16. These contradictory results suggest that there may be a *trans*-acting gene(s) that affects expression of the $GABA_A$-R α6 subunit. That is, another gene(s) located on a different chromosome may affect *Gabra6* expression. We did not perform interval mapping using cerebellar expression data for *Gabra6* because there is a low number of replicate studies across the BXD RI strains, which would greatly reduce the power to detect any significant associations. Together, these results suggest that *Gabra6* is not a promising candidate gene for *Alcw3*.

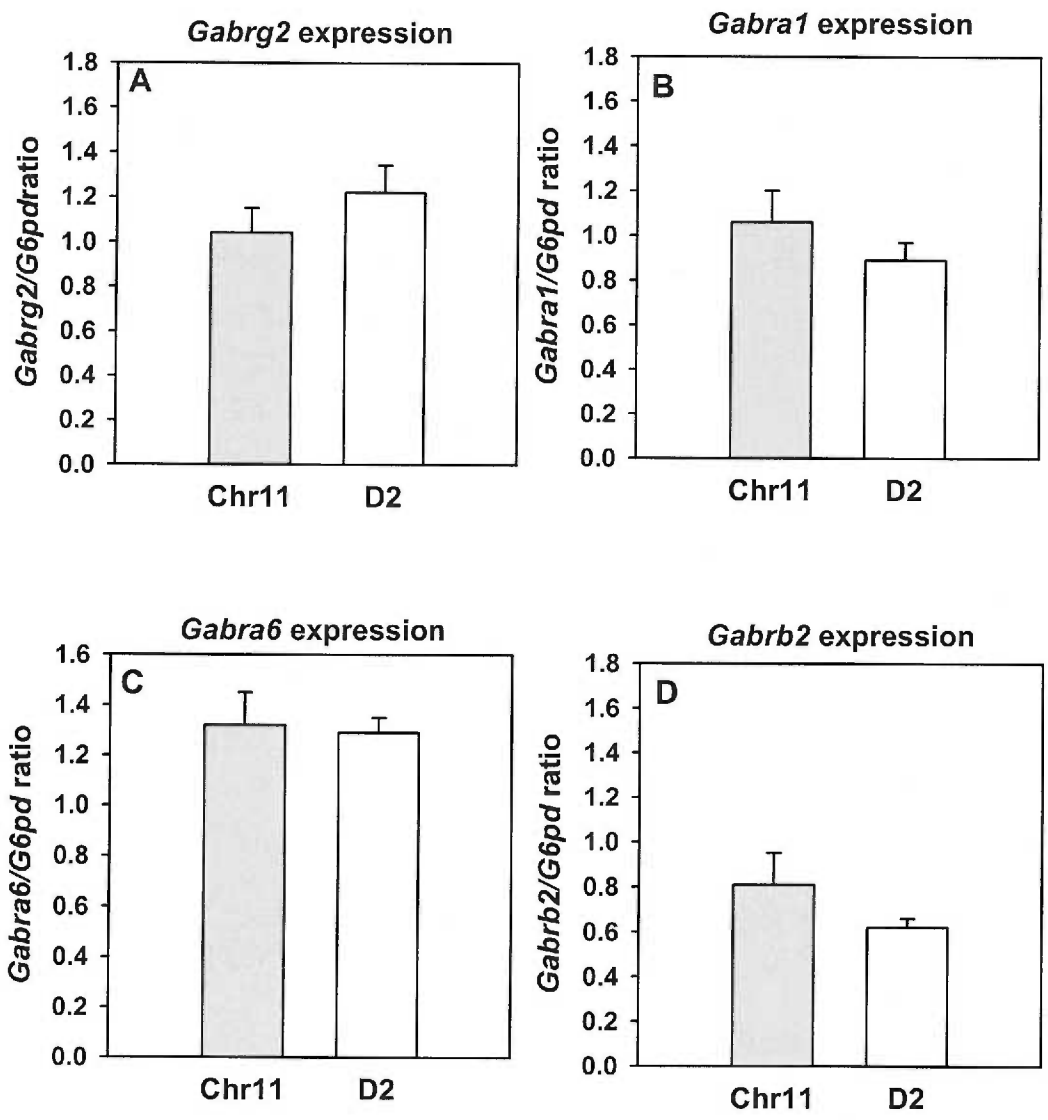

Figure 19. Whole brain GABA$_A$-R subunit gene expression in D2.B6-*D11Mit174* congenic verses DBA/2J background strain. (A) *Gabrg2* expression between Chromosome 11 congenic and D2 mice does not differ at the whole brain level ($t_{[1,4]}$ = -1.08, p = 0.34). **(B)** D2 and congenic mice have similar *Gabra1* whole brain expression ($t_{[1,4]}$ = 1.07, p = 0.35). **(C)** *Gabra6* expression does not differ between strains ($t_{[1,4]}$ = 0.19, p = 0.86). **(D)** *Gabrb2* expression is similar between congenic and their genetic background strain ($t_{[1,4]}$ = 1.26, p = 0.28).

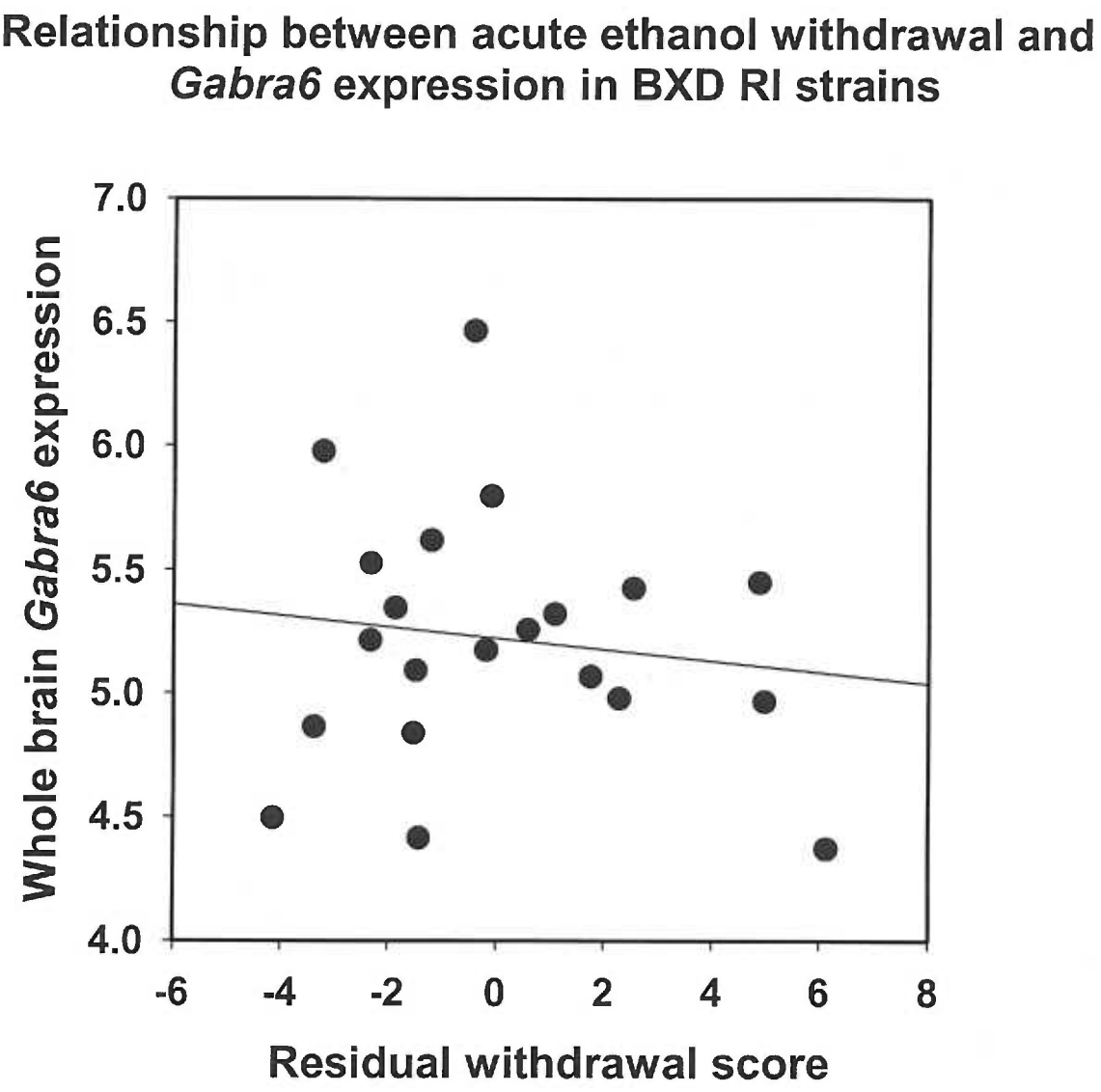

Figure 20. Lack of correlation between acute ethanol withdrawal severity and *Gabra6* whole brain expression in BXD RI mice. Acute ethanol withdrawal scores were from Buck et al., 1997 and *Gabra6* expression data were from http://www.WebQTL.org. These two measures were not significantly correlated with each other (r = -0.13, p = 0.57, two-tailed). Additionally, BXD RI whole brain *Gabrg2* expression was not correlated with residual withdrawal score (r = 0.03, p = 0.88, two-tailed; data not shown).

III.K. $GABA_A$-R Subunit Gene Expression between D1 and D2 Whole Brain.

Lastly, we measured *Gabrg2*, *Gabra1*, *Gabra6*, and *Gabrb2* whole brain expression between naïve D1 and D2 mice. Our results showed that at the whole brain level these genes did not differ between these DBA strains (Figure 21). These results were consistent with our whole brain expression data between D2 and D2.B6-*D11Mit174* strain mice. It remains to be determined if expression of *Gabrg2* or other candidate genes differs in the frontal cortex (as observed for *Gabrg2*, section IV.E).

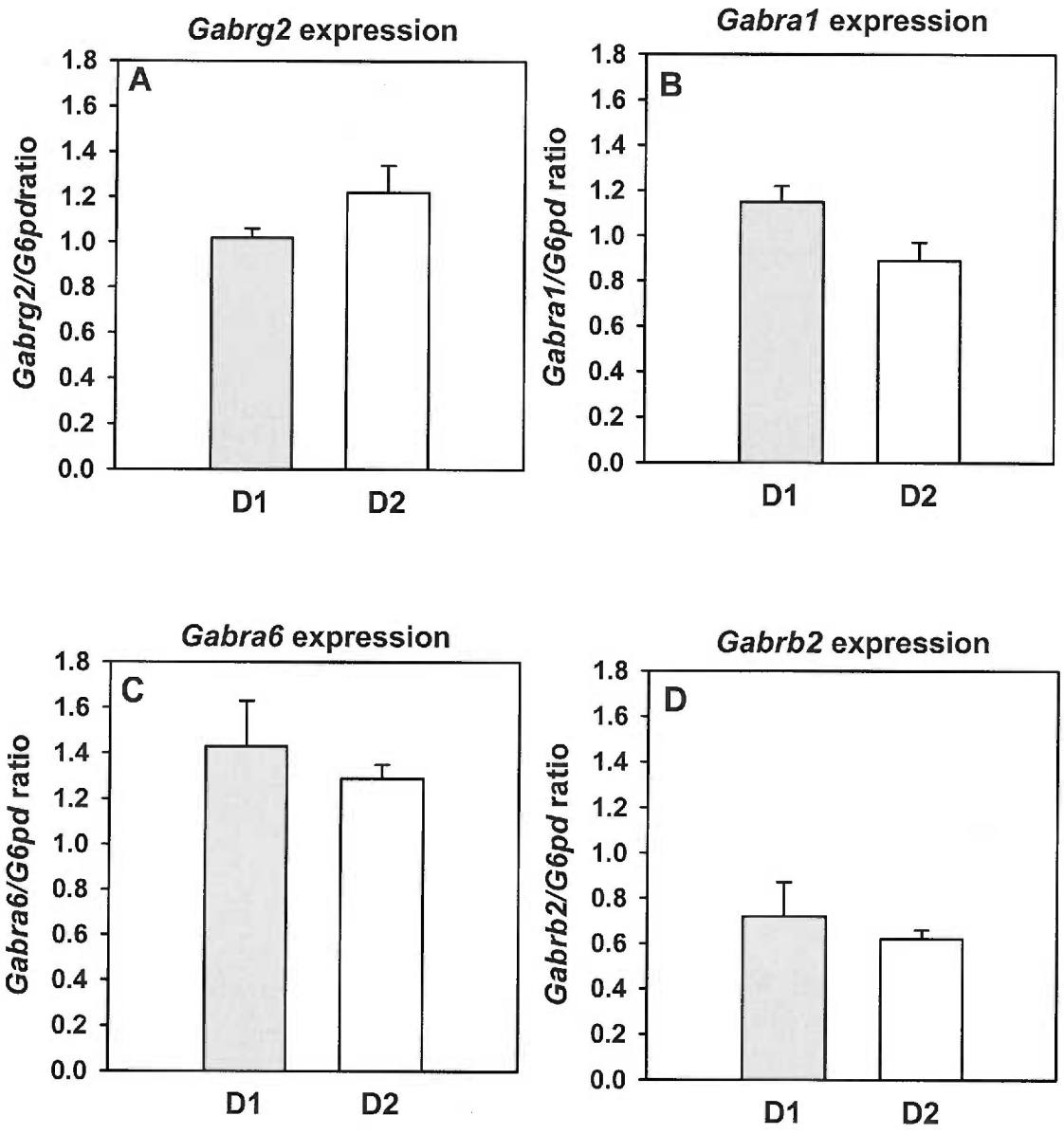

Figure 21. GABA$_A$-R subunit expression in DBA/2J and DBA/1J whole brain. (A) *Gabrg2* expression was not different between D1 and D2 mice ($t_{[1,4]}$ = -1.58; p = 0.19). **(B)** We did not observe differential *Gabra1* expression ($t_{[1,4]}$ = 1.67; p = 0.17). **(C)** D2 and D1 mice expressed similar levels of *Gabra6* ($t_{[1,4]}$ = 0.66; p = 0.54). **(D)** *Gabrb2* expression did not differ ($t_{[1,4]}$ = 0.64 p = 0.55).

IV. Discussion

IV.A. Potential effects of variation of the $GABA_A$-R γ2 polypeptide

Acute ethanol withdrawal severity is a heritable trait in mice derived from B6 and D2 mice (Belknap et al., 1993; Buck et al. 1997) and in mouse populations derived from D1 and D2 inbred strains (Hood et al., in preparation #1). Using B6D2 F2 and D1D2 F2 mouse populations, we detected a locus on mouse Chromosome 11 that maps within a cluster of $GABA_A$-R subunit genes, including *Gabrg2*, which to date is the most promising candidate gene for this QTL. Therefore, in these experiments we examined the relationship between the $GABA_A$-R γ2 subunit and acute ethanol withdrawal severity in several different populations of mice. These studies provide strong and converging evidence that *Gabrg2* is a compelling candidate gene for a QTL modifying acute ethanol withdrawal severity on mouse Chromosome 11. We first identified three single nucleotide polymorphisms (SNPs) in *Gabrg2* between D2 and B6 mice (Buck and Hood, 1998). One of these SNPs produces a structural change within the $GABA_A$-R γ2 subunit. At residue 11 of the mature polypeptide, there is a threonine in the D2 variant (T11), whereas B6 mice possess an alanine (A11). This nonconservative amino acid substitution is predicted to disrupt secondary structure in a putative helical motif in the D2 strain (Buck and Hood, 1998). This change in local peptide confirmation may therefore alter receptor function or expression, potentially via differential glycosylation. Furthermore, this amino acid exchange is correlated with acute ethanol withdrawal severity and other ethanol-related traits in BXD RI strains derived from D2 and B6 progenitors (Hood and Buck, 2000).

However, it is unclear whether amino acid substitution in the γ2 subunit between D2 and B6 mice leads to altered $GABA_A$-R function. Mihic and Buck (in press) investigated whether variation at residue 11 of the γ2 subunit modulated $GABA_A$-R responses to ethanol and multiple allosteric modulators as assessed by recombinant receptors expressed in oocytes. The sole difference observed in their study was an attenuation of the direct effect of pentobarbital on $\alpha1\beta2\gamma2_{A11}$ receptors in comparison to recombinant receptors containing γ2T11. From this study, however, we cannot conclude that amino acid exchange at residue 11 of the γ2 subunit does not affect ethanol modulation of $GABA_A$-R function for several reasons. For instance, the effect of structural differences in the γ2 subunit has not been tested with other subunit combinations (e.g., α6 and β3). Moreover, lower concentrations of ethanol (10-100 mM) failed to potentiate these recombinant receptors (Mihic and Buck, in press), whereas these intoxicating ethanol concentrations affect brain tissue (Weiner et al., 1994). This suggests that there may be other cellular components needed for ethanol potentiation of $GABA_A$-R not contained within *Xenopus* oocytes.

Variation in the $GABA_A$-R γ2 protein-coding sequence may alter cell surface receptor expression and/or ligand binding through alterations in post-translational modifications. The amino acid substitution at residue 11 of the mature γ2 polypeptide occurs near a putative N-linked glycosylation consensus site (Arg-X-Thr/Ser; x ≠ Pro; NXT/S). This sequence (NKT) is conserved across several species including mouse, human, rat, bovine, and chick (Buck and Hood, 1998; Pritchett et al., 1989; Shivers et al., 1989; Whiting et al., 1990; Glencorse et al., 1990). Moreover, this glycosylation site it located within the putative helical

region that is disrupted in the B6 strain. According to Dwek (1995), overall and local protein conformation is important for the addition of carbohydrate moieties to peptides. Therefore, if there is inference with glycosylation of the γ2 subunit, protein function or receptor expression could be compromised. To our knowledge there have not been any site-directed mutational studies to date investigating whether the γ2 subunit is truly glycosylated at this site.

For example, Ramanathan and Hall (1999) mutated three consensus glycosylation sites on the δ subunit of the muscle nicotinic acetylcholine receptor (nAChR). Mobility shifts observed on Western blots suggested that the first two putative sites were glycosylated, whereas they did not see a difference in molecular weight of the native peptide verses the third mutated site. When expressed in COS cells, the mutated δ subunit did not readily form heterodimers with the α subunit and there appeared to be less cell surface bungarotoxin binding when cells expressed the mutated form of the δ subunit compared to wildtype. Other studies of cell-surface receptors have also shown alterations in functions following mutation of putative glycosylation sites. α-bungarotoxin binding in an oocyte system was greatly reduced when glycosylation consensus sites were mutated in the α7 nAChR, although cell surface expression was observed (Chen et al, 1998). Connolly and coworkers (1996) examined assembly and cell-surface expression of $GABA_A$-R and demonstrated that the $GABA_A$-R is assembled in the endoplasmic reticulum (ER) and that assembly was not affected by lack of glycosylation following treatment with tunicamycin (prevents glycosylation) in A293 cells. However, these authors did not assess whether there were functional alterations such as changes in ligand binding.

However, tunicamycin treatment may not be the optimal method for assessing the role of glycosylation in receptor function because it appears to change the morphology of the cell (Connolly et al., 1996). Buller and colleagues (1994) mutated the rat $GABA_A$-R α1 subunit to eliminate two putative glycosylation sites. Western blot analysis indicated that both sites on the α1 subunit are most likely glycosylated due to shifts in mobility. However, this study also demonstrated the importance of expression systems and that they are sensitive to experimental conditions. That is, when the mutated α1 subunit was expressed with β2 and γ2 subunit in an oocyte system, there were no differences in [^{3}H]Ro15-1788 binding (a benzodiazepine antagonist). However, when a mammalian cell system was used (HEK cells), binding was dramatically reduced in the α1 mutant containing cells. This reduction could also be reversed when the HEK cells were maintained at a different (lower) temperature. Thus, it is unknown whether glycosylation of the α1 subunit affects $GABA_A$-R function due to complications that exist for heterologous cell expression systems.

One potential study that could be performed to determine whether cell surface expression of the $GABA_A$-R is different between D2 and B6 (or D1 mice) would be to test brain slices (or membrane preparations) for differences in benzodiazepine binding. Additionally, we could look at the expression of the γ2 subunit using cortical primary cultures to test whether altered secondary structure influences cell surface expression of $GABA_A$-R. Although these experiments indirectly test whether γ2 secondary structure affects receptor expression and/or assembly, I would predict that decreased receptor expression is associated with increased acute ethanol withdrawal severity.

IV.B. Possible ramifications of decreased *Gabrg2* expression

Although, amino acid exchange in the $GABA_A$-R $\gamma 2$ subunit is correlated with acute ethanol withdrawal severity, this particular polymorphism may be a marker for *cis*-acting regulatory elements within or nearby *Gabrg2* or a linked gene. Many investigators have suspected that the mechanism underlying most QTL is at the non-coding regulatory level and not at the structural (i.e., amino acid) level (Glazier et al., 2002; Korstanje and Paigen, 2002; Mackay, 2001). Indeed, it is predicted that only 10-20% of genes within a QTL interval will contain a non-conservative amino acid exchange and only a handful of these will result in functional alterations (Belknap et al., 2001). Thus, we hypothesized that decreased *Gabrg2* expression is associated with acute ethanol withdrawal severity. Whole brain *Gabrg2*, *Gabra1*, *Gabra6*, and *Gabrb2* expression was measured between ethanol-naïve B6 and D2 mice. *Gabra6* was differentially expressed between B6 and D2 inbred strains. However, no differences were detected for *Gabrg2*, *Gabra1*, and *Gabrb2*. Yet, when we assessed *Gabrg2*, *Gabra1*, and *Gabrb2* expression in ethanol-naïve and -withdrawn D2 and B6 mice in frontal cortex, a brain region implicated in ethanol withdrawal by FOS mapping (Buck et al., in preparation), we detected differential *Gabrg2* expression. D2 mice have greater baseline expression of *Gabg2* in frontal cortex compared to B6 mice as measured by quantitative RT-PCR. Following acute ethanol withdrawal, D2 *Gabrg2* expression is reduced in frontal cortex. We did not observe any differences between D2 and B6 mice for cortical expression of $\alpha 1$ or $\beta 2$.

Synaptic localization of $GABA_A$-R is important for overall inhibitory tone within the CNS. As well, $GABA_A$-Rs are clustered across from presynaptic terminals

(although they are also located on the soma away from the synapse; for review see Fritschy and Brunig, 2003). Recent studies have illustrated the importance of protein-protein interactions that are at least partially responsible for the clustering of the $GABA_A$-R. Essrich and colleagues (1998) demonstrated that both gephyrin and the $\gamma 2$ subunit are required for receptor clustering at the synapse. As well, $\gamma 2^{+/-}$ mice not only show decreased $\gamma 2$ content but less synaptic clustering of the $GABA_A$-R in cortex and hippocampus (Crestani et al., 1999). Thus, the decrease in *Gabrg2* expression that we observed in the frontal cortex of B6 mice compared to D2 mice may result in decreased $GABA_A$-R synaptic clustering that could lead to increased withdrawal severity. In support of this theory, $\gamma 2^{+/-}$ mice on C57BL/6 genetic background show increased acute ethanol withdrawal severity compared to $\gamma 2^{+/+}$ littermates. Confocal microscopic studies assessing the cellular distribution of $GABA_A$-R between D2 and B6 could directly test whether decreased *Gabrg2* expression leads to decreased receptor clustering. Lastly, there is another animal model that could indirectly test whether reduced $GABA_A$-R clustering results in increased acute ethanol withdrawal severity. $GABA_A$-R clusters that contain either the $\gamma 2$ or $\alpha 2$ subunits are undetectable in both brain slices and cultured hippocampal cells from gephyrin null mutant mice, although, the content of these subunits is not decreased in Western blot assays (Kneussel et al., 1999). Thus, if $GABA_A$-R clustering affects acute ethanol withdrawal severity, I would predict that gephyrin null mutant mice should have more intense withdrawal severity compared to wildtype littermates.

IV.C. Complications with null mutation experiments

Our results indicate that γ2 peptide content may influence acute ethanol withdrawal severity as measured in $\gamma2^{+/+}$ wildtype verses $\gamma2^{+/-}$ null mutant mice. Here, we report that mice heterozygous for the null mutation ($\gamma2^{+/-}$) show increased withdrawal severity (and for average baseline) compared to their wildtype counterparts. Although these mice were backcrossed for over ten generations to the C57BL/6 mice strain, $\gamma2^{+/-}$ mice are predicted to contain between 0.5 and 1% of 129/Ola genome (from the E14 ES cells; Hooper et al., 1987).

The differences observed for both baseline and withdrawal severity may be due to several different reasons. (1) 129/Ola genes linked to the null mutation increases withdrawal severity. (2) Hemizygous deletion of γ2 results in greater overall neural excitability. (3) There is a compensatory response of other genes within the GABAergic system that influences withdrawal severity. (4) There is regulatory interference of nearby genes. Lastly, (5) γ2 does contribute to acute ethanol withdrawal severity.

Scenario one above suggests that increased withdrawal severity could be related to 129/Ola genes linked to the targeted deletion of γ2. Other studies have shown that linked 129 strain genes can affect phenotypic expression (e.g., mask) For example, Kanagawa and coworkers (2000) showed that 129/Sv genes linked to null mutation of the IFN-γR, and not the mutation itself, lead to diabetes resistance in the nonobese diabetic mouse. Another dramatic example of phenotypic differences most likely due to 129 linked genes are the three different null mutant mouse lines for the corticotropin-releasing hormone receptor-2 (CRH-

excitability. Seizure phenotypes are a characteristic (unexpectedly at times) of many null mutant mice. To date, however, it has not been reported that $\gamma2^{+/-}$ mice display overt signs of seizures/convulsions. These mice are described as grossly indistinguishable from their wildtype counterparts (Günther et al., 1995; Crestani et al., 1999). In addition, there was no mention of seizures in $\gamma2^{-/-}$ mice that survived to postnatal day 18 (Günther et al., 1995), and the cause of death is still unknown. When the $\gamma2^{+/-}$ and $\gamma2^{+/+}$ mice were assessed for acute ethanol withdrawal severity, there were no overt signs of neuronal excitability such as tremor. However, this does not preclude the possibility that there are differences in neuronal discharge only measurable by EEG. Although heterozygous deletion may not lead to a noticeable seizure phenotype, conditional $GABA_A$-R $\gamma2$ subunit null mutants do display severe convulsions. The $\gamma2$ subunit is "turned-off" around the third post-natal week. Confocal microscopy shows a large decrease in post-synaptic $GABA_A$-R clustering. Preceding death at four weeks of age, these mice develop a severe seizure phenotype that includes running fits and violent convulsions (Schweizer et al., 2003). These data suggest that an extreme reduction in $\gamma2$ peptide leads to a seizure/epilepsy-like behavior, whereas deletion of $\alpha1$, $\alpha6$, or $\beta2$ does not appear to cause a severe convulsive phenotype (Homanics et al. 1997; Kralic et al., 2002; Sur et al., 2001).

One of the greatest pitfalls of null mutant studies is the possibility of compensation. That is, do other related genes change their normal expression patterns/content to pick up the role of the missing protein? There are examples of compensation in the literature concerning the ablation of other $GABA_A$-R subunit genes. For example, in $GABA_A$-R δ null mutant mice, there is an

increase in γ2 protein in cerebellar granular cells (Tretter et al., 2001) and in the forebrain (Korpi et al., 2002; Peng et al., 2002). It appears that the δ null mutation also leads to decreased $GABA_A$-R α4 content in the forebrain of these mice, which may alter the function of $GABA_A$-R in this brain region due to differential subunit composition (Peng et al., 2002). Deletion of the $GABA_A$-R β3 subunit leads to lower α2 and α3 polypeptide content affecting miniature inhibitory postsynaptic currents (Ramadan et al., 2003). $GABA_A$-R α1 subunit null mutant mice have decreased expression of β2/3 and γ2 subunit and increased α2 and α3 peptide content in the cerebral cortex compared to wildtype mice. There were no observed differences for α4, α5, or δ expression (Kralic et al., 2002). Immunoprecipipation studies using $\beta2^{-/-}$ mice, showed a decrease in all α subunits (α1-6; Sur et al., 2001). Compensation by other $GABA_A$-R subunits therefore could underlie increased acute ethanol withdrawal severity. However, Günther and colleagues (1995) did not detect alterations in whole brain protein content of the α1, α2, β1, β2/3, γ1, or γ3 subunits in $\gamma2^{-/-}$ or $\gamma2^{+/-}$ mice. This result was also reported by Crestani and coworkers (1999) for $\gamma2^{+/-}$ mice, however the data were not shown. These results suggest that there may not be compensation by other $GABA_A$-R subunits in the $\gamma2^{+/-}$ mice that could account for the observed increase in acute ethanol withdrawal severity.

The role of regulatory interference may also contribute to acute ethanol withdrawal severity in $\gamma2^{+/-}$ mice. This phenomenon is rarely discussed as a caveat of gene deletion studies, but it may play a critical role by disrupting the regulation of other genes located very close to the null mutation. One dramatic example of possible regulatory interference involves the $GABA_A$-R subunit cluster

on mouse Chromosome 11. Two research groups independently deleted the $GABA_A$-R α6 subunit gene (Homanics et al., 1997; Jones et al., 1997); within each null mutation, the neomycin (neo) resistance gene was inserted into exon eight of *Gabra6*. Both lines of α6 null mutant mice had decreased forebrain expression of the α1 and β2 subunits (Uusi-Oukari et al., 2000). The catch is – α6 is only expressed in cerebellar and cochlear nucleus granule cells (Luddens et al., 1990; Wisden et al., 1992). Uusi-Oukari and coworkers (2000) suggest that the neo gene may be disrupting common regulatory elements crucial for the concerted expression of the α1 and β2 subunits. They contend that this is plausible due to the extensive overlapping expression of the α1 and β2 subunits. However, altered γ2 expression is not observed in the α6 null mutant mice, suggesting that this putative common regulatory element is not completely shared by the $GABA_A$-R subunit gene cluster on mouse Chromosome 11. γ2 distribution, as well, does not completely overlap that of α1 and β2, suggesting independent regulation. Therefore, disruption of coordinated gene expression may underlie the increase in acute ethanol withdrawal severity observed for $\gamma2^{+/-}$ mice.

Lastly, $\gamma2^{+/-}$ mice may display increased withdrawal severity due to decreased γ2 peptide content. This is the first report where deletion of a $GABA_A$-R subunit located on mouse Chromosome 11 leads to a change in ethanol withdrawal severity following either acute or chronic ethanol exposure. α6 null mutant mice were tested for ethanol withdrawal severity using the handling-induced convulsion following chronic intermittent exposure to ethanol vapor. These null mutant mice were developed from R1 ES cells which were derived from 129/Sv

and 129cX/Sv inbred strains. These mice were a mix of 129/B6 genomes, however, it is unclear as to which 129 strain was used. Knockout mice did not display an altered withdrawal reaction compared to their wildtype counterparts (Homanics et al., 1998). This result also suggests that decreased forebrain expression of $\alpha 1$ or $\beta 2$ subunits do not contribute to ethanol withdrawal severity because the content of these subunits is reduced in this line of mice (see above; Uusi-Oukari et al., 2000). Recently, $\alpha 1$ and $\beta 2$ null mutant mice were tested for ethanol withdrawal severity following 5 days of 6% ethanol liquid diet consumption. $\alpha 1$ null mutant mice do not show altered withdrawal severity compared to wildtype mice, whereas $\beta 2$ knock out mice show elevated withdrawal reaction that is mostly due to high baseline measurement (Blednov et al., 2003). These mice were developed using AB2.2 ES cells derived from the 129/SvEv strain. Therefore, it is possible that heterozygous deletion of the $\gamma 2$ subunit, which decreases $\gamma 2$ expression, is associated with increased acute ethanol withdrawal severity.

Because there are limitations to gene deletion studies as outlined above, one potential experiment that could be used to test the hypothesis that decreased $\gamma 2$ expression is associated with increased acute ethanol withdrawal severity is to temporarily decrease expression with antisense oligodeoxynucleotides (ODNs). In this technique, ODNs designed to complement the $\gamma 2$ gene are injected into the brain. They bind to the $\gamma 2$ mRNA preventing its translation into protein. This approach has been called "knock down" verses knock out. Therefore, acute ethanol withdrawal severity can be tested without developmental compensation and genetic background confounds. This type of functional test has been

suggested as part of the burden of proof for going from QTL to QTG (Belknap et al., 2001). Based on literature relating to animal models of epilepsy, infusion of γ2 antisense ODNs into the hippocampus leads to increased electrographic limbic status (Karle et al., 1997; 1998). Therefore, it is plausible that decreased γ2 peptide content can lead to increases in withdrawal severity.

IV.D. D1D2 haplotype mapping narrows the genomic interval on mouse Chromosome 11

Acute ethanol withdrawal severity is regulated in a D1D2 F2 population by a gene(s) on mouse Chromosome 11 linked to the genetic marker *D11Mit174*. In order to obtain higher-resolution of this genomic region, we performed haplotype mapping across Chromosome 11. We assessed the entire chromosome for genomic segments that are common between D1 and D2 inbred mice and for regions that are polymorphic. Our data suggest that the majority of Chromosome 11 is similar between these two DBA strains except for ~ 8.8 Mb region (35.9-44.7 Mb) linked to *D11Mit174* (see Table 6). According to the public mouse genome database (Ensembl), there are 35 known genes (including RIKEN clones) and 11 predicted (novel) genes contained within this genomic interval on mouse Chromosome 11 that influences acute ethanol withdrawal severity. However, based on literature searches and expression information, some of these genes are highly improbable candidate genes (see below). For example some of these genes either have low levels of expression or no expression within the murine CNS. Below, is a description of the known genes (minus RIKEN clones) that are contained between 34.7 and 44.7 Mb on Chromosome 11.

IV.E Genes within the D1D2 interval

There are several other known genes contained within this QTL interval, however, most of them do not appear to be as compelling of candidate genes as the $GABA_A$-R subunit genes (although we cannot completely rule them out). For example, the gene that encodes the β1 subunit of maxiK channels, which are high conductance calcium activated potassium channels involved in vascular tone and neuronal excitability, is located within this interval (for review see Calderone, 2002). Jiang and colleagues (1999) investigated *KCNMB1* (the human ortholog) expression across multiple organ systems and show that it is highly expressed in human aorta, bladder, and uterus. Low levels of expression were detected in cortex, hippocampus and cerebellum. Also contained in this interval are genes that code for a hematopoietic cell-specific protein adaptor (*Lcp2*; for review see Myung et al., 2000), a member of the forkhead family of adaptor proteins involved mainly in lymphocyte migration (*Dock2*; reviewed by Reif and Cyster, 2002), and a gene codes a protein involved in the synthesis of coenzyme A from pantothenate (vitamin B5; Abiko, 1967). Within this interval there are also genes involved in development including a member of a family of winged helix transcription factors, which are involved with embryonic development of the nervous system (*Foxi1*; Hulander et al., 1998) and *Odz1* which is part of a family that is homologous to pair-rule genes involved in drosophila development. Most pair-rule genes code transcription factors except for *Odz* genes, which are predicted to be transmembrane molecules (Oohashi et al., 1999; Dgany and Wides, 2002). The mammalian homologs (*Odz1-4*) are

expressed in embryos and adult brain (Ben-Zur et al., 2000). To date, the function of mammalian *Odz* genes has yet to be elucidated.

Also contained within this interval are genes that have been implicated in different types of cancer such as *Hmmr* and *Pttg*. *Hmmr* encodes a cell adhesion molecule within the extracellular matrix and its protein product has been implicated in numerous forms of cancer (e.g. breast carcinoma, astrocytomas, colon cancer, and stomach cancer, for review see Entwistle et al., 1996; Toole, 1997). Pituitary tumor transforming 1 (*Pttg1*) regulates mitosis (see Zou et al., 1999) and is a transactivator that induces the expression of other growth factors (e.g. basic fibroblast growth factor; Zou et al., 1999). *PTTG* may also regulate apoptosis in the presence of p53 (Yu et al., 2000) and is considered an oncogene because it is expressed in many types of cancer (see McCabe, 2001 for review). Another gene that may be involved in the p53 cascade, *Ccng1,* is also located in this interval. This gene is part of the p53-Mdm2 stress pathway. *Ccng1* is expressed in the mammalian CNS and may be involved in early cellular differentiation during development (van Lookeren Campagne and Gill, 1998). *In situ* hybridization studies in adult rats show that *Ccng1* is abundant in the cortex, hippocampus, olfactory bulb, and thalamus (van Lookeren Campagne et al., 1999). Second step splicing factor (*Slu7*) protein product is involved in the second catalytic step of splicing pre-mRNA (Chua and Reed, 1999). Gastrotropin (fatty acid binding protein 6; (*Fabp6*) is involved in the binding of bile salts (Vodenlich et al., 1991).

Lastly, the gene which codes α1B adrenergic receptor (*Adra1b;*) is located in this interval. Norepinephrine and epinephrine are this receptor's endogenous

ligands. Norepinephrine transmission in the CNS can be anticonvulsant. Transgenic overexpression of *Adra1b* in mouse brain leads to large-scale neurodegeneration and severe convulsions in older mice (12 months). These convulsions are partially reduced following antagonism of the α1B adrenoreceptor (Zuscik et al. 2000; Kunieda et al., 2002). However, it is not clear whether this seizure phenotype is completely due to overexpression of *Adra1b* or the multiple system atrophy in these mice.

IV. F. An alternative explanation for haplotype results

We found three SNPs within the coding region of *Gabrg2* between D2 and B6 including one that changes amino acid sequence mice (Buck and Hood, 1998). This led to the hypothesis that *Gabrg2* and/or other $GABA_A$-R subunit genes underlie *Alcw3*. However, D2 is the only inbred strain that contains a threonine at residue 11 of the mature $GABA_A$-R γ2 peptide compared to 14 standard inbred mouse strains sequenced to date, including the genetically related D1 strain (Atchley and Fitch, 1993; Festing, 1994). Thus, we can speculate that the D2 allele may be a new mutation that occurred sometime between 1930 (when the DBA line was divided into D2 and D1; Russell, 1978) and the 1970's (when the BXD RI strains were being developed; Taylor, 1978). Our results could also suggest that there was some residual heterozygosity in the original DBA strain. Another possibility is that the 4 cM D2 block on mouse Chromosome 11 is itself a molecular marker linked to the true gene for *Alcw3*. Although we did not rigorously assess D1 and D2 polymorphism across Chromosome 11 (*i.e.*, SNPs), it does not appear plausible that this is *just* a marker for another gene. Chromosome 11 at the microsatellite marker level is not polymorphic between D2

and D1 mice except for an interval mapped to 17-20 cM. This suggests that there are no alternative regions in this Chromosome that could even be associated with acute ethanol withdrawal severity. However, a more rigorous aasessment of the haplotype structure between D1 and D2 strains using SNPs is warranted to conclusively eliminate other regions of Chromosome 11.

IV.G. Future Directions

The studies presented here represent only a beginning for determining whether *Gabrg2* is the gene underlying *Alcw3*. There are several other directions that this project could take to further evaluate this candidate. The studies assessing the effect of $\gamma 2$ variation on $GABA_A$-R function in *Xenopus* oocytes were inconclusive (Mihic and Buck, in press). A mammalian expression system, which might contain the necessary cellular components for proper receptor function (e.g., HEK cells), may be more informative for determining whether $\gamma 2$ variation alters ethanol potentiation of $GABA_A$-R. Moreover, receptor function should be tested between progenitor strains (or congenic verses genetic background) in neural preparations (e.g., brain slices) to determine whether $\gamma 2$ variation alters ethanol potentiation. These two systems have been used with great success for assessing the effects of relevant concentrations of ethanol (10-50 mM) on $GABA_A$-R (Weiner et al., 1994) or overall $GABA_A$ receptor function (Hamilton et al., 1993) and therefore should be useful in detecting possible alterations in receptor function produced by $\gamma 2$ variation.

Data from our lab indicates that a fronto-striatal circuit modulates acute ethanol withdrawal severity (Buck et al., in preparation). Presented here are data indicating that *Gabrg2* expression is regulated between D2 and B6 mice in the

frontal cortex. A more global gene expression approach such as *in situ* hybridization may prove fruitful in determining whether there are genotype-dependent differences in *Gabrg2* expression in other brain regions that may modulate acute ethanol withdrawal. Moreover, we should determine whether synaptic clustering of $GABA_A$-R is altered in brain regions that show differential *Gabrg2* expression. Clustering data in combination with $GABA_A$-R electrophysiological data may provide insight into the functional consequences of variation in the $\gamma 2$ subunit. Also important to determine is the nature of *Gabrg2* expression difference between D2 and B6 inbred strains. That is, does polymorphism in a regulatory region (e.g., promotor) underlie differential expression? Thus, sequencing the entire gene between D2 and B6 mice may detect the nucleotide(s) that regulate genotype-dependent differences in acute ethanol withdrawal severity.

Once the Chromosome 11 QTL for acute ethanol withdrawal severity was finer mapped, we focused on the cluster of $GABA_A$-R subunit genes and in particular the $\gamma 2$ subunit as strong candidates. However, there are other genes within this interval that are expressed in brain (e.g., *Adra1b* and *Kcnmb1*) that should be tested for sequence and/or expression differences between the D2 and B6 progenitor strains. These studies will help narrow the list of candidate genes for this QTL. Even finer mapping will also be of value to reduce the number of false candidate genes within this interval. Because D2 mice possess a unique *Gabrg2* allele, intercrosses with other inbred strains that differ in withdrawal severity may further narrow this region. For example, D2 and C3H/HeJ inbred strains possess the same alleles for *D11Mit231* (17 cM) and

D11Mit108 (18 cM), whereas D1 and D2 mice have different alleles. Thus, if a F2 intercross between the C3H/HeJ D2 strains detects a Chromosome 11 QTL as modulating acute ethanol withdrawal then the QTL interval would reduced to ~ 3 cM (6.8 Mb verses 8.8 Mb) and it would eliminate 10 known genes including ones expressed in brain (*Kcnmb1*, *Dock3*, *Foxi1*, *Pank3*, and *Odz2*) and 3 predicted (novel) genes.

Lastly, is it highly suggested that functional tests at the whole animal level should be undertaken to definitively prove that the gene is *the* gene (see review by Complex Trait Consortium, 2003). For example, one could perform a gene "knock-in" study to determine whether a particular allele (or nucleotide) may alter protein product function (see Findlay et al., 2003). A more recent technological advance that uses viral-mediated regulation of expression may prove useful for determining whether a candidate gene affects the trait in question and in what particular brain regions are important. For example Hommel and colleagues (2003) were able to produce long-lasting down-regulation (50 days) of tyrosine hydroxylase in the substantia nigra compacta of C57BL/6J mice by injecting an adeno-associated virus vector containing a short hairpin RNA that targeted the tyrosine hydroxylase gene. This reduction was also behaviorally relevant in that mice that had a less tyrosine hydroxylase compared to control mice (scrambled short hairpin RNA) showed an attenuated locomotor response to d-amphetamine (4 mg/kg, ip). Therefore, there are multiple avenues that can be used to confirm that a particular candidate gene is *the* gene.

IV.H. Summary and Conclusions

An ultimate goal for quantitative trait locus studies in mice is to identify genes and potential system networks that are relevant to the human condition. Here we used a multidisciplinary approach to identify and test candidate genes for a QTL on Chromosome 11 that modulates acute ethanol withdrawal severity in mice. First, we reduced this QTL interval using haplotype mapping to rule out plausible but false candidate genes. Next we focused on a cluster of $GABA_A$-R subunit genes that were retained after fine mapping. We determined that the $GABA_A$-R γ2 subunit gene (*Gabrg2*) is a strong candidate for the Chromosome 11 QTL modulating acute ethanol withdrawal severity in mice. The γ2 subunit is structurally different between D2 and B6 mice. Moreover *Gabrg2* expression is differentially regulated in the frontal cortex of these strains. Heterozygous γ2 null mutant mice display a more severe acute ethanol withdrawal reaction indirectly suggesting that decreased γ2 protein content is associated with increased withdrawal liability. Therefore, our fine mapping, sequencing, expression, and behavioral data indicate that *Gabrg2* is the most compelling candidate gene for an acute ethanol withdrawal QTL on mouse Chromosome 11. More work it needed (e.g., detecting the quantitative trait nucleotide and its function), to definitively say that *Gabrg* is *the* gene that underlies *Alcw3* .

V. References

Abiko Y (1967) Investigations on pantothenic acid and its related compounds. IX. Biochemical studies.4. Separation and substrate specificity of pantothenate kinase and phosphopantothenoylcysteine synthetase. J Biochem (Tokyo) 61: 290-299.

Allan AM, Harris RA (1987) Acute and chronic ethanol treatments alter GABA receptor-operated chloride channels. Pharmacol Biochem Behav 27: 665-670.

Atchley WR, Fitch W (1993) Genetic affinities of inbred mouse strains of uncertain origin. Mol Biol Evol 10: 1150-1169.

Baer K, Essrich C, Benson JA, Benke D, Bluethmann H, Fritschy JM, Lüscher B (1999) Postsynaptic clustering of γ-aminobutyric acid type A receptors by the γ3 subunit in vivo. Proc Natl Acad Sci U S A 96: 12860-12865.

Baer K, Essrich C, Balsiger S, Wick MJ, Harris RA, Fritschy JM, Lüscher B (2000) Rescue of γ2 subunit-deficient mice by transgenic overexpression of the $GABA_A$ receptor γ2S or γ2L subunit isoforms. Eur J Neurosci 12: 2639-2643.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF (2000) Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nat Genet 24: 410-414.

Bailey DW (1981) Recombinant inbred strains and bilineal congenic strains. In: The mouse in biomedical research (Foster HL, Small JD, Fox JG, eds), pp 223–239. New York: Academic.

Baulac S, Huberfeld G, Gourfinkel-An I, Mitropoulou G, Beranger A, Prud'homme JF, Baulac M, Brice A, Bruzzone R, LeGuern E (2001) First genetic evidence of $GABA_A$ receptor dysfunction in epilepsy: a mutation in the γ2 subunit gene. Nat Genet 28: 46-48

Belknap JK, Metten P, Helms ML, O'Toole LA, Angeli-Gade S, Crabbe JC, Phillips TJ (1993) Quantitative trait loci (QTL) applications to substances of abuse: physical dependence studies with nitrous oxide and ethanol in BXD mice. Behav Genet 23: 213-222.

Belknap JK, Mitchell SR, O'Toole LA, Helms ML, Crabbe JC (1996) Type I and type II error rates for quantitative trait loci (QTL) mapping studies using recombinant inbred mouse strains. Behav Genet 26: 149-160.

Belknap JK, Richards SP, O'Toole LA, Helms ML, Phillips TJ (1997) Short-term selective breeding as a tool for QTL mapping: ethanol preference drinking in mice. Behav Genet 27: 55-66.

Belknap JK, Atkins AL (2001) The replicability of QTLs for murine alcohol preference drinking behavior across eight independent studies. Mamm Genome 12: 893-899.

Belknap JK, Hitzemann R, Crabbe JC, Phillips TJ, Buck KJ, Williams RW (2001) QTL analysis and genomewide mutagenesis in mice: complementary genetic approaches to the dissection of complex traits. Behav Genet 31: 5-15.

Benke D, Fritschy JM, Trzeciak A, Bannwarth W, Mohler H (1994) Distribution, prevalence, and drug binding Profile of γ-aminobutyric acid type A receptor subtypes differing in the β subunit variant. J Biol Chem 269: 27100-27107.

Benke D, Honer M, Michel C, Mohler H (1996) $GABA_A$ receptor subtypes differentiated by their γ subunit variants: prevalence, pharmacology and subunit architecture. Neuropharmacology 35: 1413-1423.

Ben-Zur T, Feige E, Motro B, Wides R (2000) The mammalian *Odz* gene family: homologs of a Drosophila pair-rule gene with expression implying distinct yet overlapping developmental roles. Dev Biol 217: 107-120.

Bianchi MT, Song L, Zhang H, Macdonald RL (2002) Two different mechanisms of disinhibition produced by $GABA_A$ receptor mutations linked to epilepsy in humans. J Neurosci 22: 5321-5327.

Blednov YA, Walker D, Alva H, Creech K, Findlay G, Harris RA (2003) $GABA_A$ receptor α1 and β2 subunit null mutant mice: behavioral responses to ethanol. J Pharmacol Exp Ther 305: 854-863.

Blumenfeld H (2002) The thalamus and seizures. Arch Neurol 59: 135-137.

Boileau AJ, Kucken AM, Evers AR, Czajkowski C (1998) Molecular dissection of benzodiazepine binding and allosteric coupling using chimeric γ-aminobutyric $acid_A$ receptor subunits. Mol Pharmacol 53: 295-303.

Bormann J (2000) The 'ABC' of GABA receptors. Trends Pharmacol Sci 21: 16-19.

Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ (2002) International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric $acid_B$ receptors: structure and function. Pharmacol Rev 54: 247-264.

Bowser DN, Wagner DA, Czajkowski C, Cromer BA, Parker MW, Wallace RH, Harkin LA, Mulley JC, Marini C, Berkovic SF, Williams DA, Jones MV, Petrou S (2002) Altered kinetics and benzodiazepine sensitivity of a $GABA_A$ receptor subunit mutation [$\gamma 2_{(R43Q)}$] found in human epilepsy. Proc Natl Acad Sci U S A 99: 15170-15175

Browman KE, Crabbe JC (2000) Quantitative trait loci affecting ethanol sensitivity in BXD recombinant inbred mice. Alcohol Clin Exp Res 24: 17-23.

Buck KJ, Harris RA (1990) Benzodiazepine agonist and inverse agonist actions on $GABA_A$ receptor-operated chloride channels. I. Acute effects of ethanol. J Pharmacol Exp Ther 253: 706-712.

Buck KJ (1996) Molecular genetic analysis of the role of GABAergic systems in the behavioral and cellular actions of alcohol. Behav Genet 26: 313-323.

Buck KJ, Hahner L, Sikela J, Harris RA (1991a) Chronic ethanol treatment alters brain levels of γ-aminobutyric acidA receptor subunit mRNAs: relationship to genetic differences in ethanol withdrawal seizure severity. J Neurochem 57: 1452-1455.

Buck KJ, Harris RA, Sikela JM (1991b) A general method for quantitative PCR analysis of mRNA levels for members of gene families: application to $GABA_A$ receptor subunits. Biotechniques 11: 636-641.

Buck KJ, Heim H, Harris RA (1991c) Reversal of alcohol dependence and tolerance by a single administration of flumazenil. J Pharmacol Exp Ther 257: 984-989.

Buck KJ, McQuilkin SJ, Harris RA (1991d) Modulation of gamma-aminobutyric acidA receptor-operated chloride channels by benzodiazepine inverse agonists is related to genetic differences in ethanol withdrawal seizure severity. J Neurochem 57: 2100-2105.

Buck KJ, Metten P, Belknap JK, Crabbe JC (1997) Quantitative trait loci involved in genetic predisposition to acute alcohol withdrawal in mice. J Neurosci 17: 3946-3955.

Buck KJ, Hood HM (1998) Genetic association of a $GABA_A$ receptor $\gamma 2$ subunit variant with severity of acute physiological dependence on alcohol. Mamm Genome 9: 975-978.

Buck K, Metten P, Belknap J, Crabbe J (1999) Quantitative trait loci affecting risk for pentobarbital withdrawal map near alcohol withdrawal loci on mouse chromosomes 1, 4, and 11. Mamm Genome 10: 431-437.

Buck KJ, Finn DA (2001) Genetic factors in addiction: QTL mapping and candidate gene studies implicate GABAergic genes in alcohol and barbiturate withdrawal in mice. Addiction 96: 139-149.

Buck KJ, Rademacher BS, Metten P, Crabbe JC (2002) Mapping murine loci for physical dependence on ethanol. Psychopharmacology 160: 398-407.

Buller AL, Hastings GA, Kirkness EF, Fraser CM (1994) Site-directed mutagenesis of N-linked glycosylation sites on the γ-aminobutyric acid type A receptor α1 subunit. Mol Pharmacol 46: 858-865.

Burmeister M (1999) Basic concepts in the study of diseases with complex genetics. Biol Psychiatry 45: 522-532.

Cagetti E, Liang J, Spigelman I, Olsen RW (2003) Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of GABAA receptors. Mol Pharm 63: 53-64.

Calderone V (2002) Large-conductance, Ca^{2+}-activated K^{+} channels: function, pharmacology and drugs. Curr Med Chem 9: 1385-1395.

Charlton ME, Sweetnam PM, Fitzgerald LW, Terwilliger RZ, Nestler EJ, Duman RS (1997) Chronic ethanol administration regulates the expression of $GABA_A$ receptor α 1 and α5 subunits in the ventral tegmental area and hippocampus. J Neurochem 68: 121-127.

Chen D, Dang H, Patrick JW (1998) Contributions of N-linked glycosylation to the expression of a functional α7-nicotinic receptor in Xenopus oocytes. J Neurochem 70: 349-357.

Chen L, Wang H, Vicini S, Olsen RW (2000) The γ-aminobutyric acid type A ($GABA_A$) receptor-associated protein (GABARAP) promotes $GABA_A$ receptor clustering and modulates the channel kinetics. Proc Natl Acad Sci U S A 97: 11557-11562.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159.

Chou IC, Peng CT, Huang CC, Tsai JJ, Tsai FJ, Tsai CH (2003) Association analysis of γ2 subunit of γ- aminobutyric acid type A receptor polymorphisms with febrile seizures. Pediatr Res 54: 26-29.

Chua K, Reed R (1999) The RNA splicing factor *hSlu7* is required for correct 3' splice-site choice. Nature 402: 207-210.

Connolly CN, Krishek BJ, McDonald BJ, Smart TG, Moss SJ (1996) Assembly and cell surface expression of heteromeric and homomeric γ-aminobutyric acid type A receptors. J Biol Chem 271: 89-96.

Costa E (1998) From $GABA_A$ receptor diversity emerges a unified vision of GABAergic inhibition. Annu Rev Pharmacol Toxicol 38: 321-350.

Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH, Murray SE, Hill JK, Pantely GA, Hohimer AR, Hatton DC, Phillips TJ, Finn DA, Low MJ, Rittenberg MB, Stenzel P, Stenzel-Poore MP (2000) Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. Nat Genet 24: 403-409.

Crabbe JC, Janowsky JS, Young ER, Kosobud A, Stack J, Rigter H (1982) Tolerance to ethanol hypothermia in inbred mice: genotypic correlations with behavioral responses. Alcohol Clin Exp Res 6: 446-458.

Crabbe JC (1983) Sensitivity to ethanol in inbred mice: genotypic correlations among several behavioral responses. Behav Neurosci 97: 280-289.

Crabbe JC, Phillips TJ, Kosobud A, Belknap JK (1990) Estimation of genetic correlation: interpretation of experiments using selectively bred and inbred animals. Alcohol Clin Exp Res 14: 141-151.

Crabbe JC, Merrill C, Belknap JK (1991) Acute dependence on depressant drugs is determined by common genes in mice. J Pharmacol Exp Ther 257: 663-667.

Crabbe JC, Phillips TJ, Gallaher EJ, Crawshaw LI, Mitchell SR (1996) Common genetic determinants of the ataxic and hypothermic effects of ethanol in BXD/Ty recombinant inbred mice: genetic correlations and quantitative trait loci. J Pharmacol Exp Ther 277: 624-632.

Crabbe JC (1998) Provisional mapping of quantitative trait loci for chronic ethanol withdrawal severity in BXD recombinant inbred mice. J Pharmacol Exp Ther 286: 263-271.

Crabbe JC, Phillips TJ, Buck KJ, Cunningham CL, Belknap JK (1999) Identifying genes for alcohol and drug sensitivity: recent progress and future directions. Trends Neurosci 22: 173-179.

Crestani F, Lorez M, Baer K, Essrich C, Benke D, Laurent JP, Belzung C, Fritschy JM, Lüscher B, Mohler H (1999) Decreased $GABA_A$-receptor clustering results in enhanced anxiety and a bias for threat cues. Nat Neurosci 2: 833-839.

Daniels GM, Buck KJ (2002) Expression profiling identifies strain-specific changes associated with ethanol withdrawal in mice. Genes Brain Behav 1: 35-45.

Darvasi A, Soller M (1995) Advanced intercross lines, an experimental population for fine genetic mapping. Genetics 141: 1199-1207.

Darvasi A (1997) Interval-specific congenic strains (ISCS): an experimental design for mapping a QTL into a 1-centimorgan interval. Mamm Genome 8: 163-167.

Davies PA, Hanna MC, Hales TG, Kirkness EF (1997) Insensitivity to anaesthetic agents conferred by a class of $GABA_A$ receptor subunit. Nature 385: 820-823.

Demarest K, Koyner J, McCaughran J, Jr., Cipp L, Hitzemann R (2001) Further characterization and high-resolution mapping of quantitative trait loci for ethanol-induced locomotor activity. Behav Genet 31: 79-91.

Devaud LL, Smith FD, Grayson DR, Morrow AL (1995) Chronic ethanol consumption differentially alters the expression of g-aminobutryic acidA receptor subunit mRNAs in rat cerebral cortex: competitive quantitvative reverse transcriptase poymerase chain reaction analysis. Mol Pharm 48: 861-868.

Devaud LL, Fritschy JM, Sieghart W, Morrow AL (1997) Bidirectional alterations of GABA(A) receptor subunit peptide levels in rat cortex during chronic ethanol consumption and withdrawal. J Neurochem 69: 126-130.

Dgany O, Wides R (2002) The Drosophila odz/ten-m gene encodes a type I, multiply cleaved heterodimeric transmembrane protein. Biochem J 363: 633-643.

Dietrich W, Katz H, Lincoln SE, Shin HS, Friedman J, Dracopoli NC, Lander ES (1992) A genetic map of the mouse suitable for typing intraspecific crosses. Genetics 131: 423-447.

Dominguez-Perrot C, Feltz P, Poulter MO (1996) Recombinant $GABA_A$ receptor desensitization: the role of the $\gamma 2$ subunit and its physiological significance. J Physiol 497: 145-159

Dwek RA (1995) Glycobiology: more functions for oligosaccharides. Science 269: 1234-1235.

Entwistle J, Hall CL, Turley EA (1996) HA receptors: regulators of signalling to the cytoskeleton. J Cell Biochem 61:569-577.

Essrich C, Lorez M, Benson JA, Fritschy JM, Lüscher B (1998) Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the $\gamma 2$ subunit and gephyrin. Nat Neurosci 1: 563-571.

Falconer DS, Mackay TFC (1996) *Introduction to Quantitative Genetics,* 4th ed. (Essex, England: Longman Group Ltd).

Fehr C, Shirley RL, Belknap JK, Crabbe JC, Buck KJ (2002) Congenic mapping of alcohol and pentobarbital withdrawal liability loci to a <1 centimorgan interval of murine chromosome 4: identification of *Mpdz* as a candidate gene. J Neurosci 22: 3730-3738.

Festing MFW (1990) Inbred strains of mice, 12th listing, and distribution of some polymorphisms among inbred strains. Mouse Genome: 88: 19-112.

Festing MFW (1994) Inbred strains of mice. Mouse Genome 92: 420-426.

File SE, Baldwin HA, Hitchcott PK (1989) Flumazenil but not nitrendipine reverses the increased anxiety during ethanol withdrawal in the rat. Psychopharmacology 98: 262-264.

Findlay GS, Phelan R, Roberts MT, Homanics GE, Bergeson SE, Lopreato GF, Mihic SJ, Blednov YA, Harris RA (2003) Glycine receptor knock-in mice and hyperekplexia-like phenotypes: comparisons with the null mutant. J Neurosci 23: 8051-8059.

Frankel WN, Taylor L, Beyer B, Tempel BL, White HS (2001) Electroconvulsive thresholds of inbred mouse strains. Genomics 74: 306-312.

Franks NP, Lieb WR (1994) Molecular and cellular mechanisms of general anaesthesia. Nature 367: 607-614.

Friedman HJ (1980) Assessment of physical dependence on and withdrawal from ethanol in animals. In: Alcohol tolerance and dependence (Rigter H, Crabbe JC, eds), pp 93–121. Amsterdam: Elsevier.

Fritschy JM, Mohler H (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. J Comp Neurol 359: 154-194.

Fritschy JM, Meskenaite V, Weinmann O, Honer M, Benke D, Mohler H (1999) $GABA_B$-receptor splice variants GB1a and GB1b in rat brain: developmental regulation, cellular distribution and extrasynaptic localization. Eur J Neurosci 11: 761-768.

Fritschy JM, Brunig I (2003) Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. Pharmacol Ther 98: 299-323.

Fritschy JM, Schweizer C, Brunig I, Lüscher B (2003) Pre- and post-synaptic mechanisms regulating the clustering of type A γ-aminobutyric acid receptors ($GABA_A$ receptors). Biochem Soc Trans 31: 889-892.

Frye GD, McCown TJ, Breese GR (1983) Characterization of susceptibility to audiogenic seizures in ethanol-dependent rats after microinjection of γ-aminobutyric acid (GABA) agonists into the inferior colliculus, substantia nigra or medial septum. J Pharmacol Exp Ther 227: 663-670.

Gallaher EJ, Jones GE, Belknap JK, Crabbe JC (1996) Identification of genetic markers for initial sensitivity and rapid tolerance to ethanol-induced ataxia using quantitative trait locus analysis in BXD recombinant inbred mice. J Pharmacol Exp Ther 277: 604-612.

Garnier J, Osguthorpe DG, Robson B (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 120: 97–120.

Garrett KM, Haque D, Berry D, Niekrasz I, Gan J, Rotter A, Seale TW (1997) The $GABA_A$ receptor α6 subunit gene (*Gabra6*) is tightly linked to the α1- γ2 subunit cluster on mouse chromosome 11. Brain Res Mol Brain Res 45: 133-137.

Gerlai R (1996) Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype? Trends Neurosci 19: 177-181.

Glazier AM, Nadeau JH, Aitman TJ (2002) Finding genes that underlie complex traits. Science 298: 2345-2349.

Glencorse TA, Bateson AN, Darlison MG (1990) Sequence of the chicken $GABA_A$ receptor γ2-subunit cDNA. Nucleic Acids Res 18: 7157.

Goldstein DB, Pal N (1971) Alcohol dependence produced in mice by inhalation of ethanol: grading the withdrawal reaction. Science 172: 288-290.

Goldstein DB (1972) Relationship of alcohol dose to intensity of withdrawal signs in mice. J Pharmacol Exp Ther 180: 203-215.

Gonzalez LP, Hettinger MK (1984) Intranigral muscimol suppresses ethanol withdrawal seizures. Brain Res 298: 163-166.

Grobin AC, Matthews DB, Devaud LL, Morrow AL (1998) The role of $GABA_A$ receptors in the acute and chronic effects of ethanol. Psychopharmacology 139: 2-19.

Grupe A, Germer S, Usuka J, Aud D, Belknap JK, Klein RF, Ahluwalia MK, Higuchi R, Peltz G (2001) *In silico* mapping of complex disease-related traits in mice. Science 292: 1915-1918.

Günther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F, Crestani F, Aguzzi A, Arigoni M, Lang Y, Bluethmann H, Mohler H, Lüscher B (1995) Benzodiazepine-insensitive mice generated by targeted disruption of the $\gamma 2$ subunit gene of γ-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 92: 7749-7753.

Hamilton BJ, Lennon DJ, Im HK, Im WB, Seeburg PH, Carter DB (1993) Stable expression of cloned rat $GABA_A$ receptor subunits in a human kidney cell line. Neurosci Lett 153: 206-209.

Harkin LA, Bowser DN, Dibbens LM, Singh R, Phillips F, Wallace RH, Richards MC, Williams DA, Mulley JC, Berkovic SF, Scheffer IE, Petrou S (2002) Truncation of the $GABA_A$-receptor $\gamma 2$ subunit in a family with generalized epilepsy with febrile seizures plus. Am J Hum Genet 70: 530-536.

Heath AC, Bucholz KK, Madden PA, Dinwiddie SH, Slutske WS, Bierut LJ, Statham DJ, Dunne MP, Whitfield JB, Martin NG (1997) Genetic and environmental contributions to alcohol dependence risk in a national twin sample: consistency of findings in women and men. Psychol Med 27: 1381-1396.

Hedblom E, Kirkness EF (1997) A novel class of $GABA_A$ receptor subunit in tissues of the reproductive system. J Biol Chem 272: 15346-15350.

Hesselbrock V, Begleiter H, Porjesz B, O'Connor S, Bauer L (2001) P300 event-related potential amplitude as an endophenotype of alcoholism--evidence from the collaborative study on the genetics of alcoholism. J Biomed Sci 8: 77-82.

Hickman SJ, Ward NS, Surtees RA, Stevens JM, Farmer SF (2001) How broad is the phenotype of Hallervorden-Spatz disease? Acta Neurol Scand 103: 201-203.

Hitzemann R, Malmanger B, Cooper S, Coulombe S, Reed C, Demarest K, Koyner J, Cipp L, Flint J, Talbot C, Rademacher B, Buck K, McCaughran J, Jr. (2002) Multiple cross mapping (MCM) markedly improves the localization of a QTL for ethanol-induced activation. Genes Brain Behav 1: 214-222.

Holmdahl R, Bockermann R, Backlund J, Yamada H (2002) The molecular pathogenesis of collagen-induced arthritis in mice--a model for rheumatoid arthritis. Ageing Res Rev 1: 135-147.

Homanics GE, Ferguson C, Quinlan JJ, Daggett J, Snyder K, Lagenaur C, Mi ZP, Wang XH, Grayson DR, Firestone LL (1997) Gene knockout of the α6 subunit of the γ-aminobutyric acid type A receptor: lack of effect on responses to ethanol, pentobarbital, and general anesthetics. Mol Pharmacol 51: 588-596.

Homanics GE, Le NQ, Kist F, Mihalek R, Hart AR, Quinlan JJ (1998) Ethanol tolerance and withdrawal responses in $GABA_A$ receptor α6 subunit null allele mice and in inbred C57BL/6J and strain 129/SvJ mice. Alcohol Clin Exp Res 22: 259-265.

Homanics GE, Harrison NL, Quinlan JJ, Krasowski MD, Rick CE, de Blas AL, Mehta AK, Kist F, Mihalek RM, Aul JJ, Firestone LL (1999) Normal electrophysiological and behavioral responses to ethanol in mice lacking the long splice variant of the γ2 subunit of the gamma-aminobutyrate type A receptor. Neuropharmacology 38: 253-265.

Hood HM, Buck KJ (2000) Allelic variation in the $GABA_A$ receptor γ2 subunit is associated with genetic susceptibility to ethanol-induced motor incoordination and hypothermia, conditioned taste aversion, and withdrawal in BXD/Ty recombinant inbred mice. Alcohol Clin Exp Res 24: 1327-1334.

Hooper M, Hardy K, Handyside A, Hunter S, Monk M (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326: 292-295.

Hulander M, Wurst W, Carlsson P, Enerback S (1998) The winged helix transcription factor Fkh10 is required for normal development of the inner ear. Nat Genet 20: 374-376.

Jiang Z, Wallner M, Meera P, Toro L (1999) Human and rodent MaxiK channel β-subunit genes: cloning and characterization. Genomics 55: 57-67.

Joe B, Griffiths MM, Remmers EF, Wilder RL (1999) Animal models of rheumatoid arthritis and related inflammation. Curr Rheumatol Rep 1: 139-148.

Jones A, Korpi ER, McKernan RM, Pelz R, Nusser Z, Makela R, Mellor JR, Pollard S, Bahn S, Stephenson FA, Randall AD, Sieghart W, Somogyi P, Smith AJ, Wisden W (1997) Ligand-gated ion channel subunit partnerships: $GABA_A$ receptor α6 subunit gene inactivation inhibits δ subunit expression. J Neurosci 17: 1350-1362.

Kamatchi GL, Kofuji P, Wang JB, Fernando JC, Liu Z, Mathura JR, Jr., Burt DR (1995) $GABA_A$ receptor β1, β2, and β3 subunits: comparisons in DBA/2J and C57BL/6J mice. Biochim Biophys Acta 1261: 134-142.

Kanagawa O, Xu G, Tevaarwerk A, Vaupel BA (2000) Protection of nonobese diabetic mice from diabetes by gene(s) closely linked to IFN-gamma receptor loci. J Immunol 164: 3919-3923.

Kananura C, Haug K, Sander T, Runge U, Gu W, Hallmann K, Rebstock J, Heils A, Steinlein OK (2002) A splice-site mutation in *GABRG2* associated with childhood absence epilepsy and febrile convulsions. Arch Neurol 59: 1137-1141.

Kaneko S, Okada M, Iwasa H, Yamakawa K, Hirose S (2002) Genetics of epilepsy: current status and perspectives. Neurosci Res 44: 11-30.

Kannenberg K, Baur R, Sigel E (1997) Proteins associated with α1-subunit-containing $GABA_A$ receptors from bovine brain. J Neurochem 68: 1352-1360

Karle J, Witt MR, Nielsen M (1997) Diazepam protects against rat hippocampal neuronal cell death induced by antisense oligodeoxynucleotide to $GABA_A$ receptor γ2 subunit. Brain Res 765: 21-29.

Karle J, Woldbye DP, Elster L, Diemer NH, Bolwig TG, Olsen RW, Nielsen M (1998) Antisense oligonucleotide to $GABA_A$ receptor γ2 subunit induces limbic status epilepticus. J Neurosci Res 54: 863-869.

Keir WJ, Morrow AL (1994) Differential expression of $GABA_A$ receptor subunit mRNAs in ethanol-naive withdrawal seizure resistant (WSR) vs. withdrawal seizure prone (WSP) mouse brain. Brain Res Mol Brain Res 25: 200-208.

Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F, Hermanson O, Rosenfeld MG, Spiess J (2000) Deletion of *Crhr2* reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nat Genet 24: 415-419.

Kleingoor C, Ewert M, von Blankenfeld G, Seeburg PH, Kettenmann H (1991) Inverse but not full benzodiazepine agonists modulate recombinant α6β2γ2 $GABA_A$ receptors in transfected human embryonic kidney cells. Neurosci Lett 130: 169-172.

Kneussel M, Brandstatter JH, Laube B, Stahl S, Muller U, Betz H (1999) Loss of postsynaptic $GABA_A$ receptor clustering in gephyrin-deficient mice. J Neurosci 19: 9289-9297.

Kneussel M, Haverkamp S, Fuhrmann JC, Wang H, Wassle H, Olsen RW, Betz H (2000) The γ-aminobutyric acid type A receptor ($GABA_AR$)-associated protein

GABARAP interacts with gephyrin but is not involved in receptor anchoring at the synapse. Proc Natl Acad Sci U S A 97: 8594-8599.

Kofuji P, Wang JB, Moss SJ, Huganir RL, Burt DR (1991) Generation of two forms of the γ-aminobutyric acidA receptor γ2 subunit in mice by alternative splicing. J Neurochem 56: 713-715.

Korpi ER, Kleingoor C, Kettenmann H, Seeburg PH (1993) Benzodiazepine-induced motor impairment linked to point mutation in cerebellar $GABA_A$ receptor. Nature 361: 356-359.

Korpi ER, Kuner T, Kristo P, Kohler M, Herb A, Luddens H, Seeburg PH (1994) Small N-terminal deletion by splicing in cerebellar α6 subunit abolishes $GABA_A$ receptor function. J Neurochem 63: 1167-1170.

Korpi ER, Mihalek RM, Sinkkonen ST, Hauer B, Hevers W, Homanics GE, Sieghart W, Luddens H (2002) Altered receptor subtypes in the forebrain of $GABA_A$ receptor δ subunit-deficient mice: recruitment of γ2 subunits. Neuroscience 109: 733-743.

Korstanje R, Paigen B (2002) From QTL to gene: the harvest begins. Nat Genet 31: 235-236.

Kosobud A, Crabbe JC (1986) Ethanol withdrawal in mice bred to be genetically prone or resistant to ethanol withdrawal seizures. J Pharmacol Exp Ther 238: 170-177.

Kralic JE, Korpi ER, O'Buckley TK, Homanics GE, Morrow AL (2002) Molecular and pharmacological characterization of $GABA_A$ receptor α1 subunit knockout mice. J Pharmacol Exp Ther 302: 1037-1045.

Kralic JE, O'Buckley TK, Khisti RT, Hodge CW, Homanics GE, Morrow AL (2002) $GABA_A$ receptor α1 subunit deletion alters receptor subtype assembly, pharmacological and behavioral responses to benzodiazepines and zolpidem. Neuropharmacology 43: 685-694.

Kucken AM, Wagner DA, Ward PR, Teissere JA, Boileau AJ, Czajkowski C (2000) Identification of benzodiazepine binding site residues in the γ2 subunit of the γ-aminobutyric $acid_A$ receptor. Mol Pharmacol 57: 932-939.

Kunieda T, Zuscik MJ, Boongird A, Perez DM, Luders HO, Najm IM (2002) Systemic overexpression of the α1 β-adrenergic receptor in mice: an animal model of epilepsy. Epilepsia 43: 1324-1329.

Laurie DJ, Seeburg PH, Wisden W (1992a) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. J Neurosci 12: 1063-1076.

Laurie DJ, Wisden W, Seeburg PH (1992b) The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci 12: 4151-4172.

Levine A, Bashan-Ahrend A, Budai-Hadrian O, Gartenberg D, Menasherow S, Wides R (1994) Odd Oz: a novel Drosophila pair rule gene. Cell 77: 587-598.

Lewohl JM, Wang L, Miles MF, Zhang L, Dodd PR, Harris RA (2000) Gene expression in human alcoholism: microarray analysis of frontal cortex. Alcohol Clin Exp Res 24: 1873-1882.

Lister RG, Karanian JW (1987) RO 15-4513 induces seizures in DBA/2 mice undergoing alcohol withdrawal. Alcohol 4: 409-411
Loh EW, Ball D (2000) Role of the $GABA_A$- β2, $GABA_A$- α6, $GABA_A$- α1 and $GABA_A$- γ2 receptor subunit genes cluster in drug responses and the development of alcohol dependence. Neurochem Int 37: 413-423.

Lu J, Chen Y, Zhang Y, Pan H, Wu H, Xu K, Liu X, Jiang Y, Bao X, Ding K, Shen Y, Wu X (2002) Mutation screen of the $GABA_A$ receptor γ2 subunit gene in Chinese patients with childhood absence epilepsy. Neurosci Lett 332: 75-78

Luddens H, Pritchett DB, Kohler M, Killisch I, Keinanen K, Monyer H, Sprengel R, Seeburg PH (1990) Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. Nature 346: 648-651.

Lynn BD, Li X, Cattini PA, Turley EA, Nagy JI (2001) Identification of sequence, protein isoforms, and distribution of the hyaluronan-binding protein RHAMM in adult and developing rat brain. J Comp Neurol 439: 315-330.

Macdonald RL, Olsen RW (1994) $GABA_A$ receptor channels. Annu Rev Neurosci 17: 569-602.

Mackay TF (2001) The genetic architecture of quantitative traits. Annu Rev Genet 35: 303-339.

Madia F, Gennaro E, Cecconi M, Buti D, Capovilla G, Dalla Bernardina B, Elia M, Ferrari A, Fontana E, Gaggero R, Giannotta M, Giordano L, Granata T, La Selva L, Luisa Lispi M, Santucci M, Vanadia F, Veggiotti P, Vigliano P, Viri M, Dagna Bricarelli F, Bianchi A, Zara F (2003) No evidence of *GABRG2* mutations in severe myoclonic epilepsy of infancy. Epilepsy Res 53: 196-200.

Mahmoudi M, Kang MH, Tillakaratne N, Tobin AJ, Olsen RW (1997) Chronic intermittent ethanol treatment in rats increases GABA(A) receptor alpha4-subunit expression: possible relevance to alcohol dependence. J Neurochem 68:2485-2492.

Marks MJ, Stitzel JA, Collins AC (1989) Genetic influences on nicotine responses. Pharmacol Biochem Behav 33: 667-678.

Marley RJ, Wehner JM (1987) Correlation between the enhancement of flunitrazepam binding by GABA and seizure susceptibility in mice. Life Sci 40: 2215-2224.

Matthews DB, Devaud LL, Fritschy JM, Sieghart W, Morrow AL (1998) Differential regulation of $GABA_A$ receptor gene expression by ethanol in the rat hippocampus versus cerebral cortex. J Neurochem 70: 1160-1166.

Mayfield RD, Lewohl JM, Dodd PR, Herlihy A, Liu J, Harris RA (2002) Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. J Neurochem 81: 802-813.

McCabe (2001) Genetic targets for the treatment of pituitary adenomas: focus on the pituitary tumor transforming gene. Curr Opin Pharmacol 26: 620-625.

McCaul ME, Turkkan JS, Svikis DS, Bigelow GE (1991) Familial density of alcoholism: effects on psychophysiological responses to ethanol. Alcohol 8: 219-222.

McKernan RM, Whiting PJ (1996) Which $GABA_A$ receptor subtypes really occur in the brain? Trends Neurosci 19: 139–143.

McQuarrie DG, Fingl E (1958) Effects of single doses and chronic administration of ethanol on experimental seizures in mice. J Pharmacol Exp Ther 124: 264 – 271.

Mehta AK, Ticku MK (1999) An update on $GABA_A$ receptors. Brain Res Brain Res Rev 29: 196-217.

Metten P, Crabbe JC (1994) Common genetic determinants of severity of acute withdrawal from ethanol, pentobarbital and diazepam in inbred mice. Behav Pharmacol 5: 533-547.

Metten P, Crabbe JC (1999a) Genetic determinants of severity of acute withdrawal from diazepam in mice: Commonality with ethanol and pentobarbital. Pharmacol Biochem Behav 63: 473-479.

Metten P, Crabbe JC (1999b) Dependence and Withdrawal. In: Pharmacological effect of ethanol in the nervous system (Deitrich RA, Erwin VG, eds), pp 269-290. Boca Raton: CRC Press.

Meyer G, Kirsch J, Betz H, Langosch D (1995) Identification of a gephyrin binding motif on the glycine receptor β subunit. Neuron 15: 563-572.

Mhatre MC, Ticku MK (1992) Chronic ethanol administration alters γ-aminobutyric acidA receptor gene expression. Mol Pharm 42:415-422.

Mhatre MC, Pena G, Sieghart W, Ticku MK (1993) Antibodies specific for GABAA receptor alpha subunits reveal that chronic alcohol treatment down-regulates alpha-subunit expression in rat brain regions. J Neurochem 61:1620-1625.

Mihic SJ, Harris RA (1996) Alcohol actions at the $GABA_A$ receptor/ chloride channel complex. In: Pharmacological Effects of Ethanol on the Nervous System, (RA Deitric, VG Erwin, eds), pp 51–72. Boca Raton: CRC Press.

Mihic SJ (1999) Acute effects of ethanol on $GABA_A$ and glycine receptor function. Neurochem Int 35: 115-123.

Mihic SJ, Buck KJ (200X) Electrophysiological characterization of $GABA_A$ receptor γ2 subunit variants genetically correlated with behavioral responses to pentobarbital and ethanol, submitted to Alcohol Clin Exp Res.

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16: 1215.

Montpied P, Morrow AL, Karanian JW, Ginns EI, Martin BM, Paul SM (1991) Prolonged ethanol inhalation decreases γ-aminobutyric acidA receptor α subunit mRNAs in the rat cerebral cortex. Mol Pharm 39:157-163.

Morrow AL, Montpied P, Lingford-Hughes A, Paul SM (1990) Chronic ethanol and pentobarbital administration in the rat: effects on $GABA_A$ receptor function and expression. Alcohol 7:237-244.

Moss SJ, Smart TG (2001) Constructing inhibitory synapses. Nat Rev Neurosci 2: 240-250.

Mossier B, Togel M, Fuchs K, Sieghart W (1994) Immunoaffinity purification of γ-aminobutyric $acid_A$ ($GABA_A$) receptors containing γ1-subunits. Evidence for the presence of a single type of γ2 subunit in $GABA_A$ receptors. J Biol Chem 269: 25777-25782.

Mott R, Talbot CJ, Turri MG, Collins AC, Flint J (2000) A method for fine mapping quantitative trait loci in outbred animal stocks. Proc Natl Acad Sci U S A 97: 12649-12654.

Moy SS, Knapp DJ, Criswell HE, Breese GR (1997) Flumazenil blockade of anxiety following ethanol withdrawal in rats. Psychopharmacology 131: 354-360.

Myung PS, Boerthe NJ, Koretzky GA (2000) Adapter proteins in lymphocyte antigen-receptor signaling. Curr Opin Immunol 12: 256-266.

Nakayama J, Hamano K, Noguchi E, Horiuchi Y, Iwasaki N, Ohta M, Nakahara S, Naoi T, Matsui A, Arinami T (2003) Failure to find causal mutations in the $GABA_A$-receptor $\gamma 2$ subunit (*GABRG2*) gene in Japanese febrile seizure patients. Neurosci Lett 343: 117-120.

Newlin DB, Pretorius MB (1990) Sons of alcoholics report greater hang-oversymptoms than sons of nonalcoholics: a pilot study. Alcohol Clin Exp Res 14: 713–716.

Oohashi T, Zhou XH, Feng K, Richter B, Morgelin M, Perez MT, Su WD, Chiquet-Ehrismann R, Rauch U, Fassler R (1999) Mouse ten-m/Odz is a new family of dimeric type II transmembrane proteins expressed in many tissues. J Cell Biol 145: 563-577.

Park YG, Clifford R, Buetow KH, Hunter KW (2003) Multiple cross and inbred strain haplotype mapping of complex-trait candidate genes. Genome Res 13: 118-121.

Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, Houser CR (2002) $GABA_A$ receptor changes in δ subunit-deficient mice: altered expression of $\alpha 4$ and $\gamma 2$ subunits in the forebrain. J Comp Neurol 446: 179-197.

Peters A, Palay SL (1996) The morphology of synapses. J Neurocytol 25: 687-700.

Petrie J, Sapp DW, Tyndale RF, Park MK, Fanselow M, Olsen RW (2001) Altered $GABA_A$ receptor subunit and splice variant expression in rats treated with chronic intermittent ethanol. Alcohol Clin Exp Res 25:819-828.

Phillips TJ, Crabbe JC, Metten P, Belknap JK (1994) Localization of genes affecting alcohol drinking in mice. Alcohol Clin Exp Res 18: 931-941.

Phillips TJ, Lessov CN, Harland RD, Mitchell SR (1996) Evaluation of potential genetic associations between ethanol tolerance and sensitization in BXD/Ty recombinant inbred mice. J Pharmacol Exp Ther 277: 613-623.

Phillips TJ, Belknap JK (2002) Complex-trait genetics: emergence of multivariate strategies. Nat Rev Neurosci 3: 478-485.

Phillips TJ, Belknap JK, Hitzemann RJ, Buck KJ, Cunningham CL, Crabbe JC (2002) Harnessing the mouse to unravel the genetics of human disease. Genes Brain Behav 1: 14-26.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101: 815-850.

Powell SB, Paulus MP, Hartman DS, Godel T, Geyer MA (2003) RO-10-5824 is a selective dopamine D4 receptor agonist that increases novel object exploration in C57 mice. Neuropharmacology 44: 473-481.

Prescott CA, Kendler KS (1999) Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. Am J Psychiatry 156: 34-40.

Pritchett DB, Luddens H, Seeburg PH (1989) Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. Science 245: 1389-1392

Ramadan E, Fu Z, Losi G, Homanics GE, Neale JH, Vicini S (2003) $GABA_A$ receptor $\beta 3$ subunit deletion decreases $\beta 2/3$ subunits and IPSC duration. J Neurophysiol 89: 128-134.

Ramanathan VK, Hall ZW (1999) Altered glycosylation sites of the δ subunit of the acetylcholine receptor (AChR) reduce $\alpha\delta$ association and receptor assembly. J Biol Chem 274: 20513-20520.

Reggiani A, Barbaccia ML, Spano PF, Trabucchi M (1980) Acute and chronic ethanol administration on specific ^{3}H-GABA binding in different rat brain areas. Psychopharmacology 67: 261-264.

Reif K, Cyster J (2002) The CDM protein DOCK2 in lymphocyte migration. Trends Cell Biol 12: 368-373.

Reilly MT, Buck KJ (2000) $GABA_A$ receptor $\beta 2$ subunit mRNA content is differentially regulated in ethanol-dependent DBA/2J and C57BL/6J mice. Neurochem Int 37: 443-452.

Reilly, MT, Fehr, C, Buck KJ (2001) Alcohol and gene expression in the central nervous system. In: Nutrition gene interaction in health and disease. (Moustaid-Moussa, N, ed), pp 131-162. Boca Raton: CRC Press.

Risbrough VB, Brodkin JD, Geyer MA (2003) $GABA_A$ and 5-HT_{1A} receptor agonists block expression of fear-potentiated startle in mice. Neuropsychopharmacology 28: 654-663.

Risinger FO, Cunningham CL (1998) Ethanol-induced conditioned taste aversion in BXD recombinant inbred mice. Alcohol Clin Exp Res 22: 1234-1244.

Robertson HA (1980) Audiogenic seizures: increased benzodiazepin receptor binding in a susceptible strain of mice. Eur J Pharmacol 66: 249-252.

Rodgers DA, McClearn GE (1962) Mouse strain differences in preference for various concentrations of alcohol. Q J Stud Alcohol 23: 26-33.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H (1999) Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. Nature 401: 796-800.

Russek SJ (1999) Evolution of $GABA_A$ receptor diversity in the human genome. Gene 227: 213-222.

Russell ES (1978) Origins and history of mouse inbred strains: Contributions of Clarence Cook Little. In: Origins of inbred mice (Morse, HC III, ed), pp 33-43. New York: Academic Press.

Sassoe-Pognetto M, Fritschy JM (2000) Mini-review: gephyrin, a major postsynaptic protein of GABAergic synapses. Eur J Neurosci 12: 2205-2210.

Saunders PA, Ho IK (1990) Barbiturates and the $GABA_A$ receptor complex. Prog Drug Res 34: 261-286.

Scheffer IE, Berkovic SF (1997) Generalized epilepsy with febrile seizures plus. A genetic disorder with heterogeneous clinical phenotypes. Brain 120 (Pt 3): 479-490.

Schuckit MA (1994) The 1994 Isaacson Award Lecture: a prospective study of sons of alcoholics. Alcohol Alcohol Suppl 2: 1-6.

Schuckit MA (1994) Low level of response to alcohol as a predictor of future alcoholism. Am J Psychiatry 151: 184-189.

Schuckit MA (1998) Biological, psychological and environmental predictors of the alcoholism risk: a longitudinal study. J Stud Alcohol 59: 485-494.

Schuckit MA, Edenberg HJ, Kalmijn J, Flury L, Smith TL, Reich T, Bierut L, Goate A, Foroud T (2001) A genome-wide search for genes that relate to a low level of response to alcohol. Alcohol Clin Exp Res 25: 323-329.

Schuckit MA, Mazzanti C, Smith TL, Ahmed U, Radel M, Iwata N, Goldman D (1999) Selective genotyping for the role of 5-HT_{2A}, 5-HT_{2C}, and GABA α6 receptors and the serotonin transporter in the level of response to alcohol: a pilot study. Biol Psychiatry 45: 647-651.

Schweizer C, Balsiger S, Bluethmann H, Mansuy IM, Fritschy JM, Mohler H, Lüscher B (2003) The γ2 subunit of GABA(A) receptors is required for maintenance of receptors at mature synapses. Mol Cell Neurosci 24: 442-450.

Serikawa T, Kuramoto T, Hilbert P, Mori M, Yamada J, Dubay CJ, Lindpainter K, Ganten D, Guenet JL, Lathrop GM, et al. (1992) Rat gene mapping using PCR-analyzed microsatellites. Genetics 131: 701-721.

Shivers BD, Killisch I, Sprengel R, Sontheimer H, Kohler M, Schofield PR, Seeburg PH (1989) Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. Neuron 3: 327-337.

Sieghart W, Sperk G (2002) Subunit composition, distribution and function of $GABA_A$ receptor subtypes. Curr Top Med Chem 2: 795-816.

Sigvardsson S, Bohman M, Cloninger CR (1996) Replication of the Stockholm Adoption Study of alcoholism. Confirmatory cross-fostering analysis. Arch Gen Psychiatry 53: 681-687.

Smith GB, Olsen RW (1995) Functional domains of $GABA_A$ receptors. Trends Pharmacol Sci. 16: 162-168.

Somogyi P, Fritschy JM, Benke D, Roberts JD, Sieghart W (1996) The γ2 subunit of the $GABA_A$ receptor is concentrated in synaptic junctions containing the α1 and β2/3 subunits in hippocampus, cerebellum and globus pallidus. Neuropharmacology 35: 1425-1444.

Span SA, Earleywine M (1999) Familial risk for alcoholism and hangover symptoms. Addict Behav 24: 121-125.

Sur C, Wafford KA, Reynolds DS, Hadingham KL, Bromidge F, Macaulay A, Collinson N, O'Meara G, Howell O, Newman R, Myers J, Atack JR, Dawson GR, McKernan RM, Whiting PJ, Rosahl TW (2001) Loss of the major $GABA_A$ receptor subtype in the brain is not lethal in mice. J Neurosci 21: 3409-3418.

Taylor BA (1978) Recombinant inbred strains: Use in gene mapping. In: Origins of Inbred Mice (Morse HC III ed), pp 423–438. New York: Academic Press.

Taylor BA, Wnek C, Kotlus BS, Roemer N, MacTaggart T, Phillips SJ (1999) Genotyping new BXD recombinant inbred mouse strains and comparison of BXD and consensus maps. Mamm Genome 10: 335-348.

Terdal ES, Crabbe JC (1994) Indexing withdrawal in mice: matching genotypes for exposure in studies using ethanol vapor inhalation. Alcohol Clin Exp Res 18: 542-547.

Threadgill DW, Yee D, Matin A, Nadeau JH, Magnuson T (1997) Genealogy of the 129 inbred strains: 129/SvJ is a contaminated inbred strain. Mamm Genome 8: 390-393.

Ticku MK (1980) The effects of acute and chronic ethanol administration and its withdrawal on γ-aminobutyric acid receptor binding in rat brain. Br J Pharmacol 70: 403-410.

Treiman DM (2001) GABAergic mechanisms in epilepsy. Epilepsia 42 Suppl 3: 8-12.

Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J Neurosci 17: 2728-2737.

Tretter V, Hauer B, Nusser Z, Mihalek RM, Hoger H, Homanics GE, Somogyi P, Sieghart W (2001) Targeted disruption of the $GABA_A$ receptor δ subunit gene leads to an up-regulation of γ2 subunit-containing receptors in cerebellar granule cells. J Biol Chem 276: 10532-10538.

Toole BP (1997) Hyaluronan in morphogenesis. J Intern Med 242:35-40.

Uchida I, Li L, Yang J (1997) The role of the $GABA_A$ receptor α1 subunit N-terminal extracellular domain in propofol potentiation of chloride current. Neuropharmacology 36: 1611-1621.

Uusi-Oukari M, Heikkila J, Sinkkonen ST, Makela R, Hauer B, Homanics GE, Sieghart W, Wisden W, Korpi ER (2000) Long-range interactions in neuronal gene expression: evidence from gene targeting in the $GABA_A$ receptor β2- α6- α1- γ2 subunit gene cluster. Mol Cell Neurosci 16: 34-41

van Lookeren Campagne M, Gill R (1998) Tumor-suppressor p53 is expressed in proliferating and newly formed neurons of the embryonic and postnatal rat brain: comparison with expression of the cell cycle regulators p21Waf1/Cip1, p27Kip1, p57Kip2, p16Ink4a, cyclin G1, and the proto-oncogene Bax. J Comp Neurol 397: 181-198.

van Lookeren Campagne M, Okamoto K, Prives C, Gill R (1999) Developmental expression and co-localization of cyclin G1 and the B' subunits of protein phosphatase 2a in neurons. Brain Res Mol Brain Res 64: 1-10.

Vodenlich AD, Jr., Gong YZ, Geoghegan KF, Lin MC, Lanzetti AJ, Wilson FA (1991) Identification of the 14 kDa bile acid transport protein of rat ileal cytosol as gastrotropin. Biochem Biophys Res Commun 177: 1147-1154.

Wade CM, Kulbokas EJ, 3rd, Kirby AW, Zody MC, Mullikin JC, Lander ES, Lindblad-Toh K, Daly MJ (2002) The mosaic structure of variation in the laboratory mouse genome. Nature 420: 574-578.

Wafford KA, Burnett DM, Leidenheimer NJ, Burt DR, Wang JB, Kofuji P, Dunwiddie TV, Harris RA, Sikela JM (1991) Ethanol sensitivity of the $GABA_A$ receptor expressed in Xenopus oocytes requires 8 amino acids contained in the γ22L subunit. Neuron 7: 27-33.

Wallace RH, Marini C, Petrou S, Harkin LA, Bowser DN, Panchal RG, Williams DA, Sutherland GR, Mulley JC, Scheffer IE, Berkovic SF (2001) Mutant $GABA_A$ receptor γ2-subunit in childhood absence epilepsy and febrile seizures. Nat Genet 28: 49-52.

Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW (1999) $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton. Nature 397: 69-72

Wang H, Olsen RW (2000) Binding of the $GABA_A$ receptor-associated protein (GABARAP) to microtubules and microfilaments suggests involvement of the cytoskeleton in GABARAP$GABA_A$ receptor interaction. J Neurochem 75: 644-655.

Wang JB, Burt DR (1991) Differential expression of two forms of $GABA_A$ receptor γ2-subunit in mice. Brain Res Bull 27: 731-735.

Wang JB, Kofuji P, Fernando JC, Moss SJ, Huganir RL, Burt DR (1992) The α1, α2, and α3 subunits of $GABA_A$ receptors: comparison in seizure-prone and -resistant mice and during development. J Mol Neurosci 3: 177-184.

Waterston RH et al. (2002) Initial sequencing and comparative analysis of the mouse genome. Nature. 420: 520-562.

Weiner JL, Zhang L, Carlen PL (1994) Potentiation of $GABA_A$-mediated synaptic current by ethanol in hippocampal CA1 neurons: possible role of protein kinase C. J Pharmacol Exp Ther 268: 1388-1395.

Whiting P, McKernan RM, Iversen LL (1990) Another mechanism for creating diversity in gamma-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 phosphorylation site. Proc Natl Acad Sci U S A 87: 9966-9970.

Williams-Hemby L, Porrino LJ (1997) I. Functional consequences of intragastrically administered ethanol in rats as measured by the 2-[^{14}C]deoxyglucose method. Alcohol Clin Exp Res 21: 1573-1580.

Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci 12: 1040-1062.

Witmer PD, Doheny KF, Adams MK, Boehm CD, Dizon JS, Goldstein JL, Templeton TM, Wheaton AM, Dong PN, Pugh EW, Nussbaum RL, Hunter K, Kelmenson JA, Rowe LB, Brownstein MJ (2003) The development of a highly informative mouse Simple Sequence Length Polymorphism (SSLP) marker set and construction of a mouse family tree using parsimony analysis. Genome Res 13: 485-491.

Wu CH, Frostholm A, De Blas AL, Rotter A (1995) Differential expression of $GABA_A$/benzodiazepine receptor subunit mRNAs and ligand binding sites in mouse cerebellar neurons following in vivo ethanol administration: an autoradiographic analysis. J Neurochem 65: 1229-1239.

Yu O, Ito M, Chiu TH, Rosenberg HC (1986) GABA-gated chloride ion influx and receptor binding studies in C57BL6J and DBA2J mice. Brain Res 399: 374-378.

Yu R, Heaney AP, Lu W, Chen J, Melmed S (2000) Pituitary tumor transforming gene causes aneuploidy and p53-dependent and p53-independent apoptosis. J Biol Chem 275: 36502-36505.

Zhou B, Westaway SK, Levinson B, Johnson MA, Gitschier J, Hayflick SJ (2001) A novel pantothenate kinase gene (*PANK2*) is defective in Hallervorden-Spatz syndrome. Nat Genet 28: 345-349.

Zou H, McGarry TJ, Bernal T, Kirschner MW (1999) Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science 285: 418-422.

Zuscik MJ, Sands S, Ross SA, Waugh DJ, Gaivin RJ, Morilak D, Perez DM (2000) Overexpression of the α1 β-adrenergic receptor causes apoptotic neurodegeneration: multiple system atrophy. Nat Med 6: 1388-1394.