ELUCIDATING THE ROLE OF ALPHA1-CONTAINING GABA(A) RECEPTORS IN ETHANOL ACTION

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Alcohol (ethanol) has a prominent role in society and is one of the most frequently used and abused drugs. Despite the pervasive use and abuse of ethanol, the molecular mechanisms of ethanol action remain unclear. What is well known is that ethanol intoxication elicits a range of behavioral effects. These effects most likely occur through the direct action of ethanol on targets in the central nervous system. By studying behavioral effects, the role of individual targets can be determined. The function of γ-amino butyric acid type A ($GABA_A$) receptors is altered by ethanol, but due to multiple receptor subunits the exact role of individual $GABA_A$ receptor subunits in ethanol action is not known. This dissertation focused on the role of α1-containing $GABA_A$ receptors in ethanol action using gene knockin mice with ethanol insensitive α1 $GABA_A$ receptors.

In the second chapter, knockin mice were molecularly characterized and ethanol-induced behavioral effects were assessed. α1 was found to mediate acute tolerance to the motor ataxic effects of ethanol. In the third chapter, α1 involvement in ethanol induction of neuronal activity was assessed in discrete neuroanatomic regions using the immediate early gene c-fos. Specifically, c-fos immunohistochemistry was characterized after acute ethanol exposure, after chronic ethanol exposure, and finally during the ethanol withdrawal phase. α1 was found to be involved in ethanol-mediated effects in the dentate gyrus.

In the fourth chapter, α1 involvement in chronic tolerance to ethanol as well as physical dependence on ethanol was characterized. Results demonstrated that α1-$GABA_A$-Rs play a role in the development of tolerance to chronic ethanol in motor ataxia. Intriguingly, α1 was implicated in dependence as assessed with ethanol withdrawal-related hyperexcitability. Knockin mice were more sensitive to ethanol's withdrawal-related hyperexcitability effects.

In summary, this dissertation further supports α1 $GABA_A$-Rs in the mechanism of ethanol action. By chiseling away at the various components of ethanol action we are beginning to elucidate the mechanism of ethanol action. Further elucidation of the mechanism of action of α1 $GABA_A$-Rs in tolerance and dependence could deepen our understanding of the molecular mechanisms behind alcohol abuse and alcoholism. By understanding the molecular mechanisms of ethanol, alcohol abuse may be lessened and alcoholism could potentially be cured.

FORWARD

"Never never never give up."
-Winston Churchill

"Nothing in the world can take the place of persistence.
Talent will not; nothing is more common than unsuccessful men with talent.
Genius will not; unrewarded genius is almost a proverb.
Education will not; the world is full of educated derelicts.
Persistence and determination alone are omnipotent. The slogan 'press on' has solved and always will solve the problems of the human race."
-Calvin Coolidge

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PREFACE

Part of one chapter of this thesis has been published.

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LIST OF ABBREVIATIONS

ABC	Avidin Biotin Complex
ACTH	Adreno-Cortcotropic Hormone
ADH	Alcohol Dehydrogenase
AFT	Acute Functional Tolerance
BEC	Blood Ethanol Concentration
cDNA	Complimentary DNA
CNS	Central Nervous System
DAB	3,3- di-amino-benzidine
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
DSM-IV	Diagnostic & Statistical Manual – IV
Erk 1/2	Extracellular Signal-Regulated Kinase 1/2
EW	Edinger Westphal
Fos-IR	c-Fos Immunoreactivity
GABA	γ-Aminobutyric Acid
$GABA_A$-R	γ-Aminobutyric Acid Type A Receptor
HEK293	Human Embryonic Kidney
IEG	Immediate Early Gene
i.p.	Intraperitoneal
IPSC	Inhibitory Postsynaptic Current
LORR	Loss of Righting Reflex
MEK 1/2	Mitogen-Activated Protein Kinase 1/2
mRNA	Messenger RNA
NaCl	Sodium Chloride
NGS	Normal Goat Serum
NMDA	N-Methyl-D-Aspartate
NMDA-R	N-Methyl-D-Aspartate Receptor
PB	Phosphate Buffer Solution
PBS	Phosphate Buffer Saline Solution
PBST	PBS-containing 0.1% Tween-20

PBSX	PBS-containing 0.025% Triton-X100
PCR	Polymerase Chain Reaction
PVN	Paraventricular Nucleus of the Hypothalamus
RNA	Ribonucleic Acid
RT-PCR	Reverse-Transcriptase PCR
Stat-3	Signal Transducer and Activator of Transcription 3

1.0 INTRODUCTION & BACKGROUND

1.1 OVERVIEW

Alcohol (ethanol) has a prominent role in society. Despite its widespread use, alcohol misuse and abuse is such a persistent problem that the National Institutes of Alcohol Abuse and Alcoholism reported ethanols' annual 'personal, social and economic toll' in the United States in excess of $180 billion [1]. Worse off, it is estimated that approximately 14 million (7.4 percent) Americans can be categorized as alcohol abusers or alcoholics [2]. Even teetotalers and individuals who do not abuse ethanol are indirectly affected. The fact that over half of all Americans are related to an individual who is or has dealt with alcoholism is startling [3]. If this is not enough to make people take notice, the likelihood that ethanol can result in personal trauma is high – three out of every ten people will be involved in an alcohol-related traffic crash in their lifetime [4].

While the sociological impact of ethanol is known, much remains unknown about ethanol's molecular mechanism of action – for instance, differences in individual responses to ethanol, and the observation that much of the population consumes alcohol yet only a portion become addicted. Nonetheless, it is widely regarded that the intoxicating and addictive properties of alcohol are mediated by the central nervous system. Also, the intoxicating effects of alcohol result in well characterized behavioral responses such as reduced anxiety, impaired motor coordination, tolerance and withdrawal [DSM-IV, 5]. By using these behaviors as outcome measurements, we can begin to determine which molecular targets in the brain are involved in ethanol's intoxicating effects as well as tolerance and dependence. By determining ethanol's mechanism of action, we may better combat its sociological impact. For starters, we may more effectively use already established pharmacologic agents for treatment. This potentially could be done using a cocktail of drugs working on various molecular targets. Secondly, more selective or novel agents may be developed for previously established targets. Benzodiazepines are currently used for alcohol withdrawal [6, 7], but due to unwanted side effects, non-classical benzodiazepines may be more efficacious. Additionally, pharmacologic intervention may be used to treat the side effects associated with drunk driving thereby potentially reducing traffic-

related incidences. Lastly, because ethanol action results in many behavioral alterations, the mechanisms of specific behaviors may potentially be extrapolated to give insight into other disorders. For instance, ethanol-related effects could aid in elucidation of mechanisms of action of behavioral disorders such as seizures/epilepsy, anxiety, tremor, motor ataxia, cognitive impairment and insomnia.

1.2 ALCOHOL PHARMACOKINETICS

As described in Goodman and Gillman's Pharmacological Basis of Therapeutics [8], upon consumption, ethanol is absorbed via the small intestine and subjected to first-pass metabolic effects. Gastric and liver alcohol dehydrogenases (ADH) convert ethanol into aldehyde. Aldehyde is further broken into acetic acid by aldehyde dehydrogenase and ultimately into water and carbon dioxide. The alcohol dehydrogenase reaction is the 'rate-limiting' step, and thus determines how quickly ethanol is eliminated from the body. During increased alcohol consumption, this first-pass metabolism is typically saturated, and ethanol is distributed to the rest of the body where it exerts its intoxicating effects. As a result, clearance of ethanol is similar to zero-order kinetics – only a constant amount is eliminated in a set period of time. Zero-order elimination is constant except at low concentrations of ethanol; because of this, others have questioned assay limitations [9] and have tried to model elimination with a nonlinear fit across the entire ethanol concentration curve [10]. Nonetheless, the latter study still concluded classical 'zero-order kinetics' is justified for studying a limited portion of the ethanol concentration curve, i.e., at high concentrations on the descending phase of the blood ethanol concentration curve where ADH is saturated.

1.3 ACUTE ETHANOL-INDUCED BEHAVIORAL EFFECTS

Ethanol is primarily consumed for its positive behavioral attributes such as an increased overall sense of well-being, reduced anxiety and even as a sleep aide (e.g., night cap). However, ethanol

consumption is not without side effects. Acute and long-term ethanol exposure can affect judgment, motor coordination, memory, pain sensation, sexual function and can cause many other health related issues [As reviewed in DSM-IV, 5]. Excessive amounts of ethanol can even result in unconsciousness or possibly death. Individuals may even develop physical tolerance and/or dependence and become more susceptible to alcohol abuse and alcoholism. Alcohol has a relatively large therapeutic window, but tolerance possibly decreases the therapeutic window, thereby narrowing the gap between desired effects (e.g., sedative), and toxic effects. Alternatively, tolerance may possibly cause a rightward shift in ethanol's therapeutic window, thereby causing the effects to occur at higher blood ethanol concentrations. While all of the above behaviors are important to fully understanding ethanol action, some behaviors have more relevance to ethanol's societal impacts. Namely, why people self-medicate for anxiety, insomnia, and pain and how these relate to addiction. Other factors such as cognition and motor coordination have relevancy to driving while intoxicated.

While it is thought that sensitivity to alcohol's effects and predisposition to developing alcoholism are believed to have a strong genetic component (~50%, [11, 12]), environmental factors can exert a fair amount of influence [13-16] and this complex interplay between genes and the environment should not be ignored. As such, many ethanol-induced behavioral phenotypes listed above may be assessed experimentally in animals. By using animal models to assess ethanol action, in controlled laboratory environments (thereby limiting environmental influences) we can begin to dissect the genetic component and molecular targets of alcohol action and alcoholism. Additionally, evidence also suggests that various neuroanatomical regions may be important to specific behaviors. By combining genetic analysis with implicated brain regions, a more detailed understanding of the molecular targets of ethanol that mediate the drug's behavioral effects can by obtained. While ethanol has numerous acute behavioral effects, this introduction is limited to those behaviors that were investigated in this thesis – namely anxiolysis, sedation/hypnosis, analgesia and motor impairment.

1.3.1 Ethanol-Induced Anxiolysis

Ethanol is mainly consumed for pleasurable effects, including its anxiety relieving, or anxiolytic component. This effect, bar none, may drive more people to try ethanol, to keep seeking it

socially, and to develop dependence. While it is not exactly known which genes mediate ethanol's anxiolytic effects, numerous studies have suggested different neuroanatomic regions are involved such as the hippocampal area, cortex, amygdala and hypothalamus to name a few [17-21]. While one can't exactly ask a mouse if it is anxious, behavioral assays to assess anxiety-like behavior in rodents have been validated using known pharmacologic agents that reduce or increase (benzodiazepine agonists [22], benzodiazepine inverse-agonists [23] , respectively) anxiety in humans. Anxiety-related behaviors are commonly assessed in rodent models by use of light/dark exploration and the elevated plus maze assays. The light-dark apparatus was perhaps the first widely used assay for measuring anxiety-related behaviors [24, 25]. The apparatus is comprised of a brightly lit compartment separated by a small opening from an enclosed dark compartment. This assay plays on the inherent nature of rodents for exploration, but avoidance of bright open areas. Administration of anxiolytic drugs such as ethanol or benzodiazepines results in increased time in the light area and/or increased exploratory activity [e.g., 26, 27].

The elevated plus maze also relies on the same inherent exploratory/ avoidance nature as in the light-dark test. The apparatus is made of two opposing 'open' platforms and two opposing 'closed' (i.e., walled) platforms. Similar to the light-dark test, the plus maze mimics safety in closed arms and avoidance/exploration conflict in the open arms. All platforms are at right angles to each other (forming a plus sign) connected by a center area. All platforms are raised off the ground thereby adding a height and potential fear component to potentially increase anxiety. In this assay, rodents are placed in the center of the apparatus and allowed to explore. Factors that may be used to measure anxiety-like behavior or ethanol changes therein include open arm entries and time spent in open arms [28, 29]. Others have reported using changes in locomotor activity as anxiety-related measurements, given the fact that both assays are based on motor activity [30, 31].

Aside from these two assays, other tests have been used for assessing anxiolytic-like behaviors. For instance, some studies have used social interaction (i.e., sniffing, grooming) [32, 33] in ethanol-related behavior. Others have used the center portion of an open field test apparatus (see below) as another measurement of anxiety-like behavior, even modifying the assay to use food as motivation [34]. Other anxiety-related assays include the Geller-Seifter conflict, Vogel conflict, elevated zero maze, emergence test, mirrored chamber, staircase test and

defensive burying to name a few [35]. Although none of these assays are as prevalent as the light-dark apparatus or elevated-plus maze, it should be noted that anxiety is a complex behavior. Thus, because many of these assays exploit different aspects of anxiety (e.g., aversive to open areas or height) no single assay can be considered the end-all for assessing anxiety. Analysis of the role of a gene and/or drug in anxiety should be consistent across multiple assays.

1.3.2 Ethanol-Induced Sedation/Hypnosis

Ethanol has biphasic effects [for instance, see 30, 36, 37, 38]. At lower concentrations, ethanol may result in a locomotor stimulatory effect (i.e., increased activity), but at higher concentrations, ethanol acts as a sedative (i.e., decreased activity). At even higher doses, ethanol can display anesthetic-like properties by causing unconsciousness, or hypnosis. Although typically considered to be similar, sedation and hypnosis are two separate behavioral endpoints. Sedation is more synonymous with decreases in locomotor activity observed at lower blood ethanol concentrations (BEC), while hypnosis is associated with unconscious states at higher BECs. One possible brain region that may be implicated in the hypnotic effects of ethanol is the thalamus [39], although other regions thought to regulate consciousness such as the cortex may be involved [40].

Two of the most common behavioral tests used to assess sedation and hypnosis are the open field and righting reflex (also referred to as sleep time), respectively. Examples of the effects of ethanol on sedative and hypnotic responses can be found throughout the ethanol literature [e.g., 41, 42, 43]. The open field test involves placing the rodent in a large brightly lit arena and measuring locomotor activity by counting the number of line crossings or photocell beam breaks for a set period of time. It should be noted that analysis of the center activity (defined as locomotor activity in center area versus perimeter area) of the open field could be a measure of anxiety-like behavior. As with the light-dark assay described above, rodents tend to avoid bright open areas and therefore prefer to stay close to the walls of the apparatus. However, this assay isn't as prevalent as the aforementioned for anxiety related tasks. Vice versa, behavioral assays for anxiety can also be used for analysis of locomotor effects (i.e., increases in arm entries in the plus maze assay or transitions in the light-dark box apparatus).

The righting reflex may be assessed by two methods. The first is by administering a large dose of ethanol and placing the animal on its back and measuring the time required for the animal to turn over and right itself [e.g., 44]. The second method involves administering different doses and determining the concentration at which 50% of subjects, or EC_{50} lose the righting reflex [e.g., 45].

1.3.3 Ethanol-Induced Analgesia

Perhaps lesser known is ethanol's effects on nociception and potential to alleviate pain sensation (i.e., analgesia). Nonetheless, clinical studies have shown that individuals consuming the equivalent of two drinks are able to withstand experimentally-induced pain better than control subjects [46]. Furthermore, the historical attempted use of ethanol in patient care for alleviating pain has been documented [47]. Pain is a complex process which is believed to involve the interplay between numerous neuroanatomic regions including the spinal cord and supraspinal sites [48-54].

Experimentally, two methods are typically used to assess the analgesic effects of ethanol – the tail flick and the hot plate [e.g., 55, 56, 57]. The tail-flick test is carried out by placing the end of the tail in either a hot-water bath or by focusing a beam of light at a set distance from the end of the tail. Latency is then measured as the time it takes the animal to move, or flick its tail. The hotplate is conducted by placing the rodent on top of a heated surface and measuring latency till the animal reacts (lifting or licking the hind foot).

1.3.4 Ethanol-Induced Motor Ataxia

Apart from ethanol's pleasurable effects, motor incoordination is perhaps one of the most well known adverse effects of ethanol intoxication – particularly for its correlation to automobile infractions [58]. Even low amounts of ethanol can impair fine motor coordination with progressive increases in ethanol resulting in further impairment [8]. Ethanol impaired motor coordination is classically believed to be cerebellarly mediated [59]; although other regions such as the thalamus and primary motor cortex are quite possibly involved in this complex process [60, 61]. Intriguingly, there is remarkable similarity between ethanol impairment to impairment

by other pharmacologic agents [62], which may suggest shared putative molecular targets. Dependent on which assay is used, this complex behavior involves the combination of several behavioral processes such as balance, myoreflex and sensory perception [63, 64].

The rotarod device, first describe in the 1950's [65, 66] involves testing an animal on a rotating drum and has been used in numerous ethanol-related ataxia studies and even the assay itself may have several parameters. For instance, latency to fall may be measured at either a constant (fixed) speed, or the apparatus may accelerate over a period of time [63, 67]. Other variations may be employed such as assessing time to recover from drug-induced ataxia [68].

Aside from the rotarod, other assays may be employed to assess ethanol-induced ataxia. These include, but are not limited to: balance beam walking; the balancing dowel test; the tilting-plane, grid test, and footprint analysis. The balance beam and the grid test may be used to assess minor motor defects such as 'missteps' [64, 69]. The footprint, or gait analysis is more subtle and may measure differences in walking patterns [70, 71]. Even simpler still, the balancing dowel test measures the ability to balance in place [72]. As stated above, motor coordination is an incredibly complex process involving numerous factors such as balance, walking, coordinated movement, sensory processing, and myoreflex to name a few. Therefore, like anxiety no one experimental paradigm can be used to deduce ethanol's ataxic effects. Rather, multiple assays should be used to delineate ethanol's involvement on motor ataxia.

1.4 ETHANOL-INDUCED TOLERANCE AND DEPENDENCE

Tolerance is defined as a reduced response to a constant amount of ethanol or an increase in the amount of ethanol needed to elicit the same effect [5]. Conversely, ethanol dependence is classically defined by a characteristic withdrawal syndrome upon cessation of ethanol exposure [5, 73, 74]. It is not clear why those who self medicate eventually need more ethanol to achieve their desired effects. Conversely, it is not known why some are more sensitive to withdrawal-related effects. Overall, these two factors may lead to the development of alcoholism [5], and are believed to be prognostic indicators of alcoholism under the DSM-IV [75, 76]. Although simple in definition, many types of tolerance and withdrawal exist, possibly produced through either convergent or divergent molecular mechanisms, or possibly some combination therein.

Nonetheless, by determining molecular targets responsible for tolerance and withdrawal, pharmacological interventions may be used to reduce the societal impact of alcohol abuse and addiction could be dramatically decreased.

Several types of tolerance have been described including acute tolerance, rapid tolerance, and chronic tolerance [77], and each type of tolerance can impact a wide variety of ethanol-induced behaviors such as those listed above (e.g., sedation, hypnosis, ataxia, etc.) [78-80]. Similarly, withdrawal takes a variety of forms, such as convulsions and anxiety [74, 81-83]. Aspects of these differences in tolerance and withdrawal are discussed below.

1.4.1 Acute Tolerance

Acute tolerance is the ability to adapt in a relatively short period of time (i.e., same drinking bout), and is thought to involve molecular neuro-adaptations to counteract the initial ethanol insult. Acute tolerance is believed to occur so rapidly that most experimental paradigms are not able to tease it apart from initial sensitivity. This is graphically portrayed in figure 1A. Initial sensitivity for a specific behavioral phenotype is defined where the phenotype is lost on the rising phase of the ethanol concentration response curve. Tolerance occurs as the ability to regain the behavioral phenotype at a higher ethanol concentration on the descending portion of the ethanol response curve [reviewed in, 84]. However, even if adaptations do indeed develop during the same ethanol challenge, these adaptations may still be considered a component of the initial response. As suggested by the definition of tolerance, in this paradigm no subsequent exposure to ethanol is present, thus it may be argued whether this is a true measure of acute tolerance.

Indeed, in line with the definition of tolerance, others have suggested acute tolerance be measured over a short period of time and involve more than one ethanol exposure. In doing so, acute tolerance to a specific phenotype may be assessed on similar descending phases of the blood alcohol concentration response curve [85]. Better known as acute functional tolerance (AFT), Erwin and Deitrich [86] adapted this into a behavioral paradigm to assess acute tolerance to the ataxic effects of ethanol as well as the hypnotic effects [87]. This is depicted in figure 1B. Because the latter involves two or more periods of ethanol intoxication and occurs over a longer period of time, some have considered this to involve aspects of both acute tolerance and rapid

tolerance. However, because this method involves repeated testing of animals, certain tests where the animals should be experimentally naïve (i.e., anxiety-related assays) need to be assessed using other methods.

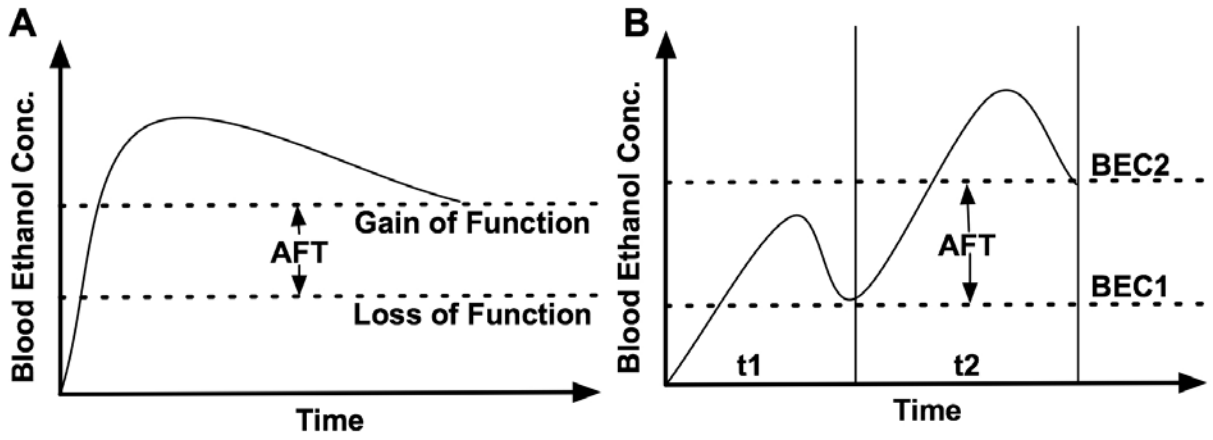

Figure 1.1. Acute tolerance in relation to blood alcohol concentration curves.
Acute tolerance based on adaptions during a single dose (A). Acute functional tolerance (AFT) is defined as the difference in the blood ethanol concentrations when the behavior/phenotype was lost and subsequently regained. Figure adapted from Ponomarev and Crabbe [88]. Acute tolerance based on more than one dose (B). AFT in this paradigm compares the regaining of a phenotype twice from two subsequent ethanol exposures. Adapted from Erwin and Deitrich [86]. The difference in BEC at the two time points is a measure of AFT.

1.4.2 Rapid and Chronic Tolerance

Rapid tolerance and chronic tolerance differ from acute tolerance (and from each other) in the length of time necessary for adaptation to occur. Rapid tolerance is believed to occur during subsequent ethanol exposure after the first/previous exposure has been cleared from the body. This may be seen for up to twenty-four hours after the previous exposure, and the tolerant effects dissipate about as fast as they develop without subsequent ethanol exposures [89, 90]. Chronic tolerance develops after days or weeks of repeated ethanol administration [91] and unlike rapid tolerance, the effects may be seen for an extended period of time after cessation of ethanol exposure. Tolerance may be seen in numerous ethanol-related behavioral paradigms such as those listed below.

1.4.2.1 Ethanol-Related Sedation/Hypnosis

Repeated ethanol exposure has been shown to alter ethanol's sedative and hypnotic effects. With respect to sedation, individuals commonly self-medicate with ethanol to aide in sleep. However, in rodent models instead of resulting in a sedative effect, chronic administration of ethanol causes a stimulatory effect seen as increases in locomotor activity [92-94]. Thus, instead of resulting in sedative effects (the sought after effect), ethanol causes a reciprocal event by increasing activity (restlessness). This could create a potentially vicious cycle where more ethanol is consumed to achieve a sedative effect, thereby leading to more severe consequences such as unconsciousness (hypnosis). The effects of chronic ethanol on sedation may be assessed using the open-field apparatus [e.g., 93], although other assays such as the elevated plus maze or light-dark apparatus primarily used for anxiety may also be used.

With respect to the hypnotic effects of ethanol, animal models have also demonstrated tolerance to the hypnotic effects of ethanol in both rapid and chronic tolerance paradigms. For instance, some studies have reported rapid tolerance to the hypnotic effects of ethanol in different inbred strains of mice as well as in rats [95, 96]. Additionally, chronic tolerance to ethanol-induced hypnosis has been demonstrated using rats and mice with different treatment methods [97-99].

1.4.2.2 Ethanol-Related Ataxia

Because of the correlation between consumption of ethanol and the development of tolerance to the psychomotor affects of ethanol [100, 101], many people incorrectly assume that tolerance enables them to drive drunk without consequence. However, this often leads to serious repercussions (i.e., decreased mental acuity).

Nonetheless, tolerance to the motor impairing effects of ethanol from multiple exposures has been readily demonstrated. Because of the complexity of motor tasks, numerous different methods have been used to demonstrate tolerance to different motor related tasks in rodents. For instance, simple motor tasks such as balancing on a dowel used in the acute functional tolerance test described above have been done [72, 79, 102-104]. Other tests assaying coordinative movement such as the rotarod are more readily employed [105-110]. However, tolerance may not be readily observed by increasing the complexity of the task such as increased sensory perception found in the accelerating rotarod [106]. However, others have demonstrated tolerance

to ethanol's ataxic effects on the accelerating rotarod but only after using a 6 week exposure paradigm to high dose ethanol concomitant with training [111]. Other tests such as the grid test, tilt plane, and treadmill/ moving belt have also been used to assess tolerance to ethanol on motor coordination [90, 112, 113].

It should be stressed that acquiring tolerance to a task while intoxicated (e.g., motor coordination) may involve a learning component. For instance, studies have shown that failure to practice a motor-related task while in an intoxicated state results in failure to develop tolerance [113-115]. Additionally, introducing external environmental factors can increase ataxic sensitivity even after tolerance develops [116, 117]. For instance, while people may learn to drive while intoxicated after doing so a few times (thereby giving themselves a false sense of security), extrinsic factors such as weather may impede this newfound tolerance.

1.4.2.3 Ethanol-Related Hypothermia

Although not mentioned above in the acute effects of ethanol, one of the effect's ethanol has on the autonomic nervous system is suppression of thermal regulation. Interestingly enough, because rapid/chronic tolerance is readily observed in subjects using different treatment methods [79, 89, 118], this assay has been previously established as a model for assessing tolerance. For example, the development of tolerance differs in relation to age – while young adult rats develop tolerance to ethanol's hypothermic effects, older rats do not [119]. Other studies using selectively inbred lines of mice that differ in their hypothermic response to ethanol have supported the hypothesis that tolerance has a genetic component [120-122]. Additional work has also extended this viewpoint to suggest the possibility that there is a common genetic component to tolerance in this behavior and motor ataxia and hypnosis [123, 124].

1.4.2.4 Ethanol-Related Anxiety

Although alcohol has well known affects in alleviating anxiety, divergent hypotheses exist as to the effects of alcohol during repeated/chronic ethanol exposure. The first possibility is that tolerance to the anxiety-relieving effects can occur, thereby forcing individuals to consume more to achieve the same state of decreased anxiety, ultimately resulting in a negative reinforcing effect [125]. This may be an important reason for increased consumption and may lead to alcoholism. In fact, increased anxiety is comorbid in alcohol dependent patients [126]. There

are some rodent studies utilizing the anxiolytic measures described previously that would agree with this phenomenon. For instance, studies assessing rapid and chronic tolerance have demonstrated that the anxiety-relieving effects of ethanol are ablated in tolerant animals [127-129]. Other studies have also concluded that tolerance develops to the anxiolytic effects when compared to acutely treated animals, but anxiolytic-like effects were still observed in comparison to controls [130]. It is important to point out with respect to anxiolytic assays that repeat testing of animals takes into account a fear-component, and assessments could result in a false-negative assessment of the drug effect [131, 132]. Interestingly, in assessment of social anxiety in people, tolerance was observed using heart rate in which male subjects were asked to interact with a female based on their classification of tolerance with respect to ataxia [133]. Tolerance was observed but only in people classified already as 'high tolerant' on a separate unrelated ataxia measure. Conversely, this was not seen in other related studies [134].

Alternatively, a second suggestion is that tolerance does not develop to the anxiolytic component of ethanol and may therefore reinforce drinking and lead to alcoholism. In people, self-medication with alcohol may also be done as an attempt to reduce tension or alleviate stress [135, 136]. Others have suggested that decreases in anxiety may be perceived as a positive pleasurable effect, thereby facilitating ethanol consumption [137-139]. Ultimately, continual maintenance of anxiolysis during ethanol exposure leads to alcohol dependence and alcoholism. Numerous rodent studies report anxiolytic-like effects of ethanol after repeated ethanol exposure using various treatment conditions, length of ethanol exposure, and using different experimental setups [140-144].

It is possible the discrepancy in the above studies may be due to length of ethanol exposure and dose of ethanol employed. In essence, the anxiolytic-potential of ethanol may wane over time. However, anxiolytic-like effects of ethanol are still observed in a binge-drinking model where high amounts of ethanol are consumed for extended periods [142]. Overall, although both hypotheses differ in how anxiety-related effects attributed to chronic ethanol exposure and alcohol addiction neither negates the potential role of anxiety in alcohol addiction.

1.4.2.5 Ethanol-Related Nociception

Because ethanol possesses analgesic properties, in a subset of people, a potential reinforcing effect of ethanol is its analgesic properties. People who suffer from chronic pain often self-medicate with ethanol [145, 146]. Unfortunately, tolerance to ethanol-induced analgesia can develop thereby leading people to consume more ethanol to achieve the same level of effect. Although not as heavily studied as the aforementioned behaviors, many studies have demonstrated the development of tolerance to ethanol-induced analgesia in rodent models. Thermal and chemical (formalin) nociception have been used to assess tolerance to the analgesic effects of ethanol as well as in inbreed as selectively bred lines.[147-153].

1.4.2.6 Metabolic and Tolerance

Metabolic tolerance occurs where increases in pharmacokinetic elimination of ethanol decrease the overall amount of ethanol and/or presence of ethanol in the body. It should be noted that metabolic tolerance is suggested to play a role in the development of chronic tolerance. However, this typically occurs after substantially long periods of high amounts of ethanol exposure. For instance, ethanol metabolism was modestly increased after 9 days, and a much higher increase was observed after 20-plus days [154]. Changes in ethanol metabolism were also observed in animal models of binge drinking [97]. Other models using high doses of ethanol have replicated these findings [154, 155]. Conversely, other studies indicate metabolic tolerance is not observed when ethanol is given for shorter periods of time and or at lower doses, thereby suggesting that this effect my only be seen under extreme exposure criteria [89, 92, 104, 156]. Thus, metabolic tolerance may not be a factor in the majority of the population who consume moderate amounts of ethanol. Therefore, tolerance related affects observed in the general population may be attributed to altered pharmacodynamic effects of ethanol. By conducting chronic behavioral experiments with moderate doses of ethanol, it is possible that metabolic tolerance may not confound observations.

1.4.3 Ethanol Dependence

As defined above, ethanol dependence is defined by withdrawal-related behaviors. The classical definition of withdrawal was determined by seizure-related activity after cessation of ethanol

exposure [157]. However, this activity falls at one extreme end of the withdrawal-related spectrum. The spectrum of withdrawal-related effects can range from having minimal effects after a night of social drinking to hangovers, irritability, nausea, sluggishness, increased tension and anxiety, hyperalgesia (increased pain sensitivity), to having tremors/convulsions and in worse cases, *delirium tremens* and possibly death [8, 74, 81, 83, 158]. Withdrawal-related cases presented to emergency rooms and hospitals are typically treated with benzodiazepines which have helped decrease ethanol withdrawal-related mortalities [7].

The possibility exists that physical withdrawal-related experiences could precipitate alcoholism [82]. Although some of these behavioral aspects are hard to experimentally delineate, rodent assays specific to ethanol withdrawal hyperexcitability have been developed. Additionally, alterations to previous experimental paradigms such as those related to anxiety and nociception have been used. It should be noted that although alcoholism may be manifested, in part, by *physical dependence*, the behaviors described below in no way constitute or attempt to correlate with *psychological dependence* – or the drug craving/drug seeking behaviors. Nonetheless, negative factors attributed to ethanol withdrawal may be enough of a motivator to trigger relapse. Three readily measured rodent behaviors related to ethanol withdrawal – specifically hyperexcitability, anxiety and hyperalgesia are described below.

1.4.3.1 Alcohol Withdrawal-Related Hyperexcitability

Alcohol withdrawal hyperexcitability is perhaps the most prevalent assay used to assess ethanol withdrawal-related effects in rodents. Originally described by Goldstein and Pal [157], the assay involves continuously administering high amounts of vaporized alcohol and then assessing withdrawal-related seizure-like activity (e.g., clonus seizures) following cessation of treatment. Using this assay, much work has been done in establishing that withdrawal severity has a definite genetic component either through the use of selectively bred lines or differences in mouse strains [159-162]. Additional work using quantitative trait loci studies have suggested specific regions of the genome where factors related to withdrawal hyperexcitability may be located [163-167]. Studies have directly demonstrated the amount of alcohol exposure can increase withdrawal severity [168]. Additionally, the length of exposure can have dramatic effects. For instance, acute withdrawal hyperexcitability may be seen after only one binge session of ethanol, although the severity of responses is typically less than that seen following termination of chronic

exposure [e.g. 169, 170]. Extensive work has also shown that previous exposure can dramatically enhance seizure severity during ethanol withdrawal, thereby causing a 'kindling' effect [83, 171].

1.4.3.2 Alcohol Withdrawal-Related Anxiety

Although ethanol withdrawal-related seizures are severe, these effects usually dissipate within 24 hours following cessation of ethanol exposure. However, other components of ethanol dependence such as anxiety tend to be more long lasting (see below, section 1.4.5). It is more widely accepted that hedonic components of ethanol dependence such as anxiety likely lead to alcoholism or relapse in those abstaining from drinking [172-174].

Behaviorally, in rodent models increased anxiety resulting from ethanol withdrawal can be assessed with several of the above-described assays. For instance, the anxiogenic effects of withdrawal have been observed on the elevated plus maze and light/dark assays [144, 169, 175-180]. Other additional assays have also demonstrated this effect using the mirror test, social interaction and the elevated-zero maze [178, 181-184].

Aside from the withdrawal-related effects, additional work has shown that while no differences were detected with respect to gender, there may be an age related effect, with adolescents experiencing less severe withdrawal-related anxiety compared to adults [185, 186]. There are also significant time and dose related effects as well. For instance, persistent anxiogenic effects have been observed at least four weeks after cessation of ethanol [187, 188]. This result potentially lends support as to why abstainers relapse. Significant anxiogenic effects can also be seen one day after a single ethanol exposure, reminiscent of an acute hangover effect [189]. More recently, experiments have demonstrated that single exposure to moderate doses of ethanol (that don't typically result in acute withdrawal hyperexcitability) can also potentiate withdrawal-related anxiogenesis in periods less than 24 hours after exposure, and subsequent exposures can increase the magnitude and duration of increased anxiety [190]. A more extensive review of withdrawal-related anxiety has been described elsewhere [191].

1.4.3.3 Alcohol Withdrawal-Related Hyperalgesia

Although alcohol consumption can have a concomitant analgesic effect, tolerance can develop and events opposite to the acute effects, known as hyperalgesia – increased pain sensitivity – can

occur during withdrawal [e.g., 56, 192]. Even worse, disorders such as alcoholic neuropathy may develop [193-195]. Although originally thought to be due to thiamin deficiency and/or neurotoxic effects of ethanol on peripheral nerves [196, 197], more recent work suggests the possible involvement of more centrally-mediated molecular mechanisms [198, 199]. Studies in animal models have been able to replicate the ethanol withdrawal-related hyperalgesic (increased pain sensitivity) effects using thermal, mechanical and chemical nociception measures [56, 149, 192, 200, 201]. Moreover, additional work has shown that hyperalgesic effects may persist long after ethanol has been cleared from the system [199, 202]. Surprisingly, neonatal ethanol exposure can result in hyperalgesic states postnatally and may extend well into adulthood [203, 204].

1.4.4 Relationship of Ethanol Dependence and Addiction: Allostasis

The majority of people do not become addicted after their first bout of ethanol consumption or exposure. Perhaps the most relevant model for alcoholism to date is the allostatic hypothesis proposed by Koob and Moal [125] in which the hedonic factors (mood, anxiety, stress, etc.) of alcohol push individuals from social use to abuse, and eventually to dependent states [reviewed in: 21, 205]. In normal individuals, mood will tend to fluctuate about a set homeostatic point, and ethanol usage can increase the fluctuations about this set point (refer to figure 2). Eventually, when alcohol is taken in larger quantities than anticipated, withdrawal occurs at which point a potentially new, altered homeostatic set point (allostasis) occurs. Individuals may eventually rebound back to their set point after a finite period of time, but those who seek ethanol to alleviate these negative affects may start a negative, downward spiral of seeking, excessive consumption and withdrawal eventually leading to alcoholism.

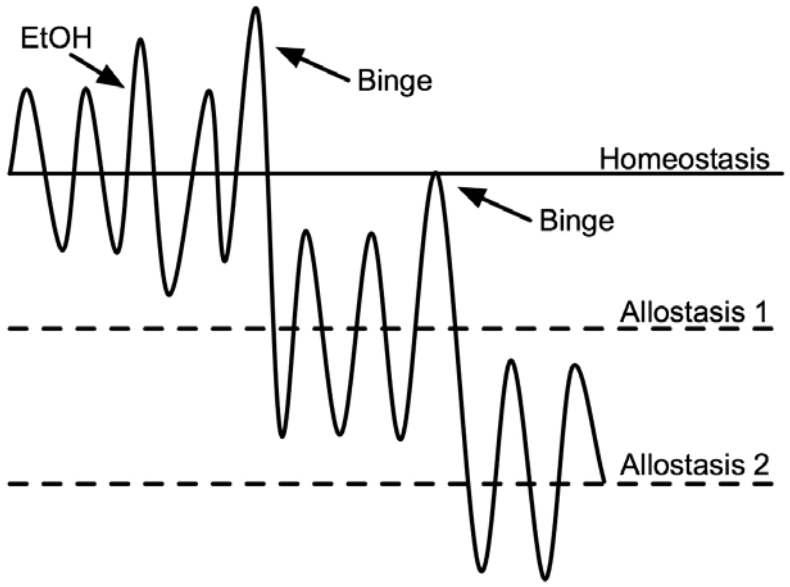

Figure 1.2. Allostasis.
Allostasis occurs when normal homeostatic mood is altered either from a psychopathologic state or pharmacologic agent (i.e., alcohol). Peaks represent positive affects of ethanol such as increased overall well-being (decreased anxiety, relaxation) whereas troughs represent withdrawal like states of ethanol such as increased anxiety, tension and irritability. Adapted from Koob [205].

One such hedonic factor, anxiety, may be used as an example to illustrate this hypothesis. While ethanol consumption may decrease anxiety and increase overall well-being (positive affect), larger than anticipated consumption may lead to withdrawal-related anxiogenic states (negative affect) which are more commonly manifested during hangover periods and is observed more often than not in the absence of severe withdrawal states (convulsions). Continual, repeated ethanol consumption and withdrawal precipitates this yo-yo pattern between the positive and negative affects. Although anxiolysis may still be evident with earlier allostatic set points, eventually, with new allostatic set points the previous mood alleviating effects of ethanol are absent to which ethanol is necessary to even operate normally, thereby precipitating the development of alcoholism. Allostasis may explain why the aforementioned discrepancy in tolerance to ethanol's anxiolytic effects may indeed be dependent on the amount of alcohol administered and the length of ethanol exposure. Repeated ethanol use may continue to elicit an anxiolytic effect. Eventually a new set point is established, after which ethanol no longer decreases anxiety, but rather is needed to maintain normal anxiety-like behavior.

1.4.5 Relationship Between Sensitivity, Tolerance, and Dependence

Much work has been done to evaluate the relationship between initial sensitivity and tolerance and dependence to determine if there is any causal link that may predicate alcoholism. However, despite numerous attempts, any linkage with these ethanol-related effects has been at best ambiguous. For instance, one of the most impressive studies involving a 400-plus cohort population, of which half included 'sons-of-alcoholics', determined that decreased initial sensitivity to alcohol's effects (subjective feelings, physiologic measures, motor performance) was a prognostic indicator for the development of alcoholism later in life [206]. Interestingly, although anxiety and depression were co-morbid with alcoholism, anxiety and depression were more likely to be substance-induced rather than a causal role [207, 208]. Other factors such as history of 'blackouts' (amnesia, unconsciousness) from alcohol abuse do not have any causal link to addiction [209]. Overall, these results suggest that initial sensitivity to drug-related effects is more important than other extrinsic behaviors and factors.

In animal models, much work has also been done to determine a relationship between ethanol sensitivity and tolerance. Studies have shown that subjects that are more sensitive to the acute effects of ethanol on specific behaviors develop rapid/chronic tolerance faster compared to those that are less sensitive [95, 210-213]. In support of this relationship between sensitivity and tolerance, Bowers et al. [214] reported that mice with decreased initial sensitivity for sedative-hypnotic and hypothermic effects failed to develop tolerance after chronic ethanol exposure. Others have reported similar findings and posited that experience of the consequences from drug exposure is necessary for tolerance to develop [215, 216]. Work by Khanna et al. [217] has also suggested that there is a direct relationship between sensitivity and tolerance, although it remained to be determined if there was a distinct genetic linkage. Conversely, studies done in selectively bred lines of rats and mice have reported either decreased acquisition of tolerance or even sensitization instead of tolerance upon subsequent ethanol exposures [218-220]. Another study has suggested that no correlation exists with respect to initial sensitivity and chronic tolerance [103].

Other studies have suggested a possible linkage between sensitivity and withdrawal. Crabbe et al. [221] studied inbred strains of mice and suggested the possibility that severity of withdrawal symptoms is negatively correlated with initial sensitivity and magnitude of tolerance;

but other studies using selectively bred mice have suggested the opposite in that sensitivity, tolerance and withdrawal are separate entities [222].

Apart from correlations with sensitivity, other studies have assessed the relationship between various forms of tolerance. Studies have indicated that acute and rapid tolerance are predictive of chronic tolerance and even exposure to alcohol during ontogeny can have repercussions in adulthood [96, 112, 223]. However, others have suggested otherwise. For instance, studies using pharmacologic agents that attenuated the development of chronic tolerance had no effect on acute tolerance [224]. Recent work using *Drosophila* has implicated rapid and chronic tolerance are mechanistically distinct [225]. In fact early work by Ritzmann and Tabakoff [226] had suggested a dissociation between tolerance and dependence.

Overall, while studies suggest that sensitivity and tolerance are directly related, further studies are needed to further validate this relationship. Likewise, further work is necessary to determine the relationship between sensitivity and withdrawal as well as the various forms of tolerance. Nonetheless, what is known is that tolerance and dependence can be seen on multiple measures and can develop at different rates, depending on the measures employed as well as treatment and dosages used [79, 227].

1.4.6 Summary of Ethanol Sensitivity, Tolerance and Dependence

Despite the complexity of the interplay between sensitivity, tolerance and withdrawal, assessment of the interplay between these and also with respect to specific behaviors most likely suggests that multiple targets are associated with ethanol's pharmacologic effects. More importantly, the above evidence hints that no one gene product is responsible for all of ethanol's actions; rather, it is most likely the result of a complex interplay of multiple targets. It is possible, given the evidence presented above, that different neuroanatomic regions mediate different behavioral responses. Nonetheless, it remains to be determined which genes or molecular mechanisms can exert more influence than others with respect to any or all of ethanol's effects. What is well known is that ethanol-related effects most likely occur through its direct or indirect action on targets in the central nervous system. Therefore, to more accurately dissect the role of specific molecular targets, numerous different behavioral markers should be

assessed with respect to sensitivity, tolerance and physical dependence in addition to their cellular and neuroanatomic effects.

1.5 NEUROANATOMIC & MOLECULAR TARGETS FOR ETHANOL

1.5.1 Neuroanatomic Regions Via Immediate Early Genes

Although the CNS is a complex association of multiple gene products in various neuroanatomic regions, it is quite possible that specific regions of the brain control specific behaviors. By determining which regions play a role in specific ethanol-related behaviors, we can better determine specific genes and/or neurocircuitry in ethanol behavior. While numerous neuroanatomic regions may be involved in mediating ethanol's behavioral effects, trying to pinpoint which regions – or more specifically, which cell types are involved is a daunting task. Nonetheless, for more than a decade studies have investigated the expression of immediate early genes (IEGs) to help identify which regions may be of high importance. IEGs are advantageous because they typically have low basal expression. Exposure to stimuli (i.e., pharmacologic agent, stress, etc.) subsequently activates IEGs in neurons [228]. This IEG induction in various brain structures has yielded valuable insight into regions that may play a role with respect to ethanol action. The majority of IEGs employed are inducible transcription factors (ITFs; such as c-fos, c-jun, fos-B, and zif-268) that propagate the stimuli into a cellular response. It's tantalizing to speculate that the resultant products may aide in neuroadaptations (i.e., acute tolerance) to the stimulus.

While numerous different ITFs exist that may yield valuable insight into ethanol action, the most commonly employed is c-fos, a component of the AP-1 dimer transcription factor. Currently, it is suggested that c-fos is activated via a MEK1/2-Erk1/2-Stat3 pathway [229], although compartmentalization of the scaffolding protein RACK1 may play a role [230]. Much work utilizing different treatment paradigms has shown that ethanol results in bidirectional changes in c-fos [231-234]. Transient increases have been observed in various regions such as the Edinger-Westphal nucleus and paraventricular nucleus of the hypothalamus while resulting in concomitant decreases in other regions such as hippocampal areas [231, 235-237]. Many

studies have also correlated brain regions with specific behaviors. These include the hippocampus in memory and anxiety, the Edinger Westphal in hypothermia, ethanol consumption as well as decreased stress-induced expression of c-fos [228, 236, 238-240]. Other studies have used this technology to determine which cell types may be involved in Fos-IR (e.g., urocortin neurons, γ-amino-butyric acid neurons [241-243]) as well as potential genetic differences [237, 240, 244, 245]. Still, other studies have implicated changes in various regions during tolerance as well as acute and chronic withdrawal [231, 233, 235, 246-253]. While tolerance to c-fos induction may potentially be long-lasting, even from short exposure periods [246], the time-course for withdrawal-related effects tend to return to normal after about a day [234]. Overall, the use of immediate early genes to identify neuroanatomic regions in ethanol action may aide in determining which neuronal targets are involved in ethanol-induced behavioral effects.

1.5.2 Putative Molecular Targets For Ethanol Action

Ethanol is thought to elicit its behavioral effects by influencing multiple molecular targets in the brain such as neurotransmitter systems [e.g., voltage-gated calcium and potassium channels, N-methyl-D-aspartate (NMDA), gamma-amino-butyric-acid (GABA), glycine, serotonin (5-HT), adenosine, dopamine, acetylcholine second messenger systems (PKC, cAMP/PKA, nNOS), and neurosteroids to name a few [as reviewed in: 254, 255-258]. By understanding the role of specific gene products in the molecular mechanism of ethanol action, we can begin to tease apart which gene products are responsible for specific ethanol related effects.

While numerous molecular targets have been assessed, much evidence suggests that the primary action of ethanol is via ion channels [259], such as increasing or decreasing ion flux at specific receptors. The most likely ion channel targets are those that mediate rapid excitatory and inhibitory actions in the nervous system. For instance, ethanol has been shown to modulate the main receptors for excitation and inhibition –NMDA- and GABA type A ($GABA_A$)-receptors [260]. It is well known that acute ethanol exposure can alter the fine balance between the excitatory and inhibitory neurotransmitter systems (refer to figure 1.3A and 1.3B, reviewed in [261]) by inhibiting NMDA function while enhancing function of $GABA_A$-receptors. During chronic ethanol exposure, these neurotransmitter systems adapt by enhancing and blunting,

respectfully, NMDA- and $GABA_A$-receptor function (figure 3C). When ethanol is removed, there is an overall net increased excitatory function in the CNS - directly correlating with the enhanced hyperexcitability observed during ethanol withdrawal (figure 3D).

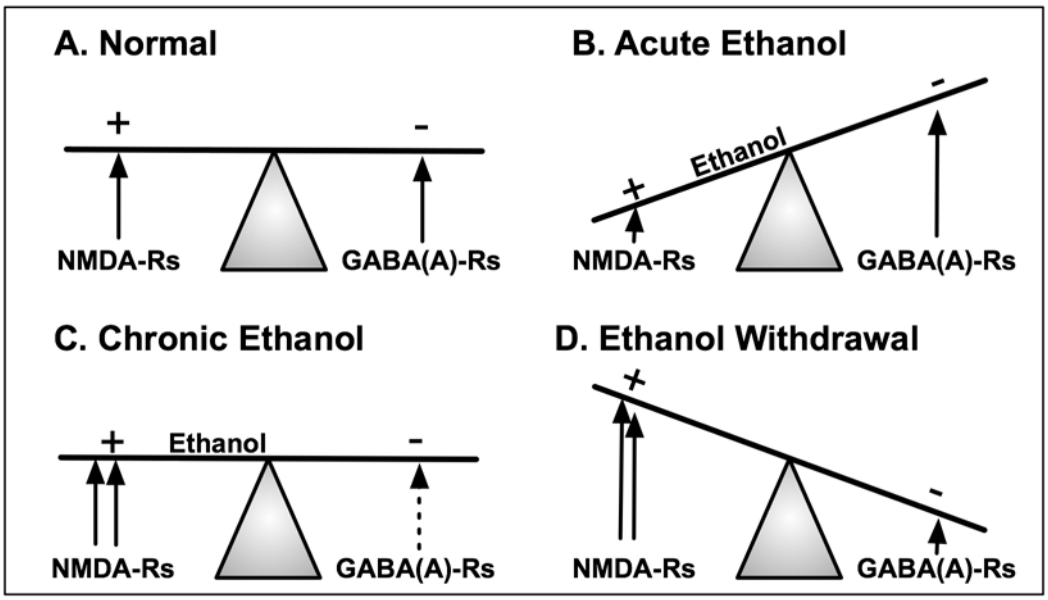

Figure 1.3. Ethanol Effects on Excitatory and Inhibitory Function.
(A) A delicate balance exists between excitatory and inhibitory neurotransmitter systems in the CNS. (B) Addition of ethanol results in a net inhibitory effect by decreasing excitation and enhancing inhibition. (C) Adaptation of neurotransmitter systems (e.g., enhancing NMDA-R function and decreasing $GABA_A$-R function) restores the delicate balance. (D) Removal of ethanol from the system results in a net excitatory effect. Adapted from deWitte [261].

$GABA_A$-receptors ($GABA_A$-Rs) may be considered a primary target for ethanol action for a number of reasons. These include: 1) ethanol is pharmacologically considered to be a sedative/hypnotic drug and is thought to depress neuronal function; 2) $GABA_A$-receptors mediate the majority of rapid synaptic inhibition, thereby further depressing the central nervous system; 3) much evidence suggests ethanol directly modulates $GABA_A$-R function by potentiating the inhibitory effects of GABA at $GABA_A$-Rs; 4) ethanol behavioral responses are altered by $GABA_A$-R pharmacologic agents; 5) chronic ethanol regulates $GABA_A$-R function as well as expression of specific subunits, and; 6) genetic analysis has implicated $GABA_A$-Rs in tolerance and dependence, to name a few. These are discussed in detail below.

1.6 ETHANOL AND $GABA_A$-RECEPTORS

1.6.1 Acute Ethanol and $GABA_A$-Receptors

Neurochemical and electrophysiologic studies demonstrate that $GABA_A$-Rs are sensitive to physiologic concentrations of ethanol. Ethanol potentiates muscimol (a $GABA_A$-R agonist) stimulated chloride uptake [262-264] that is blocked by $GABA_A$-R antagonists [262, 265] and inverse agonists [266]. Ethanol also increases chloride currents in neurons which is reversed by the $GABA_A$-R inverse agonist Ro15-4513 [267]. Ethanol potentiation has also been shown in slice recordings [268], with differences seen in various brain regions [269, 270], cell types in the same region [270] as well as in different areas of the same neuron [271]. This variability suggests that specific $GABA_A$-R subtypes on specific neuronal circuits may mediate ethanol action. What is incredible is that specific $GABA_A$-R subtypes have been potentiated by ethanol concentrations as low as 1-3mM, or about half a beer [272, 273].

Ethanol-induced behavioral effects are remarkably similar to other drugs that are known to function through $GABA_A$-Rs [e.g., benzodiazepines (BZs), barbiturates and certain anesthetics] [274-279]. Even more important, $GABA_A$-R modulators have identified these receptors as key molecular targets for ethanol-induced behavioral effects. $GABA_A$-R antagonists and inverse agonists reduce ethanol intoxication [280-282] and the motivational aspects of ethanol [283]. Also, the $GABA_A$-R modulator Ro15-4513, not only reduces ethanol enhancement in vitro [267], but also reduces ethanol-induced behavioral effects [280, 284]. $GABA_A$-R modulators have also implicated $GABA_A$-Rs as key targets of ethanol's action in mice selectively bred for ethanol-related behaviors [285].

1.6.2 $GABA_A$-Receptors and Ethanol Tolerance and Dependence

Numerous studies have also implicated $GABA_A$-Rs in having a major role in tolerance and dependence. For instance, GABAergic agonists reduce withdrawal symptoms associated with ethanol [177, 286, 287] and are a first line of treatment (e.g., diazepam, lorazepam) for patients [6, 7]. In vitro studies have demonstrated that $GABA_A$-Rs are desensitized after chronic ethanol treatment [263, 264], while the efficacy of inverse-agonists are enhanced [288]. In vivo, chronic

exposure alters expression of some $GABA_A$-R subtypes [289, 290]. Additionally, quantitative trait locus (QTL) studies which map regions of chromosomes involved in certain phenotypes suggest specific $GABA_A$-R subtypes (see secton 1.7) are involved in tolerance and dependence in animal and clinical studies [291-294]. Behaviors associated with ethanol dependence have also been linked to $GABA_A$-Rs by facilitation with diazepam [295]. Diazepam also accelerates the development of tolerance to alcohol [296]. Studies done with neurosteroids (potent $GABA_A$-R modulators) have also suggested that $GABA_A$-Rs are involved in tolerance, and animals treated with certain neurosteroids can develop cross-tolerance to ethanol [297, 298]. Interestingly, specific $GABA_A$-R subtypes are necessary for the development of tolerance to the sedative effects of diazepam [299]. Even more importantly, changes in $GABA_A$-R subtypes have been noted in human alcoholics [292, 300-307]. Thus, evidence from a variety of approaches suggests that tolerance and dependence are mediated in part by $GABA_A$-Rs. It is possible that different aspects of tolerance and/or dependence may be mediated by different $GABA_A$-R subtypes. More so, what $GABA_A$-R subunits mediate tolerance and dependence to ethanol?

1.7 $GABA_A$-RECEPTOR MOLECULAR PHARMACOLOGY

While it was generally recognized early on that GABA activated a chloride channel, the exact structure and function of these channels remained unknown until work identified different subunits and isoforms of the $GABA_A$ to their heterogeneity [308-310]. $GABA_A$-Rs are now known to be pentameric chloride channels composed of multiple subunits (α1-6, β1-3, γ1-3, δ, ε, π, ρ1-2 and θ [311]) each of which is made of four transmembrane domains. Incorporation of all possible different subunit combinations would result in an astronomical variety of different $GABA_A$-R subtypes. However, the majority of $GABA_A$-Rs are comprised of 2α, 2β and a γ or δ subunit (Figure 4) [312], of which the most common is α1β2/3γ2 [313].

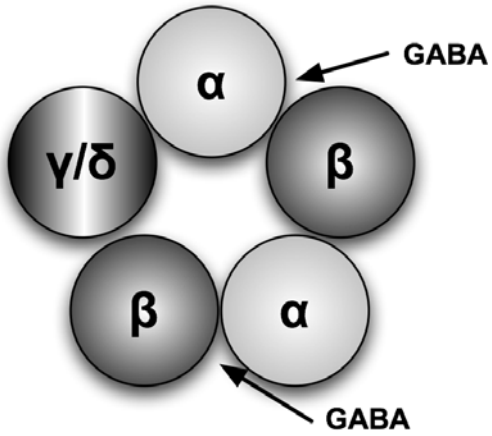

Figure 1.4 Pentameric $GABA_A$-R Composition
$GABA_A$-Rs are typically composed of 2 alternating α and β subunits in conjunction with either a γ or δ subunit. Together, these subunits form a 'donut-like' structure around a middle pore through which chloride ions may pass.

Interestingly, the incorporation of different subunits into the pentameric complex can result in different pharmacologic responses. For instance the sleep aide zolpidem (Ambien®) is preferentially selective for α1-containing receptors, [314], whereas those containing α4 or α6 are insensitive to typical benzodiazepines [276], while those containing the delta subunit have the highest affinity for the inverse-agonist Ro15-4513 [315]. Besides differences in ligand sensitivity, subunits can also dictate different channel kinetics [e.g., 314, 316], neuronal localization (e.g., synaptic versus extrasynaptic) [317], and also temporal expression [318]. Basically, different $GABA_A$-R subtypes contribute differently to GABAergic inhibition in the central nervous system. Unique roles of individual $GABA_A$-R subunits have been defined for BZs and intravenous anesthetics [319-323]. From these studies, it is clear that different subunits mediate different drug-induced behavioral responses. Therefore, it is possible that different subunits give rise to specificity in ethanol's mechanism of action. However, the question remains, if ethanol exerts its effects through $GABA_A$-Rs, then which subunits underlie specific ethanol-induced behavioral responses? Unfortunately, while there are pharmacologic agents that are 'selective' for different $GABA_A$-R subtypes, they are not specific, making a pharmacologic dissection of individual subunits in alcohol action markedly difficult. Alternatively, the use of genetically modified rodent models is a tool that can be used to better address this interaction.

1.8 $GABA_A$-RECEPTOR TRANSGENICS AND GENE TARGETED MICE IN ETHANOL ACTION

1.8.1 Transgenics and Global Knockouts

Transgenic and gene targeted mice represent an excellent resource to study putative molecular targets of ethanol-related behaviors. Transgenic mouse models are those animals with an extra copy of a gene or a novel gene that is randomly inserted into the genome resulting in increased gene expression [Reviewed in: 324]. Conversely, gene targeted mouse models contain a modified endogenous gene thereby resulting in a mutant gene product or ablation of the gene product. Global knockouts are typically the most common of the latter group. While transgenic and knockout models have been used to address the role of $GABA_A$-R subtypes in ethanol action (refer to Table 1), strikingly, most have only addressed the acute effects of ethanol and not adequately assessed the role of tolerance and/or withdrawal with the exception of acute functional tolerance to motor coordination and withdrawal-related hyperexcitability.

Unfortunately, knockouts are not an ideal model for assessing ethanol-related behaviors. By removing a specific subunit, the brain often tries to restore the altered neurotransmitter system to homeostasis by altering levels of other isoforms thereby masking potential differences [316, 336-340]. Also, these compensatory changes may result in subunit substitution and assembly of functional receptors as well as reorganization of the GABAergic circuitry [341, 342]. Therefore, it is unclear whether the observed phenotype is due to absence of the subunit in question or changes in other subunits. It has even been demonstrated that non-$GABA_A$-R protein levels (e.g., K^+ channels) are altered by global knockout of a $GABA_A$-R subunit [343]. Even if compensation does not occur, because of so many different isoforms within each subtype, this redundancy may mask the true role of specific subunits. Although useful, global knockouts are not ideal for studying ethanol-induced behavioral effects.

Table 1.1.1. $GABA_A$-R Subunits in Ethanol-Related Behavioral Responses.
Numerous studies have assessed the role of different $GABA_A$-R subunits in ethanol action using transgenics and knockout mouse models. =, no change with respect to genotype; ↑, increased in comparison to controls; ↓, decreased in comparison to controls; Tg, Transgenic; KO, knockout. For a more detailed interpretation, refer to [325, 326].

Subunit	Genetic Alteration	Acute Response	Tolerance	Withdrawal
α1	KO	=Anxiolysis [327] = Hypnosis [327] = Ataxia [327] = Anticonvulsant [327] ↑ Motor Activity [327] ↓ Hypnosis, Males [328]	= AFT [327]	= Chronic HIC [329]
α2	KO	↓ Hypnosis [326] = Anxiolysis [326]		=↓ Acute HIC [326]
α6	KO	= Hypnosis [330]	= AFT [331] =Tolerance to Hypnosis [331]	= Chronic HIC [331]
β2	KO	↓ Hypnosis, Males [328]		=↑ Chronic HIC [329] ↑ Acute HIC in Males [325]
β3	KO	= Hypnosis [332]		
γ2s	Tg	= Hypnosis [333]	↓ AFT [333]	= Acute HIC [333]
γ2L	Tg	= Hypnosis [333]	↓ AFT [333]	= Acute HIC [333]
γ2L	KO	= Hypnosis [334] = Anxiolysis [334] = Motor Activity [334]	= AFT [334]	=Chronic HIC [334]
δ	KO	↓Anticonvulsant [335] = Hypnosis [335] =Hypothermia [335] = Anxiolysis [335]	= AFT [335] = Tolerance to Hypnosis (background strain effects) [335]	↓ Chronic HIC [335]

1.8.2 Gene Knockins

An alternative strategy not limited by these caveats is the gene knockin approach. Knockin animals possess a mutation in an endogenous gene. The mutation is typically a point mutation(s) involved in drug responses, phosphorylation, or a residue for protein-protein interactions to name a few. Because of being under the control of the endogenous promoter, the mutant gene should have normal expression as well as levels of distribution. More importantly, if designed properly, the mutant protein will have a normal response to endogenous ligands.

Although knockin technology may be used for any gene, proof of principle was demonstrated for $GABA_A$-R involvement in BZ action. A histidine at position 101 conferred sensitivity of α subunits to BZs [344, 345]. In vitro, receptors could be "switched" from sensitive to insensitive by mutating histidine 101 to arginine [344-346] without affecting GABA response. By using this molecular switch in gene knockin mice, the role of individual $GABA_A$-R subunits in BZ-induced behaviors was addressed. α1 conferred the sedative and amnestic effects [276, 347], whereas α2 mediated the anxiolytic effects [348], and α2, α3 and α5 were involved in the myorelaxant properties [349, 350]. The knockin approach has also defined the role of $GABA_A$-R β subunits in the mechanism of action of etomidate and propofol [322, 323].

A molecular switch has been identified that is sufficient to eliminate $GABA_A$-R enhancement by ethanol and some inhaled anesthetics [351]. Specifically, mutating serine(S)270 in the α1 subunit abolished enhancement of $GABA_A$-R function by ethanol and isoflurane [351, 352]. Mutating S_{270} to histidine (H) appeared to be a good molecular switch to assess $GABA_A$-R involvement in ethanol-related behaviors. However, α1 S_{270}H knockin mice had abnormal behaviors and died prematurely [353]. Upon further analysis it was found that this mutation resulted in hypersensitivity of the receptor to its natural ligand, GABA by a left shift in the GABA concentration-response-curve [353, 354]. Thus, the S_{270}H mutation by itself was not a perfect switch for investigating ethanol action. However, more recent work has shown that incorporation of a second mutation [leucine$(L)_{277}$] in the 2-3 transmembrane linker domain restored GABA sensitivity to near normal affinity [355]. Therefore, by using mouse models harboring the S_{270}H and L_{277}A mutations, we may gain a more accurate representation of α1-containing $GABA_A$-Rs in ethanol action. Indeed, such a mouse was created by the Homanics'

lab for use as a model to investigate α1 $GABA_A$-Rs in alcohol action. A targeting construct harboring the above mutations (see Figure 1.5) was created and gene targeting technology in embryonic stem cells was used to create gene targeted knockin mice [355]. These mice were overtly normal and brain $GABA_A$-Rs had a relatively normal response to GABA.

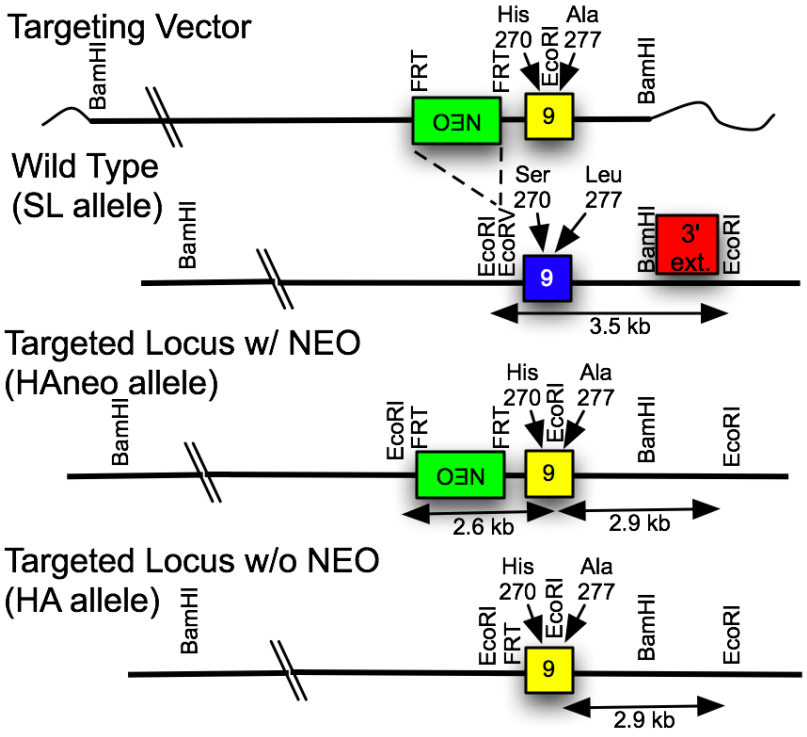

Figure 1.5. Gene targeting strategy used in mouse embryonic stem cells.
Exon 9 corresponds to nucleotides 1308 to 1510 of published mouse α1 cDNA [356] and is color coded to indicate differences in wildtype (blue) and knockin (yellow). A neomycin cassette was use as a positive selection marker. 3' Ext is the 3' external probe used for genotyping and refers to a portion of DNA that was not used in the original targeting construct (hence external). Note the incorporation of the silent mutation results in a smaller restriction fragment (3.5kb to 2.9kb) thereby allowing for genotyping via Southern blot technique. Published in Borghese et al. [355].

1.9 IMPORTANCE OF α1-CONTAINING $GABA_A$-RS AND ETHANOL ACTION

The $GABA_A$-R α1 subunit is worth focusing on for several reasons. For starters, α1 is the most abundant α subunit in adult brain, present in approximately 50% of all $GABA_A$-Rs [357, 358].

α1 mediates BZ-induced sedation [320] and memory impairment [276] and gives merit to α1 potentially mediating similar behavioral responses to ethanol. Studies with the α1-selective agonist zolpidem have suggested a prominent role of this subunit in ethanol sensitivity. Criswell et al. [359] demonstrated that zolpidem sensitivity of $GABA_A$-Rs also predicted ethanol sensitivity of $GABA_A$-Rs. Zolpidem studies also identified specific brain regions that were differentially sensitive to the effects of ethanol [360]. These same regions may contribute to seizures associated with alcohol withdrawal [summarized in 361]. Even more importantly, QTL studies have identified chromasome 11 which contains α1 in ethanol tolerance [123] and withdrawal [291] and have even narrowed in on a 5 centimorgan region containing α1 for withdrawal. Pharmacologic studies also implicate α1 in drug seeking behavior [362]. Other studies show chronic ethanol decreases α1 levels [289, 363] via receptor internalization [364]. It is postulated that these changes in $GABA_A$-R plasticity may result in tolerance and withdrawal [20]. Overall, studies in tissue obtained from alcoholics suggests α1 may be involved in drinking behavior, alcoholism, and alterations in protein levels [292, 302, 306]. Thus, α1 potentially mediates aspects of initial sensitivity, tolerance and dependence. While many biochemical, pharmacological, and behavioral data implicate α1 $GABA_A$-Rs in ethanol action, they have not definitively addressed the role of α1 in ethanol-induced effects. Utilization of the previously created α1 ethanol-insensitive knockin mice will help address the involvement of α1 in ethanol-induced effects.

THE DISSERTATION

While the physiologic actions of ethanol are well known, the role of individual molecular targets with respect to these behaviors remains elusive. Ion channels are extensively implicated targets for ethanol – especially $GABA_A$-receptors, which mediate the majority of rapid inhibition in the brain. However, the role of individual $GABA_A$-R subunits to ethanol action is unknown. Previous attempts to study the role of individual subunits via gene-knockout mouse models were confounded by compensatory mechanisms. Additionally, many of these studies did not behaviorally dissect the role of individual subunits in ethanol action in detail during multiple ethanol-related stages – specifically tolerance and dependence, of which $GABA_A$ –Rs are highly implicated. Thus, the goals of this thesis are two-fold: 1) Molecularly characterize and assess the role of a novel α1-$GABA_A$-R knockin mouse line harboring mutations that render α1-containing $GABA_A$-Rs insensitive to ethanol, and 2; determine the role of this subunit with respect to ethanol-induced behavioral responses, including sensitivity, tolerance and dependence. *I hypothesize that α1-containing receptors mediate ethanol-induced behavioral responses – specifically acute responses, tolerance, and withdrawal.*

In chapter two, the α1 knockin mice were molecularly characterized as well as some acute ethanol-induced behavioral responses. In chapter three, c-fos immunohistochemistry was used to examine ethanol-related responses in specific brain regions. Specifically, c-fos expression was characterized in wildtype and knockin mice after acute ethanol exposure, after chronic ethanol exposure, and during ethanol withdrawal. Finally, in chapter four, ethanol-induced responses were characterized in controls and α1 knockin mice with respect to tolerance and physical dependence. From these results, the role of α1-$GABA_A$-Rs with respect to ethanol action is discussed as well as their contribution to identifying a putative relationship between sensitivity, tolerance and dependence.

2.0 CHAPTER TWO: CHARARACTERIZATION OF α1 $GABA_A$-RECEPTOR GENE KNOCKIN MOUSE MODEL AND ASSESSMENT OF ACUTE ALCOHOL-INDUCED BEHAVIORS

2.1 INTRODUCTION

Ethanol has a prominent role in society and is one of the most frequently used and abused drugs. Despite the prevalence of alcohol use, the molecular mechanisms underlying its behavioral effects are unclear. Ethanol intoxication elicits a diverse array of behavioral effects, which are likely due to actions of ethanol on multiple brain proteins [259]. However, much evidence suggests that ethanol enhances the function of $GABA_A$-Rs [258]. However, a pharmacologic dissection of specific $GABA_A$-R subtypes in ethanol action is limited due to the various subunits and limited subunit specific drugs.

Gene knockin mouse models have proven to be an invaluable tool with which to genetically dissect the involvement of specific genes with respect to drug action and are more advantageous than gene knockout models. Previous work has identified a mutation ($S_{270}H$) in the second transmembrane domain that abolished ethanol potentiation of GABA at the $GABA_A$-R (described above). However, because this mutation resulted in enhanced sensitivity for its natural ligand [i.e., a left shift in the GABA concentration-response curve (CRC)], a second mutation was needed to restore near-normal GABA-sensitivity. Mutation of L_{277} to A resulted in a shift in the GABA CRC in the opposite direction. Recent work has shown that incorporation of both mutations in α1-containing $GABA_A$-Rs expressed in heterologous systems (*Xenopus* oocytes and HEK293 cells) resulted in receptors with near-normal GABA CRC while maintaining insensitivity to ethanol's potentiating effects [355]. Responses were also significantly blunted for the longer chain alcohol butanol and the general anesthetic isoflurane [355]. Conversely, responses to the $GABA_AR$ agonist flunitrazepam did not differ [355].

However, it should be noted that rapid application of GABA in HEK293 cells resulted in a 2-fold increase in the GABA EC50 in mutant receptors [355]. Unexpectedly, modulation of GABA responses by Zinc (Zn^{2+}) was slightly decreased in mutant receptors as was other $GABA_A$-R agonists such as pentobarbital and the intravenous anesthetic etomidate [355]. However, responses at higher concentrations of etomidate (which can directly activate $GABA_A$-Rs in the absence of GABA) did not differ [355]. The differences in responses likely indicate that the mutation on the $\alpha 1$ subunit results in allosteric effects and/or conformational changes that impede the actions of these agents.

Gene knockin mice harboring these mutations were created [355]. In this chapter, mutant mice were assessed to confirm that the desired mutations were present in vivo. Additionally, because gene modification in mouse models may result in compensatory alterations, the GABAergic system was molecularly characterized in mutant mice. Lastly, because the knockin mutations result in ethanol insensitive $\alpha 1$-$GABA_A$-Rs, ethanol-induced responses on specific behavioral measures as well as to other pharmacologic agents were also assessed.

2.2 MATERIALS AND METHODS

2.2.1 Mouse Production

Wildtype (homozygous for S_{270} and L_{277}; genotype referred to as SL/SL) and knockin (homozygous for H_{277} and A_{277}; genotype referred to as HA/HA) mice used for these experiments as well as subsequent chapters were produced from heterozygous (SL/HA) breeding pairs at the University of Pittsburgh Medical Center (Pittsburgh, PA). All mice were genotyped by Southern blot analysis of EcoRI digested DNA and hybridization with a 3' external probe (refer to figure 1.5 and 1.6) as described previously [353]. All mice were of a mixed C57BL/6J $\times$ Strain 129SvJ background of the $F_3 - F_6$ generations. All animals were maintained under specific pathogen free conditions in a photoperiod-controlled environment (lights on at 7:00 AM and off at 7:00 PM) with *ad libitum* access to standard rodent chow and water. All experiments were approved by the Institutional Animal Care and Use Committee and were conducted in

accordance with National Institutes of Health guidelines on the use of animals in research. All mice were experimentally naïve and minimally handled (cage changes) prior to experiments.

2.2.2 Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Mice were euthanized by an overdose of isoflurane and brains were rapidly dissected. Whole-brain RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). α1 mRNA was converted to cDNA and amplified using the Superscript™ One-Step RT-PCR with Platinum Taq Kit™ (Invitrogen) with primers corresponding to the coding sequence region of the α1 cDNA (see Table 2.1). RT-PCR conditions were set as follows: 1) 50°C for 30 min, 2) 94° 2 min, 3) 94° for 1 min, 4) 60° for 30 sec, 5) 70° for 1 min, 6) 39 times back to step 3, 7) 72° for 5 min, 8) 4° hold. Gel purified RT-PCR products from control and knockin brains were sequenced at a core facility using the same primers. Results were compared.

Table 2.1. α1 $GABA_A$-R RT-PCR Primers For Sequencing.
Primer sets used to amplify and sequence α1 $GABA_A$-R cDNA isolated from wildtype and knockin mice. Sequence coordinates correspond to Gabra1 cDNA sequence obtained from Pubmed complete cds: core nucleotide accession number BC132331 (gi: 124375661).

Primer Set	Forward Primer Sequence Location	Reverse Primer Sequence Location
1	GCAAGCCCGTGATGAAGA 446-463	TTGTGGAAAAATGTATCTGGAGTCC 818-842
2	TCCGTCAAAGTTGGAAGGATGA 731-752	CCACGCATACCCTCTCTTGGT 1465-1485
3	CTCCCAAGTCTCCTTCTGGCTC 1254-1275	GGTTCTGGTGGTTTTGTCTCAGG 1657-1679
4	TGGGATGGCAAAAGCGTG 1483-1500	GGACAGAGGCAGTAAAGCAGATAG 1897-1920

2.2.3 Immunohistochemistry

Wildtype and knockin mice were euthanized by an overdose of isoflurane and transcardially perfused with isotonic saline followed by 4% paraformaldehyde. Whole brains were rapidly dissected out and placed in paraformaldehyde fixative solution overnight. The next day, the

solutions were switched to a 30% sucrose solution and brains were allowed to equilibrate from 1-5 days. For α1 $GABA_A$-R immunohistochemistry, brains were cut in 30 μm sagital sections using a cryostat and sections stored at -20°C in a cryoprotectant solution (phosphate buffer with ethylene glycol, sucrose, and polyvinylpyrolidone). Free-floating sections were washed three times with PBS and endogenous peroxidase activity quenched by incubating tissue with 0.9% hydrogen peroxide for 10min. Sections were subsequently washed three times and blocked with 4% NGS in PBSX (PBS with TritonX-100, 0.025%) for one hour followed by primary antibody for α1 (1:2000, a generous gift from Dr. Jean-Marc Fritschy, University of Zurich, Zurich, Switzerland) overnight [365]. After washing with PBSX, sections were incubated for 1 hour with biotin conjugated goat anti-rabbit IgG secondary antibody (Biotin Conjugated Anti-Rabbit IgG, NB-730B, Novus, CO). Sections were subsequently washed with PBSX and incubated with the avidin-biotin complex (Vectastain ABC Kit™, Vector Labs, Burlingame, CA) for 1 hour. After washing, immunoreactions were visualized with 3,3-diaminobenzidine (DAB; Sigma, St. Louis, MO). The color reaction was terminated by washes in cold-PBS. Tissue sections were mounted on gelatin-coated slides and air-dried. Sections were dehydrated using an ascending series of ethanol concentrations and cleared using xylenes and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Sections from both wildtype and knockin mice were processed in parallel with investigator blind to genotype.

For determining cortical GABAergic interneuron staining, 30 μm coronal sections were cut and immunoreactivity was determined using antibodies for the calcium-binding proteins parvalbumin (1:2,000, p3171, Sigma), calbindin (1: 2,500, ab11426, Abcam, Cambridge, MA) and calretinin (1:1,000, ab5054, Chemicon International, Temecula, CA). Additionally, SMI-32 (1:1,000, SMI-32R, formally Sternberger Monoclonals, Lutherville, MD, now Covance, Princeton, NJ) was used to stain a subset of pyramidal neurons, glial fibrillary acidic protein (GFAP: 1:1,000, G9269, Sigma) was used to assess glial cells, and glutamic acid decarboxylase (GAD 65/67: 1:2,000, G5163, Sigma) was used to assess GABA producing cells. Sections were stained and processed in a similar fashion as described above. To assess cortical lamina, coronal sections were mounted onto slides and stained with cresyl violet (Nissl stain). Sections from wildtype and knockin mice were age and sex-matched and hippocampal architecture was used as landmarks to make valid comparisons between cortical regions. Laminar thickness from matched pairs was visually compared between genotypes.

Processed imunoperoxidase and Nissl sections were analyzed using bright-field microscopy. Assessments between wildtype and knockin animals were done using 3 animals per group. Digital photographs were obtained using a Leica DFC300 FX digital color camera attached to a Leica DM5000 B microscope and the accompanying Leica Application version 2.6 software (Leica Microsystems, Wetzlar, Germany). Parameters for image acquisition were the same for both genotypes. Minimal adjustments of brightness and contrast were made to entire images if necessary. Images were converted from color to gray-scale using Adobe Photoshop CS (v8.0, Adobe, San Jose, CA).

2.2.4 Semi-quantitative Immunoblot Analysis

Cerebral cortices and cerebella of adult mice were rapidly dissected over ice, flash-frozen on dry ice, and stored at -80°C. P2 membranes were isolated by first homogenizing samples, then centrifuging at 1,000 g for 10 min followed by spinning the supernatant at 10,000 g for 25 min. The resultant pellet was resuspended and protein concentration was determined via using a bicinchoninic acid method. P2 membrane fractions from cortex were processed and analyzed as pooled samples (three pools per genotype, eight mice per pool), whereas cerebellar samples were analyzed individually (n = 8/genotype). Aliquots of 25 μg of protein from each sample were separated by electrophoresis on precast SDS-10% polyacrylamide gels (Bio-Rad, Hercules, CA) and subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad) for detection by subunit specific antibodies. $GABA_A$-R anti-α1 (1:10,000), -α2 (1:10,000), and -α3 (1:10,000) antibodies [365] were generously donated by Dr. Jean-Marc Fritschy. Anti-β2 (1:5,000, NB 300-198), anti-β3 (1:2,500, NB 300-119), anti-γ2 (1:2,000, NB 300-151) and anti-α4 (1:2,000, NB 300-193) antibodies were obtained commercially (Novus Biologicals, Littleton, CO). Membranes incubated with α1, α3, β3 and actin were washed 3x with PBS-containing 0.1% Tween-20 (PBST) for 10 min; membranes incubated with α2, α4, β2 and γ2 were washed for 30 min with PBST. Primary antibodies were detected with either horseradish peroxidase-conjugated goat anti-rabbit (α1, α4, β2, β3, γ2 and actin; 1:5,000, NB-730H, Novus) or rabbit anti-guinea pig (α2, α3, 1:5,000, ab6771-1, Abcam) IgG polyclonal antibodies were visualized by enhanced chemiluminescence (Western Lightning; PerkinElmer Life and Analytical Sciences,

Boston, MA). To ensure equal loading, blots were stripped using Re-blot (Chemicon International) and reprobed with an anti-β-actin polyclonal antibody (1:10,000, ab8227-50; Abcam) for normalization. Multiple exposures of each membrane were used to ensure that the measured signal was within the linear range of the film. Band intensity was measured densitometrically (Kodak 1D software, version 3.6; Eastman Kodak, Rochester, NY). Each sample was analyzed on three to four different blots. Data were analyzed by Student's t-test.

2.2.5 Ethanol Metabolism and Clearance

Following injection of ethanol [(3.5 g/kg i.p.) Pharmco, Brookfield, CT], blood was collected from the retro-orbital sinus at 30, 60, 90, and 120 min postinjection. Blood ethanol concentrations (BECs) were determined as described previously[366]. In brief, blood samples were collected in heparinized capillary tubes, mixed with 3% perchloric acid, and centrifuged for 10 min at 1500g at 4°C. Supernatants were subsequently used to assess ethanol concentration via an alcohol dehydrogenase enzymatic reaction and quantified with a spectrophotometer at a 340nm. Blood concentrations were calculated using a set of ethanol standards. The rate of clearance was determined by linear regression analysis. Data were analyzed by Student's t-test.

2.2.6 Elevated Plus Maze

Mice were evaluated for basal anxiety as well as ethanol-induced anxiolysis using the elevated plus maze. The elevated plus maze was raised 38.5cm off the floor and consisted of four arms of 30 x 5cm. Two arms had 15cm high walls while the other two had no walls. All arms were connected by a 5 x 5cm center platform. The whole apparatus was constructed of clear Plexiglas, except for the floor of the arms and center platform, which was made of black Plexiglas. Mice were transported to the testing room 1 day prior to testing. Animals were tested between 9:00 AM and 11:00 AM under ambient room light. Mice were weighed and injected with 0.75, 1.0, 1.5 g/kg ethanol or saline 10 min prior to testing. Each mouse was placed on the central platform of the maze facing an open arm. Mice were allowed to freely explore the maze for 5 min during which the following measurements were manually recorded: number of open

arm entries, number of closed arm entries, total number of entries, time spent in open arms, and time spent in closed arms. A mouse was considered to be on the central platform or any arm when all four paws were within its perimeter. Data were analyzed using ANOVA with genotype and dose as the between-subject factors. Data were further analyzed with Fischer's post hoc test, or pair-wise comparisons were made with Student's t-test where appropriate.

2.2.7 Recovery From Drug-Induced Motor Ataxia

Mice were trained on a fixed speed (5.0 rpm) rotarod (Ugo Basile Rota-Rod, model 7650; rod diameter = 6 cm; Stoelting Co., Wood Dale, IL) and training was considered complete when mice were able to remain on the rotarod for 60 s. 10 min after pentobarbital administration (35 mg/kg i.p., Ovation Pharmaceuticals, Deerfield, IL), each mouse was placed back on the rotarod, and time spent on the rotarod was measured for up to 60 s. Behavior of mice as measured at 10 min intervals. Data were analyzed using two-way repeated measures ANOVA. Time was the repeated measure factor and genotype was the between-group factor.

2.2.8 Acute Functional Tolerance (AFT)

Acute/rapid tolerance to the motor ataxic as well as the loss of righting reflex effects of ethanol were measured using the two-dose method originally designed by Erwin and Deitrich [86]. For motor ataxia, ethanol naïve mice were trained to balance on a stationary dowel (2.5cm, rotarod drum, Ugo Basile) for a 60 sec period. All mice accomplished this task within 3 trials. Once basal training was accomplished, mice were injected with the first ethanol exposure (1.75 g/kg, i.p.) and placed back on the dowel until they fell off. Mice were repeatedly tested until they regained the ability to balance on the rod for 60 sec. Once this was achieved (t1), a retro-orbital blood sample was collected for BEC determination (BEC1). Mice were then immediately given the second ethanol exposure (2.0 g/kg, i.p.). After losing the ability to remain on the rod, mice were tested repeatedly (~ 5 min intervals) until they regained the ability to balance on the dowel for 60 sec (t2). Once this was achieved, a second blood sample was collected for BEC determination (BEC2). AFT is defined as the difference in BECs at t2 versus t1 (i.e., BEC2 – BEC1).

Development of AFT to ethanol-induced loss of righting reflex was also determined similarly to previous studies [87]. Mice received an initial hypnotic dose of ethanol (3.5 g/kg, i.p.). Once the mice lost the ability to right themselves, they were placed on their backs in a v-shaped trough. The duration of time until the animal could turn over (right itself) 3 times in a 30 sec period was recorded and a blood sample taken for BEC1 determination. Mice were then given the second ethanol exposure(2.0 g/kg, i.p.), and immediately placed back in the v-shaped troughs. The duration of the second loss of righting reflex was recorded and when the mice regained the ability to right themselves (t2), another blood sample taken for BEC determination (BEC2). AFT was again calculated similarly to the dowel test. All data were analyzed using student's t-test.

2.3 RESULTS

2.3.1 Gross Assessment of Knockin Mice

Knockin mice harboring the point mutations born from heterozygous parents were normal in size, growth rate, appearance, and overt behavior. For instance, the average body weight of male mice at 8 weeks of age was 24.8 ± 1.0 for wildtypes and 23.3 ± 1.0 for knockin mice and 27.8 ± 0.8 and 29.0 ± 1.0, respectively, at 12 weeks of age. Knockin mice were born at about the expected Mendelian frequency of 1:2:1 (e.g., WT – 611; Hets – 1181; KI – 579). Knockin mice had normal posture and were superficially indistinguishable from wildtype litermates.

Knockin mice generated normal litter sizes compared to heterozygous breeding pairs (6-9 versus 3-7, respectively; based on backcrossed breeding pairs). Knockin mice also did not differ from controls in basal motor coordination, anxiety-like behavior, or thermal nociception (data not shown). Knockin mice also did not display any obvious behavioral abnormalities, such as running in circles or repetitive motions or aggression. However, ~10% (52 mice of 579) premature deaths were observed in knockin mice between 7 and 12 weeks of age (possibly due to observed generalized tonic-clonic seizures in a few cases) compared to wildtype mice (~1%, 7

mice of 611), but otherwise mutants had normal life spans. Also, in work not conducted here, knockin mice had higher levels of overall motor activity [355].

2.3.2 RT-PCR

Because we had created a novel mouse line harboring point mutations, we first wanted to verify that the knockin mutations were present and that no other unintended mutations were present in the $GABA_A$-R α1 gene transcript. Brain mRNA was converted to cDNA by RT-PCR and portions of the α1 $GABA_A$-R coding region were amplified as shown in figure 2.1A and subsequently analyzed by DNA sequence analysis. As expected, only the intended mutations were present in the $GABA_A$-R α1 gene transcripts (Figure 2.1) and no other unintended mutations were introduced.

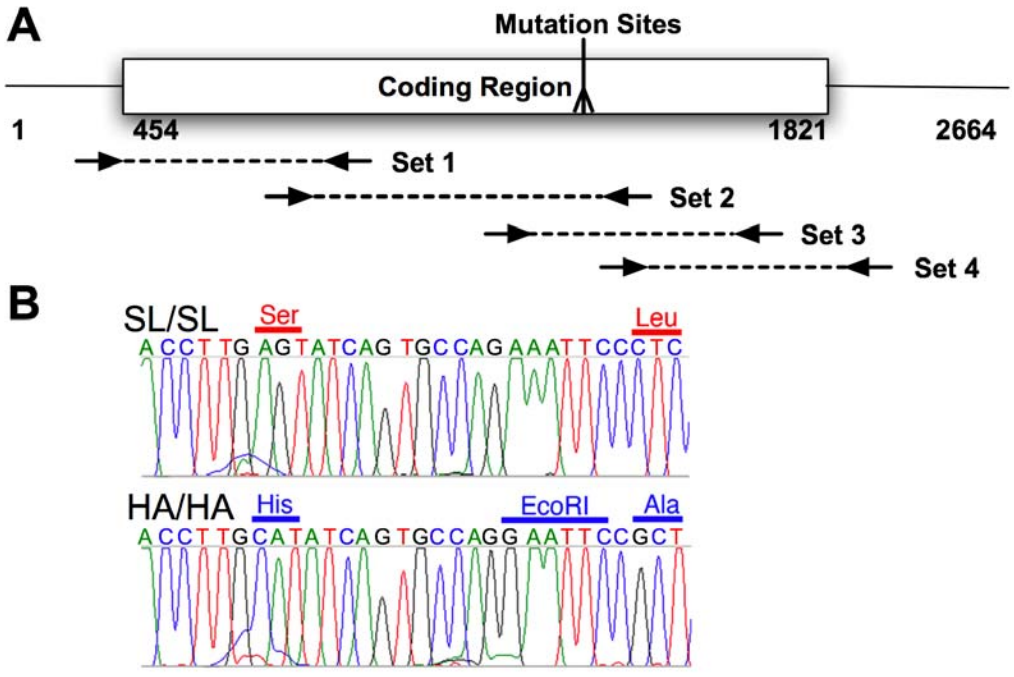

Figure 2.1 DNA Sequence of α1 $GABA_A$-R RT-PCR Products
Overlapping sections of the α1 $GABA_A$-R coding region were amplified and sequenced (A). Coding regions and primer sets are not drawn to scale. Representative sequence alignment of cDNA from RT-PCR products from whole brain of wildtype (top) and knockin (bottom) mice (B). Shown are regions of sequenced DNA where the intended mutations where the expected mutations are expressed in the α1 gene in the brains of knockin animals. Panel B is published in Borghese et al. [355].

2.3.3 α1 Immunohistochemistry

Because α1 $GABA_A$-Rs are expressed nearly uniformly through the brain, we next wanted to determine whether knockin mice expressed α1 similarly to controls. Therefore, distribution of α1 $GABA_A$-R was conducted using an α1 specific antibody. The pattern of immunohistochemical labeling in sagittal sections of wildtype and mutant mice is shown in figure 2.2. As expected based on results published elsewhere [365], α1 appeared to be normally distributed in knockins compared to wildtype controls (i.e., α1 specific staining was observed in cortex, hippocampus, midbrain, cerebellum). However, the intensity of staining appeared to be decreased in knockins in the cortex with respect to wildtypes.

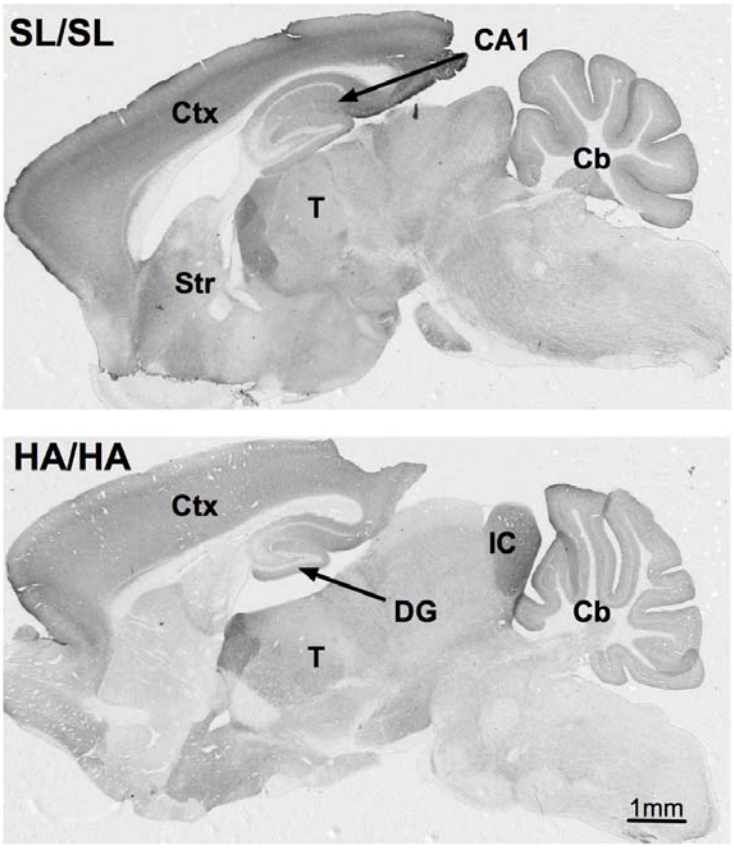

Figure 2.2. Distribution of α1 $GABA_A$-R Protein
α1 protein appears to be normally distributed throughout the brain. However, there appears to be a decrease in staining intensity in the cortex of knockin mice.

2.3.4 Semi-quantitative Immunoblot Analysis of $GABA_A$-R Subunits

Because α1 immunohistochemistry appeared to be reduced in the cortex, semi-quantitative immunoblot (western) analysis was carried out for α1 as well as several other $GABA_A$-R subunits. Analysis revealed that α1 subunit levels were decreased in the cortex of knockins compared to wildtypes by approximately 50% (Figure 2.3A). Analysis of other subunits also revealed changes in the abundance of several $GABA_A$-R subunits – specifically, α3, β2 and γ2 levels were increased whereas β3 levels were decreased. Further analysis was also done on cortical samples to determine whether α4 subunit levels were altered. While not statistically significant, there was a general trend towards an increase in α4 levels (Figure 2.3B) in knockins compared to wildtypes (123 ± 4% and 100 ± 4%, respectively).

Because subunit levels were altered in the cortex, additional analysis was carried out on cerebella. Analysis revealed that α1 subunit levels in knockins was similar to wildtypes (Figure 2.3C; 94 ± 9% and 100± 11%, respectively). Thus, introduction of these point mutations resulted in region specific alterations of α1 $GABA_A$-R subunit levels and that these alterations were accompanied by compensatory changes in other $GABA_A$-R subunit levels.

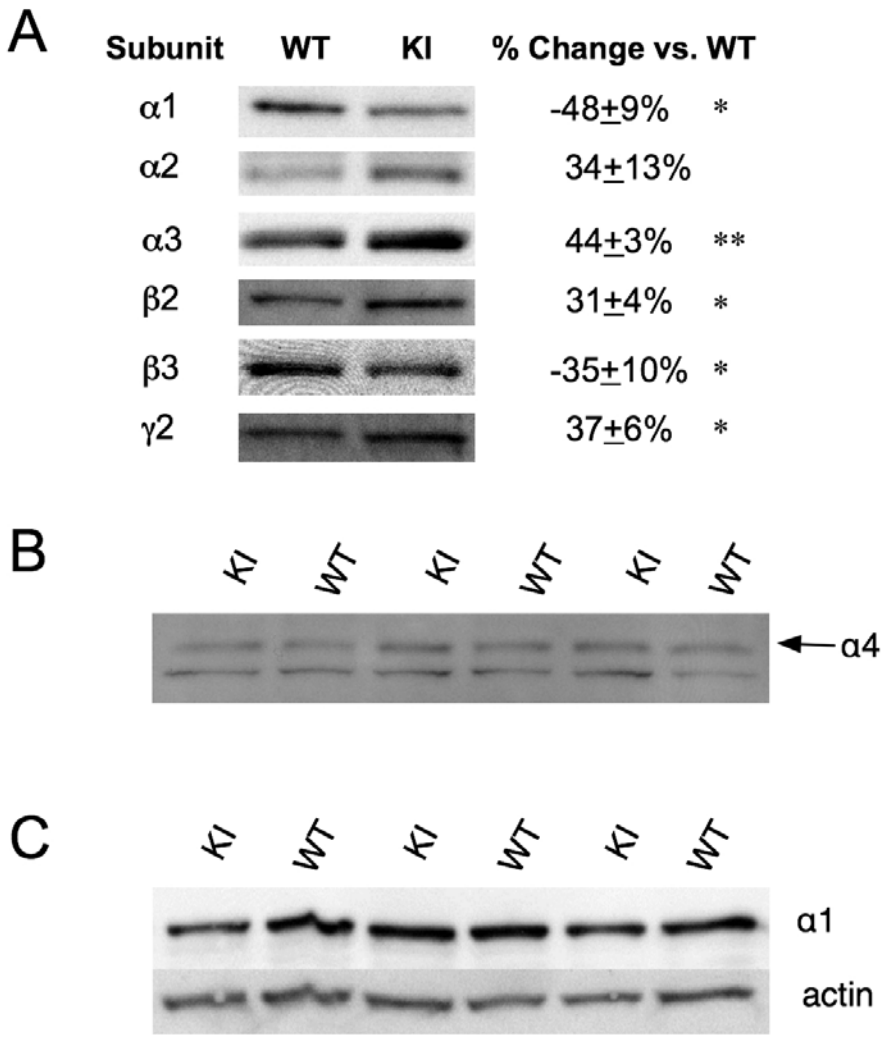

Figure 2.3 Immunoblots of $GABA_A$ Receptor Subunits
In cortical tissue, analysis revealed differential changes in a number of subunits. Specifically, α1 and β3 were reduced whereas α2, α3, β2 and γ2 were increased. Shown are representative pooled samples. Three pools per genotype, eight mice per pool were done. Also shown is the percentage of change in band intensity of knockins relative to wildtypes (A). Representative immunoblot analysis of α4 subunit levels. Additional experiments using tissue from α4 knockout mice identified the upper band as α4 (B). Analysis of α1 subunit levels in cerebellum. Shown are representative samples from individual mice (n=8 per genotype) (C). Data presented as mean ± SEM. *$p<0.05$; **$p<0.01$. Panels A and C were published in Borghese et al. [355].

2.3.5 Cortical Laminar Cytoarchitecture

Because $GABA_A$-R subunits – specifically $\alpha 1$, are temporally regulated during development in cortical lamina [367], combined with the observed alterations in $GABA_A$-R subunits in the cortex, cytoarchitecture was assessed to determine if any changes differences occurred in the cortical lamina. Cortical layers from Nissl stained sections were identified based upon the unique cytoarchitecture associated with each layer as done elsewhere [e.g., 368]. Upon gross observation, analysis via Nissl stain (Figure 2.4) suggested that the overall lamina pattern was intact in knockin mice versus controls. More specifically, no differences between genotypes were detected in laminar thickness in the deeper layers (V and VI) or superficial layers (I –III) based on visual comparison of laminar thickness in different cortical regions. Thus, it could be concluded that the point mutations did not result in gross abnormalities in cortical cytoarchitecture.

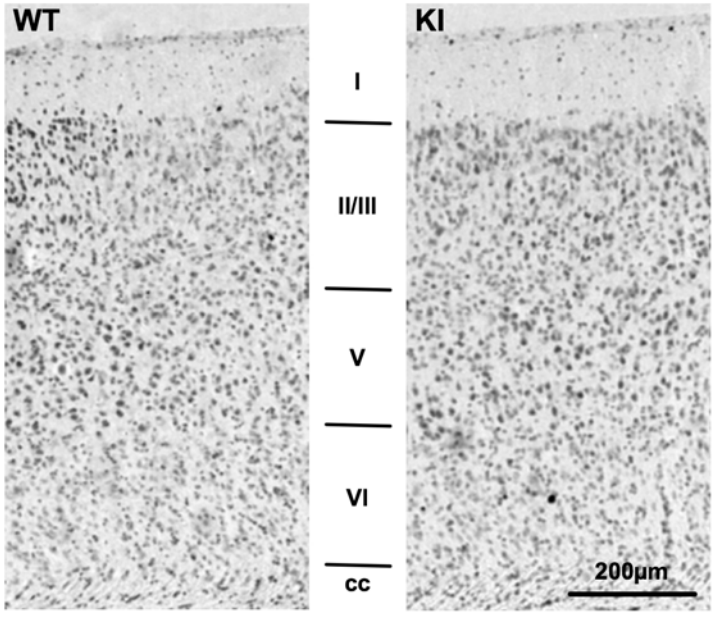

Figure 2.4. Assessment of Cortical Lamina
Panels show representative images for wildtype (WT) and knockin (KI) mice, respectively. Knockin mice appear to have normal laminar distribution. Cortical layers are indicated by Roman numerals. Layer IV, which is typically associated with barrel-like structures, is absent in the above images. n = 3 mice per genotype.

2.3.6 Distribution of GABA-Related and Cell Specific Markers

To further investigate other aspects of the GABAergic system and other cell specific markers, the expression of molecules associated with several distinct neuronal cell types were examined. Specifically, immunoreactivity for the calcium-binding proteins calbindin, calretinin and parvalbumin were used to assess GABAergic interneurons, whereas GAD65/67 was used to assess GABA producing cells. Conversely, SMI-32 was used to assess a subpopulation of pyramidal neurons and GFAP for glial cells.

For GABAergic interneurons, in both mouse lines, calbindin staining (Figure 2.5A) was restricted to layers II/III, although some sporadic staining was observed in deeper layers. There also did not appear to be any substantial differences with respect to genotype. Similar staining patterns and intensity was also observed for calretinin (Figure 2.5B) with respect to genotype. Parvalbumin (Figure 2.5C) appeared to have a less restricted staining pattern through more of the cortical layers; but no differences were evident with respect to genotype. For GABA producing cells, immunoreactivity for GAD 65/67 (Figure 2.5D), was prominent throughout the cortex, but no differences with respect to genotype were observed.

With respect to nonGABA-related molecular markers, analysis of pyramidal neurons by SMI-32 (Figure 2.6A) showed staining in deep layers (i.e., layer V) as well as in superficial layers (II/III). Processes were observed extending into the upper layers. Conversely, molecular markers for glial cells by GFAP (Figure 2.6B) indicated isolated staining to the uppermost superficial layer (I) and to the corpus callosum. No differences between genotypes were detected for either molecular marker.

Thus, these results indicate that neuroanatomic distribution of GABAergic interneurons and GABA-producing cells were normal. Additionally, no noticeable differences were observed in the distribution or morphology of a subset of pyramidal neurons or glial cells. Overall, although mutation appears to regionally alter $GABA_A$-R subunit levels in the cortex, cortical cytoarchitecture and GABAergic interneurons and GABA producing cells were conserved, as were other major cell types.

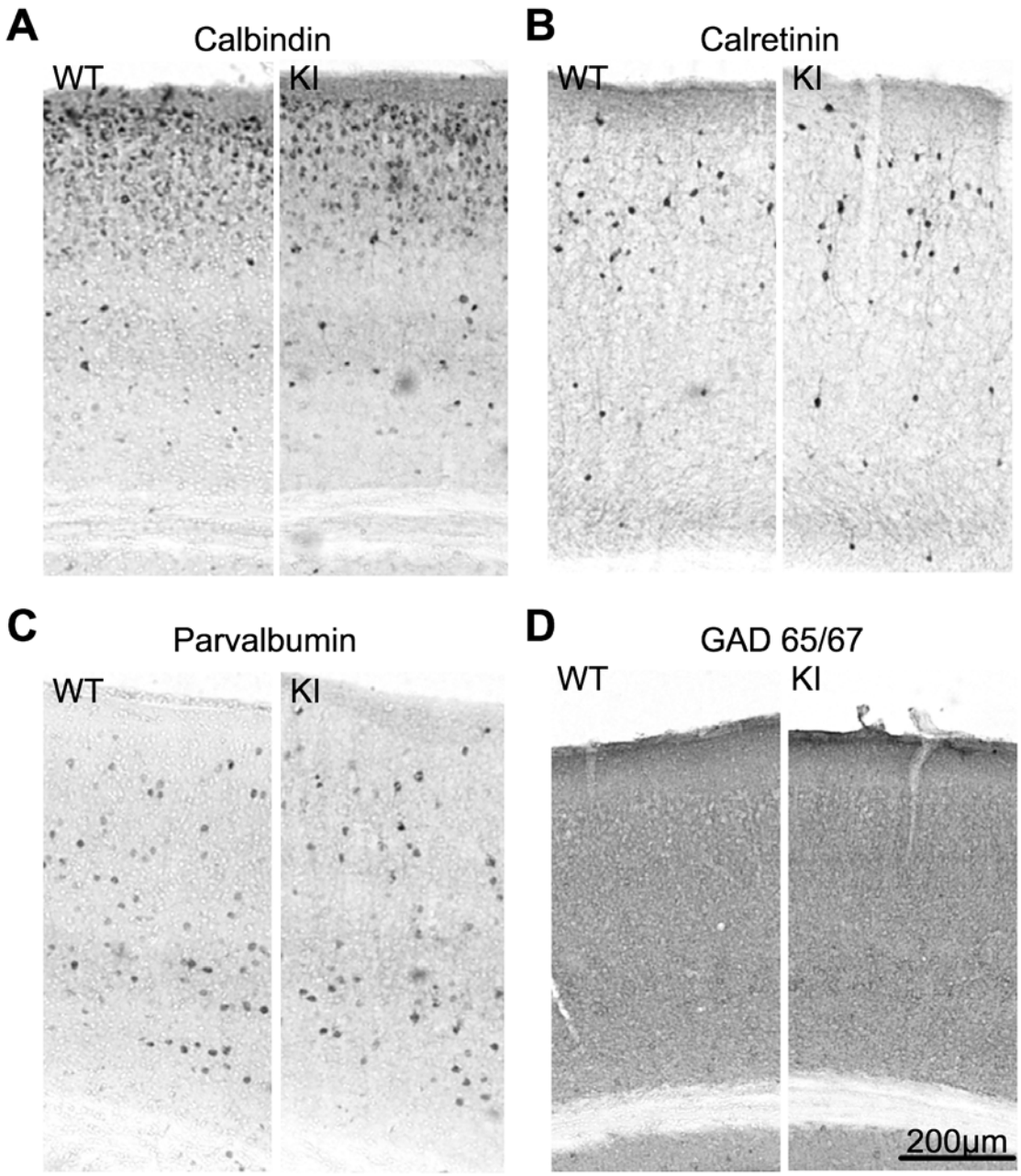

Figure 2.5. Laminar Distribution of GABAergic Cells
Molecular markers for specific calcium-binding proteins were used to assess GABAergic interneurons from wildtype (WT) and knockin (KI) mice. Calbindin (A) and calretinin (B) positive neurons were more prominent in the superficial layers (although calretinin was less intense) but had diffuse staining in deeper cortical layers. Parvalbumin (C) positive neurons were diffusely located throughout the cortical lamina. GABA-producing neurons noted by GAD 65/67 was found throughout the cortical layers. No difference between genotypes was evident. n = 3 mice per genotype.

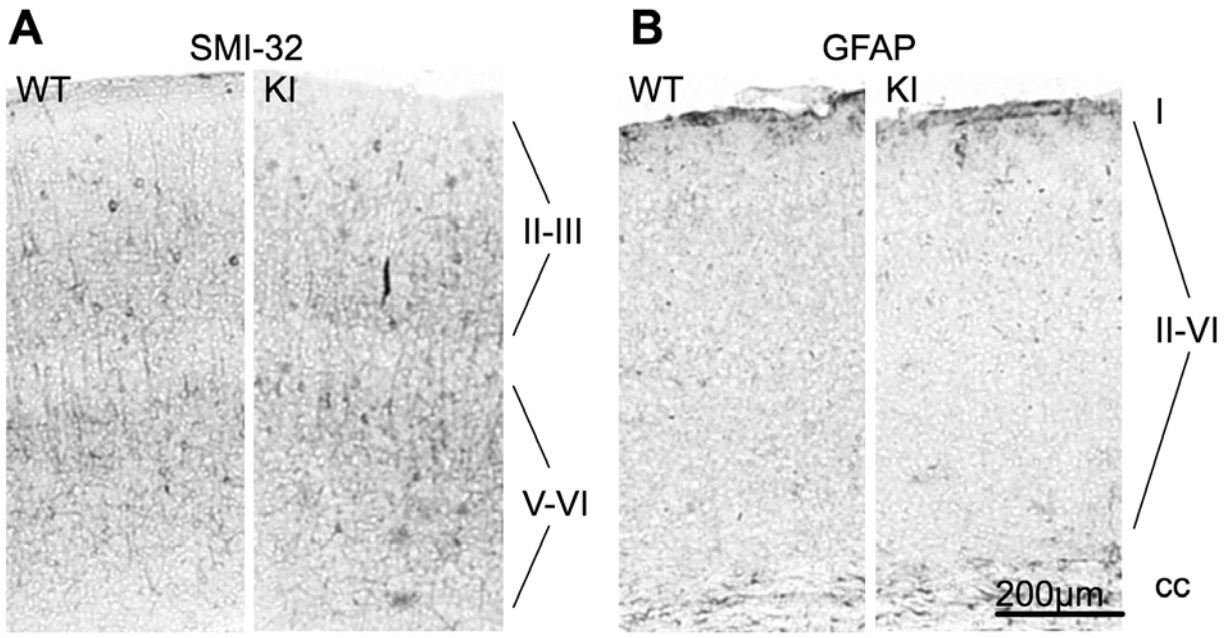

Figure 2.6. Laminar Distribution of Pyramidal and Glial Cells
SMI-32 (A) stains a subset of pyramidal neurons. Immunoreactivity appeared to be seen in deeper layers such as layer V, as well as superficial layers. Dendritic processes were also observed extending dorsally from the deeper stained neurons. GFAP (B) immunoreactivity for glial cells indicates more localized staining in layer I and in the corpus callosum. Low amounts of glial cells were observed in other cortical layers. n = 3 mice per genotype.

2.3.7 Ethanol Metabolism and Clearance

To determine whether ethanol pharmacokinetics was similar between wildtype and knockin mice, metabolism and clearance was assessed before proceeding with pharmacodynamic (behavioral) analysis. BECs were measured every 30 min for 2 h following injection of a hypnotic dose (3.5 g/kg) of ethanol. BECs and clearance rate (Figure 2.7) did not differ with respect to genotype thus allowing valid comparisons between genotypes for ethanol-related behaviors.

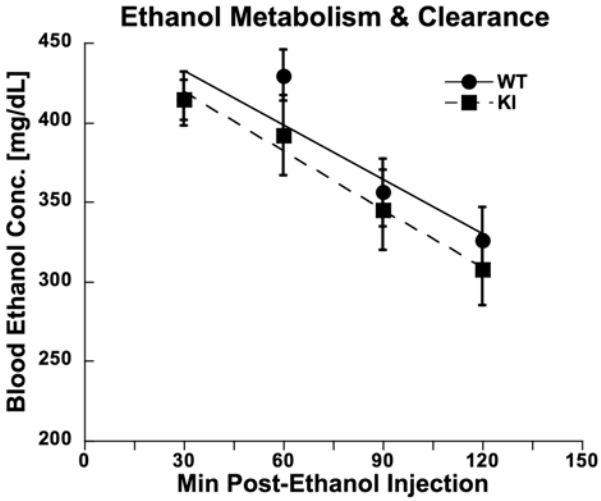

Figure 2.7. Ethanol Metabolism and Clearance
Blood ethanol concentrations were measured every 30 min following a hypnotic dose (3.5 g/kg) of ethanol. BECs did not differ with respect to genotype. For instance, at 30 min post injection, BECs for wildtype and knockin mice were 415 ± 17 and 414 ± 13, respectively. Clearance also did not differ (1.13 ± 0.22 and 1.22 ± 0.19 mg/dl/min for wildtypes and knockins, respectively. n = 5-6 mice per genotype. Data represent mean ± SEM. Published in Werner et al. [369].

2.3.8 Ethanol-Induced Locomotor Activity and Anxiolysis

The elevated plus maze was used to assess basal and ethanol-induced locomotor activity and anxiety-like behavior. Locomotor activity was assessed by total number of arm entries, while anxiety was measured by percent of time spent in open arm entries and percent of open arm entries.

Basal performance on the elevated plus maze (following saline injection) did not differ with respect to genotype for any measure (Fig 2.8A locomotor activity, B and C, anxiety-like effects). Thus, locomotor activity and indicators of anxiety-like behavior were not altered by the mutations in the $GABA_A$-R $\alpha 1$ subunit.

Using total number of arm entries to assess the effect of ethanol on locomotor activity, significant main effects of dose and genotype were observed (Fig 2.8A) (ANOVA: dose $F_{3, 77} = 6.7$, $p < 0.001$; genotype $F_{1, 79} = 4.3$, $p<0.05$), but no interaction. Subsequent pairwise comparisons revealed a stimulating effect of ethanol in both genotypes at the lowest dose of ethanol tested (0.75 g/kg). Interestingly, knock-in mice were more sensitive to the locomotor stimulant effect of ethanol at 1 g/kg when compared to wild-type mice ($p< 0.01$). At 1.5 g/kg

ethanol had a locomotor stimulant effect in knock-in mice compared to saline, while no effect was observed in wild-type mice compared to saline controls.

With respect to the anxiolytic effects of ethanol, two-way ANOVA indicated a significant main effect of dose ($F_{3, 73} = 7.0$; $p < 0.001$), and interaction of genotype x dose ($F_{3, 73} = 4.3$, $p < 0.01$), but no main effect of genotype in the percent of open arm entries (Fig 2.8B). For percent time spent in open arms (Figure 2.8C), the statistical analysis indicated a significant effect of dose ($F_{3, 73} = 6.0$, $p < 0.01$), genotype ($F_{3, 73} = 4.0$, $p<0.05$), and interaction of genotype x dose ($F_{3, 73} = 2.9$, $p < 0.05$). In all analyses, no effect of gender was observed.

Because the anxiolytic effects observed at 1.0 and 1.5 g/kg may be potentially confounded by the genotypic differences observed in total arm entries, we limited our analysis and interpretation to the 0.75 g/kg dose. At this dose, ethanol decreased anxiety-like behavior in knock-in mice, but not in controls. This is evidenced by the increase in percent open arm entries (Fig 2.8B, $p < 0.05$) and percent time in open arms (Fig 2.8C, $p < 0.01$) in knock-in mice compared to controls. These data indicate that knock-in mice are more sensitive to the anxiolytic effects of ethanol, but there is no difference in ethanol's locomotor stimulatory effect at low doses.

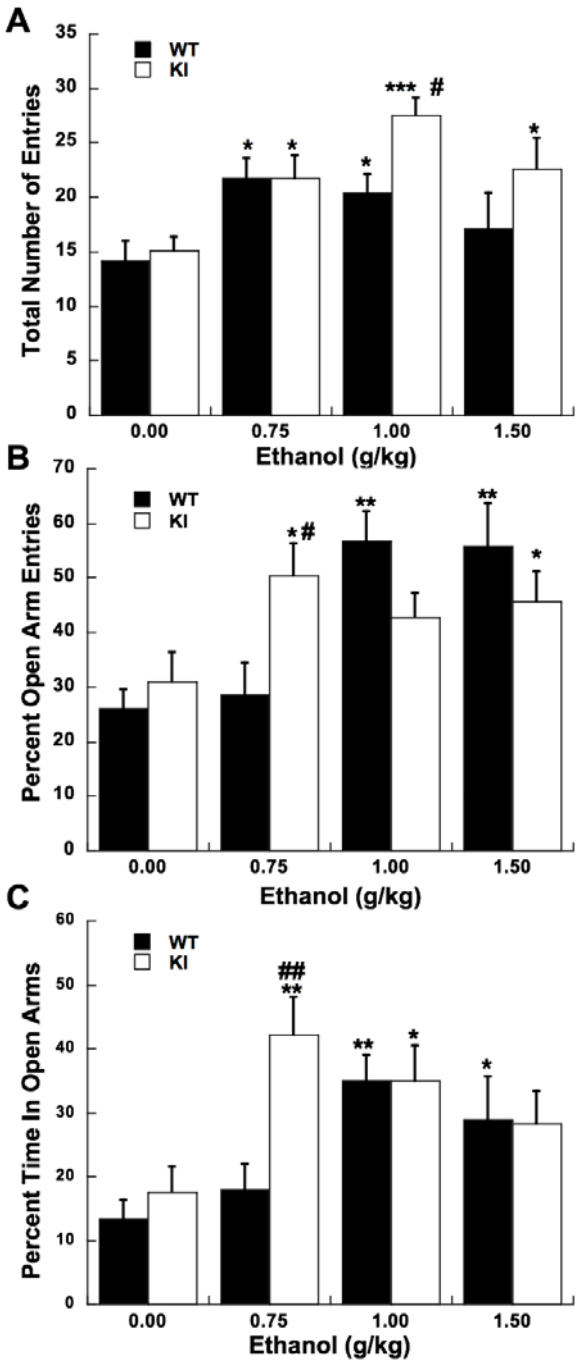

Figure 2.8. Evaluation of anxiety and activity using the elevated plus maze.
Total arm entries (A), percentage of open arm entries (B), and percent of total time spent in open arms (C) are shown. Saline or ethanol was administered 10 min prior to testing. The locomotor stimulant effect of ethanol was detected in both genotypes at 0.75 g /kg compared to saline. Knockin mice had increased locomotor stimulant effect at 1.0 g/kg compared to controls. The anxiolytic effect of ethanol was seen at 1.0 and 1.5 g/kg in both genotypes. Ethanol increased the percentage of open arm entries relative to the total number of entries and percentage of time spent in open arms relative to the total time. Knockin mice had increased responses compared with wildtype mice at specific doses 0.75 for anxiolytic effect. Data represent mean ± S.E.M., n = 10–11 per group per genotype. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ represent comparison with saline groups (within genotype). #, $p < 0.05$; and ##, $p < 0.01$ represent differences between genotypes at each ethanol dose. Published in Werner et al. [369].

2.3.9 Recovery from Pentobarbital-Induced Ataxia

Work conducted by collaborators (Y. Blednov et al., University of Texas) indicated that knockin mice recovered more quickly from ethanol's ataxic effects [369]. Subsequent experiments also revealed that knockin mice also recovered more quickly from the ataxic effects of the $GABA_A$-R agonist etomidate [369]. To determine if this phenotype generalized to other sedative/hypnotic drugs, pentobarbital (35 mg/kg) was also tested for effects on motor impairment (Figure 2.10). Following pentobarbital administration, there was a significant effect of time ($F_{6, 108} = 167$, $p < 0.0001$), but genotype and the interaction of genotype × time were not significant. Thus, while the knockin mutations affected recovery from ethanol- and etomidate-induced motor ataxia, the mutation did not affect recovery from pentobarbital-induced motor ataxia. No effect of gender was observed.

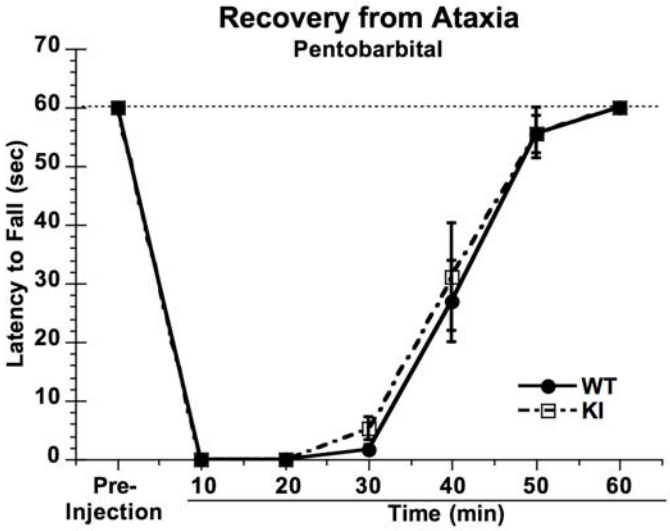

Figure 2.9 Effects of Pentobarbital On Motor Coordination Using Rotarod.
Mice were injected with sodium pentobarbital (35 mg/kg) and the ability to balance on a fixed speed rotarod (5.0 rpm) was measured at 10 min intervals. Knockin and wildtype mice were similarly impaired after pentobarbital exposure. n = 9-11 per group. Data represent mean ± SEM. Published in Werner et al. [369].

2.3.10 Acute Functional Tolerance (AFT)

To assess acute/rapid tolerance, AFT to the ataxic effects of ethanol was measured using the stationary dowel (Figure 2.10A). Time to recover from the first ethanol exposure (t1) and BEC at t1 (BEC1) did not differ between genotypes. Knockin mice displayed significantly longer time to recover from the second ethanol exposure (t2) and also had reduced BEC at t2 (BEC2) compared to controls ($p < 0.01$). This resulted in an overall decrease in AFT (defined as BEC2-BEC1) with respect to ataxia in knockins compared to controls (64.2 ± 7.9 versus 138.5 ± 11.8, respectively, $p<0.01$).

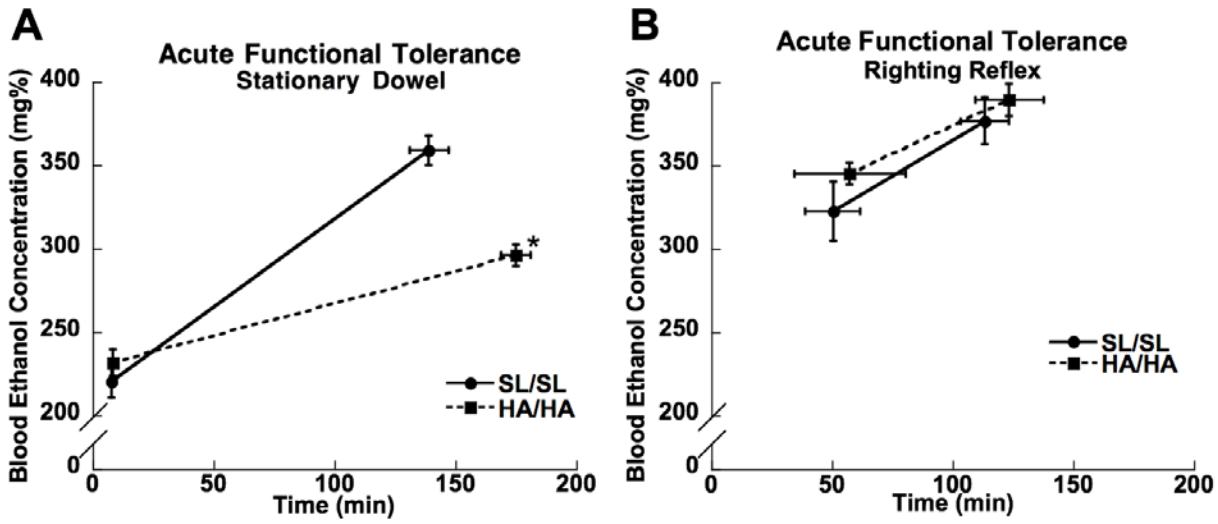

Figure 2.10. Acute Functional Tolerance
Assessment of acute/rapid tolerance to the ataxic (A) and hypnotic (B) effects of ethanol. The first set of coordinates in each panel represent BEC1 and t1 (blood alcohol concentrations at first recovery). The second set of coordinates represent BEC2 and t2 (blood alcohol concentrations at second recovery). Knockin mice displayed reduced AFT to ethanol's ataxic effects that was due to longer t2 and lower BEC2 compared to wildtype. No differences were observed in AFT to ethanol's hypnotic effects. n = 9-11 per group for stationary dowel. n = 7 per group for righting reflex. Data represent mean ± SEM. *, $p < 0.01$ for t2, and BEC2.

To determine if reduced tolerance generalized to other behavioral measures, AFT to the hypnotic effects of ethanol was assessed (Figure 2.10B). Wildtype and knockin mice did not differ on any measured parameter (i.e., t1, t2, BEC1, BEC2, AFT). AFT for wildtype and knockin mice was 54.6 ± 23.4 and 44.4 ± 15.5, respectively.

Overall, this suggests that knockin mice develop less acute/rapid tolerance to the ataxic effects of ethanol compared to wildtype mice, whereas acute/rapid tolerance to other ethanol-related behaviors such as hypnosis is similar between genotypes.

2.4 DISCUSSION

In this chapter, α1 $GABA_A$-R knockin mice harboring mutations $S_{270}H$ and $L_{277}A$ were molecularly characterized and several acute ethanol and pentobarbital induced behaviors were assessed. Aside from displaying ~10% random deaths in the knockin (thought to be due to seizures) and increased locomotor activity [355], knockin mice were overtly indistinguishable from wildtype littermates. This is in stark contrast to α1 global knockouts and knockins harboring only the $S_{270}H$ mutation. Global knockout mice are indeed viable but exhibit an intentional tremor, slightly reduced body weight, increased motor ataxia and increased pharmacologic-induced seizure susceptibility [336, 370, 371]. Mice harboring the single point mutation for ethanol insensitivity were hypoactive, hypersensitive to human contact and also displayed tremor, reduced body weight, and reduced viability [353]. Single knockin mice also had abnormal EEG activity and displayed seizure-like activity from anesthesia [372]. Paradoxically, the single point mutant mice were hyperactive in novel environments (similar to the double mutants) [353]. Apart from the spontaneous deaths and increased locomotor activity, the double mutant mice have none of these deficits. Therefore, incorporation of the second mutation, which normalizes GABA sensitivity *in vitro* [355], appeared to correct the abnormalities that were observed in the single mutant animals and eliminated the essential tremor observed in global knockouts. Thus, the double knockin mouse model is better suited than the global knockout or single knockin models for assessing the role of α1-containing $GABA_A$-Rs in ethanol action.

Work conduced by others has indicated that knockin (mutant) mice did not differ on the majority of basal behavior responses; however mutant mice did display increased locomotor activity, as well as some impaired memory function compared to controls [355, 369]. Electrophysiological recordings from hippocampal pyramidal neurons indicated mutant mice had

significantly less ethanol-induced potentiation of evoked inhibitory post-synaptic currents (IPSCs) than wildtype mice at 80mM ethanol [369]. However, no difference in ethanol's potentiating effect was detected between genotypes at 20 or 40mM ethanol [369]. Responses to flunitrazepam and pentobarbital were not different [369]. These results suggest that at the cellular level the mutation primarily alters the response to ethanol and not other GABAergic agents.

Our results indicate that although mutant α1 subunits are found regionally expressed in a similar manner to wildtype animals (Fig 2.2), there relative amounts of α1 protein appear to be reduced by approximately 50% in cortical regions (Fig 2.3). This decrease in α1 appears to result in bidirectional changes in other subunits. However, it should be noted that this reduction wasn't observed in all brain regions. For instance, knockin mice displayed similar levels of α1 in the cerebellum relative to wildtype mice (Fig 2.3). In additional work not reported here, knockin mice had normal flunitrazepam binding indicating normal amounts of benzodiazepine-sensitive receptors [355]. Taken together, it is possible that compensatory changes in other subunits account for the decrease in α1. Indeed, α3 levels were increased by 44%. The finding that changes in subunit levels were found in specific brain regions was unexpected as compensatory mechanisms are typically more commonly associated with knockout models where the gene is completely ablated. However, while some benzodiazepine knockin models (α1, α2) have reported normal expression of $GABA_A$-R subunits [276, 277], other benzodiazepine knockin models have reported altered subunit levels [350]. Additionally, work has also pointed out that although prior knockin models appeared normal on the molecular level, the resultant incorporation of point mutant(s) did not necessarily result in a silent phenotype – in other words, some changes in basal behavior were observed [373]. However, knockin models produced in other genes have also reported altered levels [374, 375]. Alterations in target proteins in knockin models may still be useful apart from their original mutations. Indeed, many diseases may be the result of abnormal changes in gene product levels and initiating other cellular pathologies thereby giving insight into other diseases [e.g., 376].

Interestingly, the observed subunit changes in the cortex did not appear to disturb the cortical cytoarchitecture, and the distribution of several specific cell types examined were preserved. Additionally, other aspects of the GABAergic system appeared to be intact, such as the distribution of GABAergic interneurons and GABA producing cells (Fig 2.5). Other cell

types (Fig 2.6) and laminar cytoarchitecture was also preserved (Fig. 2.4). This is somewhat of a surprise, given the changes seen in cortical $GABA_A$-R subtypes (Fig 2.3), especially since parvalbumin- and calretinin-immunoreactive cells, express α1-containing $GABA_A$-Rs [377]. However, other interneurons (i.e., calbindin expressing) do not express α1 [377]. Other alpha subunits appear to be absent in hippocampal interneurons [365, 378]. Nonetheless, recent work conducted by Schneider-Gasser et al. [379] suggests that in α1 knockout mice, α2 and α3 subunits substitute for α1 on interneurons. However, other studies have suggested that reorganization of neuronal circuits occurs rather than 'subunit substitution' [342]. Nonetheless, this suggests that in specific brain regions, compensatory changes are observed to allow for stable neuronal networks. Additionally, the staining pattern, regional and laminar distribution of interneurons used in this study did not differ between α1 knockouts and wildtypes [379] and is in agreement with results presented here. Taken together, changes in α1 do not result in differences in GABAergic interneurons.

Behaviorally, knockin mice were more sensitive to anxiolytic effects of ethanol as tested on the elevated plus maze assay. This effect was dependent on dose and was detected in both measurements of anxiety-like behavior at an ethanol dose of 0.75 g/kg (Fig 2.8). The increased sensitivity was the opposite of the outcome predicted if α1 $GABA_A$-Rs were responsible for this effect of ethanol. Knockin mice were predicted to be less sensitive to ethanol. However, the anxiolytic effects of benzodiazepines have been linked to $GABA_A$-Rs possessing α2 and α3 subunits, not α1 subunits [380]. Our results suggest that α3 and possibly α2 subunits are increased in the cortex of these mice (Fig 2.3). Therefore, increased expression of other ethanol-sensitive α subunits may contribute to the observed phenotype and may represent molecular targets for the anxiolytic effects of ethanol. However, studies by Popik et al. [381] using a highly selective α1-$GABA_A$-R agonist suggest that these receptors may play a role anxiety. This is not in agreement with our results, but still indicates that α1-containing $GABA_A$-Rs may have a role in anxiolytic effects. Studies have reported that basolateral amygdala (where α1 is expressed [e.g., 382]) is an important site of action for anxiety and fear-related behavior [143]. Therefore, it is possible that the neurocircuitry in this region (or other regions such as the hippocampus) may be altered thereby resulting in enhanced ethanol-induced anxiolytic effects. Further experiments would have to be conducted to further delineate this possibility.

Perturbation of $GABA_A$-Rs by ethanol or other GABA agonists is well known to alter motor coordination. Additional studies by other investigators indicate α1 knockin mice displayed a faster recovery from the ataxic effects of ethanol and etomidate than wildtype mice [369]. In response to the GABA agonist pentobarbital, ataxia was not altered by the mutation (Fig 2.9) thereby suggesting that the introduced mutations are specific for ethanol and etomidate and do not alter responses to other $GABA_A$-R agonists. However, experiments by our lab not reported here (accelerating rotarod, unpublished observations) indicate that initial sensitivity to the ethanol's ataxic effects are not altered. The discrepancy between the initial sensitivity to- and recovery from- ethanol ataxia may be due to ethanol's promiscuous effects at multiple molecular targets. Ataxia is due, at least in part, to drug effects on cerebellar function [59], and it is important to note that normal α1 protein levels were observed in the cerebellum of mutant animals (Figure 2.3). Thus, while ethanol may initially produce motor impairment via multiple molecular targets, continued motor impairment requires potentiation of α1-containing $GABA_A$-Rs by ethanol. By removing this major site of ethanol action in the cerebellum, knockin mice were able to recover more quickly.

Aside from ethanol and pentobarbital, in work done by our lab and the University of Texas (Y. Blednov), knockin mice had decreased duration of etomidate-induced loss of righting reflex [369]. The decreased sensitivity to etomidate is consistent with previously observed electrophysiological recordings [355]. The discrepancy in the responses to etomidate and ethanol may be due to the fact that etomidate has a more selective mechanism of action than ethanol. A single mutation in the β3 subunit of the $GABA_A$-R markedly reduced the loss of righting reflex duration after etomidate administration [322]. This indicates that etomidate exerts most of its effects via the $GABA_A$-R. In contrast, ethanol effects are likely determined by actions on multiple targets, including non-$GABA_A$-R targets [383, 384].

It was surprising that pentobarbital behavior was unchanged in knockin mice compared to controls. Electrophysiologic recordings in oocyctes indicated a decreased sensitivity to pentobarbital-induced potentiation [355] in the mutant $GABA_A$-R. However, *in vivo* slice recordings did not indicate any difference [369], although only a single concentration of pentobarbital was used. While the behavioral data are in agreement with the slice recordings, the experimental setup may not have been sensitive enough (i.e., dose, time points or speed or rotarod speed) to detect differences between genotypes. Decreases in the time interval and/or

increasing the motor challenge (increased time requirement) could increase the sensitivity of the motor ataxia assay.

In parallel to the decreased sensitivity of the knockin mice for ethanol-induced motor ataxia, knockin mice also displayed decreased acute functional tolerance (Fig 2.10A) – i.e., in other words, knockin mice did not recover from the second ethanol exposure as readily as controls. Other studies have indicated similar directional changes. For instance, in selectively bred lines of rats and mice, those which had decreased initial ethanol effects developed little to no tolerance whereas those lines which were more sensitive developed greater tolerance [95, 211, 213]. Using the same rationale applied to the present experiments, although knockin mice displayed decreased response to motor ataxia compared to controls (i.e., faster recovery), it is possible that since ethanol was no longer acting through α1-containing $GABA_A$-Rs, the necessary molecular neuro-adaptations did not occur thereby leaving knockin mice more susceptible to the next intoxicating state compared to controls. To determine whether decreased tolerance is observed in other ethanol-related behaviors, acute/rapid tolerance was also assessed with respect to the righting reflex (Fig 2.10B). No differences were observed between genotypes. Therefore, ethanol acting through α1-containing $GABA_A$-Rs mediated in part the development of acute/rapid tolerance.

In summary, these results demonstrate that although the α1 knockin model displays some compensatory changes in $GABA_A$-R expression, they indeed appear to be a more ideal system than global knockouts or the single $S_{270}H$ knockin model to study ethanol-related behaviors. By utilizing this knockin mouse model, α1-containing $GABA_A$-Rs were shown to mediate in part specific ethanol-induced behavioral responses (i.e., ataxia) and may participate in the development of AFT. Importantly, these results lend support to gene knockin models being a more ideal model for studying specific biological and pharmacological mechanisms than global knockouts.

3.0 CHAPTER THREE: DETERMINATION OF NEUROANATOMIC REGIONS ACTIVATED DURING ETHANOL EXPOSURE VIA ANALYSIS OF THE INDUCTION OF THE IMMEDIATE EARLY GENE C-FOS

The analysis of immediate early gene (IEG) expression has given powerful insight into which neuroanatomic regions and cell types are primarily affected from pharmacologic insults such as ethanol. Understanding which regions are involved in drug action is of critical importance to focus further investigations into the molecular mechanisms and neurocircuitry of specific biological, physiological and behavioral effects. In doing so, work may be done to prevent side effects or to harness specific effects of the drug of interest. Although many IEGs have been utilized, c-fos – a component of the AP-1 transcription factor, is the most prevalent in ethanol-related studies. C-fos immunoreactivity (Fos-IR) has not only been used to study the acute effects of ethanol action [e.g., 231], but has also been used to assess tolerance and withdrawal [e.g., 246, 253].

The GABAergic system has been suggested to have a putative role in c-fos induction and perturbation of receptor function by receptor agonists or antagonists may induce Fos-IR. Studies have suggested that low doses of ethanol may be primarily mediated through GABA receptors in the hippocampus and other brain regions, whereas higher doses of ethanol may involve a number of other targets [228]. In support of this, in certain regions of the brain, up to 70% of c-fos positive neurons are GABAergic [243]. Ethanol may also reverse c-fos activity by GABA antagonists. For instance, induction of c-fos mRNA levels after administration of pentylenetetrazole or picrotoxin, but not NMDA, was reduced by ethanol [385]. The same study showed this effect of ethanol could be reversed in part by the $GABA_A$-R inverse agonist Ro15-4513.

Direct involvement of $GABA_A$-Rs in mediating Fos-IR has been demonstrated. For instance, Batchell et al. [229] showed that Fos-IR in the Edinger-Westphal nucleus (EW) – a

highly sensitive region to ethanol-induced Fos-IR, is attenuated by the $GABA_A$-R antagonist bicuculline. Furthermore, this study identified the MEK1/2-Erk1/2-Stat3 pathway in Fos-IR activity. In other work, $GABA_A$-Rs were found to regulate the paraventricular nucleus of the hypothalamus (PVN) [386], a second region highly sensitive to ethanol-related Fos-IR [e.g., 231]. Interestingly, in work assessing PKC isoforms (which regulate $GABA_A$-R function), PKC-epsilon knockouts had increased Fos-IR in the PVN during ethanol withdrawal, whereas control mice did not [249]. Coincidentally, the pattern of c-fos staining from ethanol is suggested to be remarkably similar to nitrous oxide treated animals [387], the effects of which may be produced in part through α1-containing $GABA_A$-Rs [388]. In other studies assessing α1 $GABA_A$-Rs, exposure to the benzodiazepine diazepam increased c-fos transcripts, but not in benzodiazepine insensitive mutants [389]. Of note, diazepam induced Fos-IR was suggested to be similar to ethanol [231]. Because α1 $GABA_A$-Rs are suggested in Fos-IR coupled with α1's diverse expression [365], it is quite possible that α1 $GABA_A$-Rs may play a role in ethanol-induced Fos-IR. Therefore, it is hypothesized that α1 is involved in ethanol-induced Fos-IR.

By using ethanol-insensitive α1 $GABA_A$-R knockin mice, the direct involvement of α1 may be addressed. Additionally, neuroanatomic alterations in Fos-IR in may be used to better understand ethanol-related behavioral differences in this model. I hypothesize that α1 $GABA_A$-R's mediate in part ethanol-related changes in spatial c-fos expression. In this report α1-$GABA_A$-R involvement in ethanol-related Fos-IR was assessed in certain brain regions. Specifically, the PVN and EW – which are particularly sensitive to ethanol action was assessed as was the dentate gyrus (DG) of the hippocampus, a region with an abundance of α1-$GABA_A$-Rs expressed in interneurons [377]. Fos-IR was assessed after acute ethanol exposure, after chronic (repeated) ethanol exposure, as well as during periods of ethanol withdrawal.

3.1 MATERIALS AND METHODS

3.1.1 Ethanol Treatment

Mice were produced as described in chapter two. Mice were between 8 and 12 weeks of age at time of use. For acute ethanol studies, mice were transported to a procedure room where they were injected with either saline (0.9% NaCl i.p., n =2 per genotype) or ethanol (2.0 g/kg i.p., Pharmco, Brookfield, CT; n=2 per genotype) and placed into separate holding cages. After two hours mice were sacrificed by an overdose of isoflurane, perfused with 4% paraformaldehyde and brains dissected out for c-fos immunoreactivity (described below). The 2-hour time point and ethanol dose were chosen because they resulted in robust Fos-IR after ethanol exposure in preliminary experiments. The time and doses are in the range of methods used elsewhere [229, 231, 390].

In separate experiments, to assess repeated ethanol exposure (tolerance), mice were transported and housed in a procedure room and injected once daily for ten days with ethanol (2.0 g/kg, i.p., n=4 per genotype). On the eleventh day, mice were injected with ethanol (2.0 g/kg) and placed into holding cages in an adjacent procedure room and sacrificed two hours later.

For assessing ethanol withdrawal, mice were also treated with ethanol (2.0 g/kg, i.p., n=3 per genotype) for ten days. On the eleventh day, following ethanol (2.0 g/kg) injection mice were placed back in their cages and left undisturbed. Mice were sacrificed eight hours later. This time point was chosen based on studies assessing ethanol withdrawal-related Fos-IR elsewhere [231]. All treatments and processing of all tissue was done with investigator blinded to genotype.

3.1.2 C-Fos Immunoreactivity

Immediately after euthanasia, mice were transcardially perfused and whole brains were dissected out and preserved via fixative as described in chapter two. 30 μm coronal sections were cut in series, using a 6:6 design (six identical series with each containing six sections) and stored at -20°C in cryoprotectant. For c-fos staining, free-floating sections containing the regions of

interest were wash three times with PBS and endogenous peroxidase activity was removed by incubating with 0.9% hydrogen peroxide for 10 min. After washing with PBSX (PBS with TritonX-100, 0.025%), sections were blocked for one hour in 4% normal goat serum (NGS) in PBSX. Sections were then incubated overnight with an antibody against c-fos (1:10,000, PC38, Oncogene Science Inc., Cambridge, MA) in 4% NGS. The following day, sections were washed 3x with PBSX and incubated for one hour with biotin-conjugated goat anti-rabbit IgG secondary antibody (1:2,000, NB-730B, Novus, Littleton, CO) in 4% NGS. Sections were then washed 3x with PBSX and incubated with an avidin-biotin complex (Vectastain ABC Kit™, Vector Labs, Burlingame, CA) for one hour. After washing sections, immunoreactions were visualized with 3,3-diaminobenzidine (DAB; Sigma, St. Louis, MO). Color reactions were terminated by washes with PB. Tissue sections were mounted on gelatin-coated slides and air-dried. Mounted sections were dehydrated using an ascending series of ethanol concentrations, and cleared using xyenes and coverslipped with Permount (Fisher, Pittsburgh, PA).

Sections from wildtype and knockin mice were processed in parallel in all reactions. Sections from saline and acute ethanol-treated animals were processed simultaneously. Sections from chronic ethanol-treated mice to assess tolerance were processed separately. If further comparisons needed to be made to verify initial analysis, additional immunoreactions were done with sections from acute ethanol-treated animals as a control. Sections from chronic ethanol-treated mice for assessing withdrawal were also processed separately.

Assessments of genotype and treatment(s) were done by visually comparing digital photographs. Methods for identifying regions of interest were based on established anatomic regions based on the neuroanatomic atlas by Watson and Paxinos [391]. Digital photographs were obtained using a Leica DFC300 FX digital color camera attached to a Leica DM5000 B microscope and the accompanying Leica Application version 2.6 software (Leica Microsystems, Wetzlar, Germany). Parameters for image acquisition were the same for both genotypes. Images were converted from color to gray-scale using Adobe Photoshop CS (v8.0, Adobe, San Jose, CA).

3.2 RESULTS

3.2.1 C-Fos Levels Following Acute Ethanol Exposure

To assess ethanol-induced alterations of Fos-IR in different brain regions, Fos-IR was compared after saline and acute ethanol exposure in the PVN, EW and DG (Figures 3.1-3.3, respectively). Very low or undetectable levels of Fos-IR was observed in the PVN or the EW in saline treated animals. No differences were observed in basal activity in these two regions with respect to genotype. After ethanol administration, Fos-IR was observed in both brain regions, as observed by the increase in nuclear localized punctate staining in all ethanol-treated samples compared to saline controls. No differences in genotype were observed.

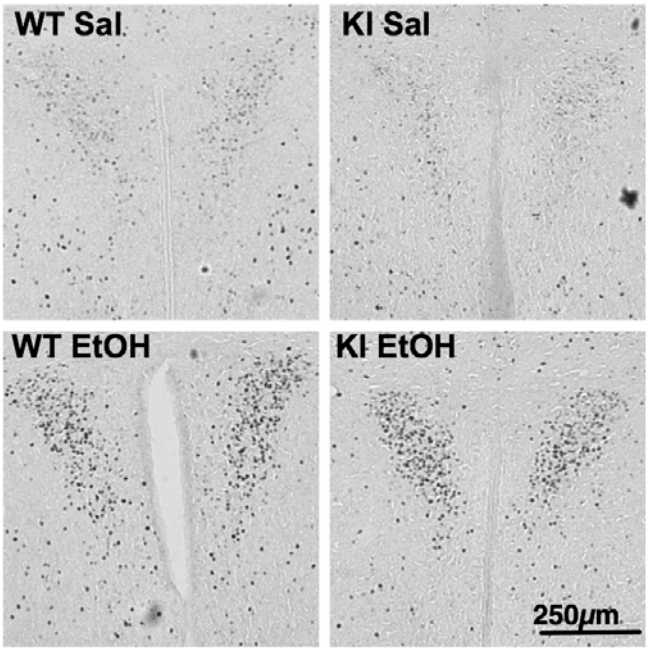

Figure 3.1. PVN Fos-IR Levels After Saline or Acute Ethanol.
Representative images of saline and acute ethanol-induced c-fos levels in the PVN. Administration of ethanol results in a robust increase in c-fos immunopositive cells. No observable genotype differences were detected. n = 2 per genotype per treatment.

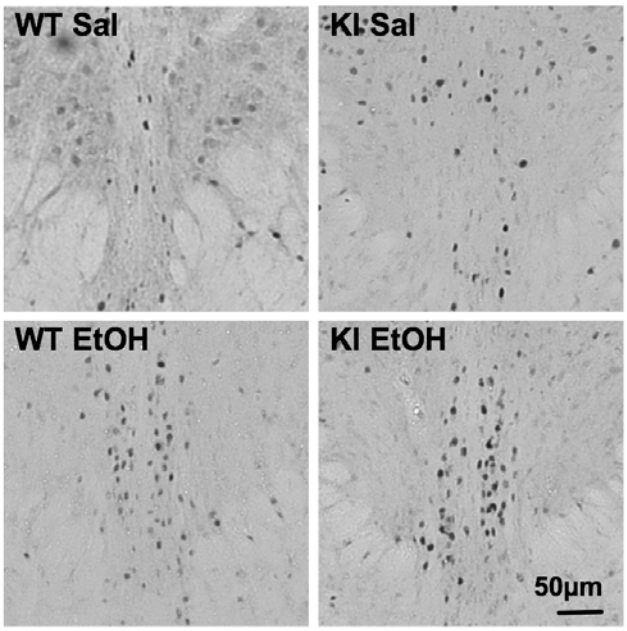

Figure 3.2. EW Fos-IR Levels After Saline or Acute Ethanol.
Representative images of saline and acute ethanol-induced c-fos levels in the EW. Administration of ethanol results in a robust increase in c-fos immunopositive cells. No observable genotype differences were detected. n = 2 per genotype per treatment.

In contrast, there appeared to be a reduced number of Fos-IR in the DG granule cell layer of saline treated knockin mice compared to wildtype mice. After acute ethanol administration, the number of Fos-IR appeared to further decrease in knockin mice compared to saline treated knockins. In fact, one of the two ethanol treated knockin mice displayed little to no c-fos immunopositive cells. In wildtype mice, it is not clear whether Fos-IR was decreased after acute ethanol administration.

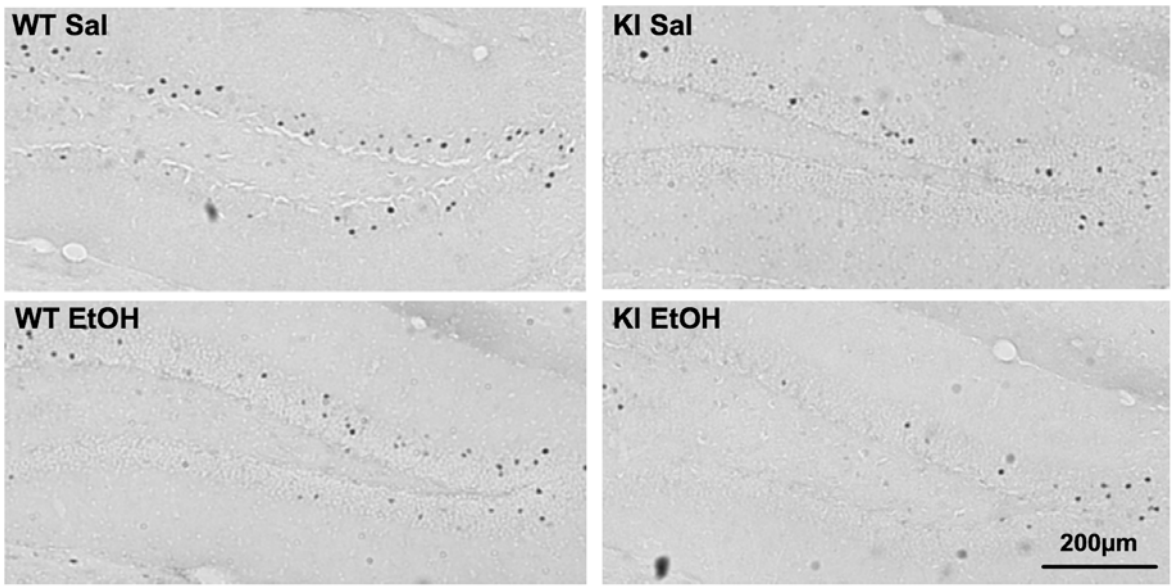

Figure 3.3. DG Fos-IR Levels After Saline or Acute Ethanol.
Representative images of saline and acute ethanol-induced c-fos levels in the DG. There appears to be a reduction in the number of cells in saline treated knockins compared to wildtypes. The number of c-fos immunopositive cells appears to be further reduced after ethanol exposure. It is not clear whether acute ethanol exposure resulted in decreased c-fos immunopositive cells. n = 2 per genotype per treatment.

Overall, these results suggest wildtype and knockin mice had similar acute ethanol-related Fos-IR in the PVN and EW, whereas in the DG knockin mice were more sensitive to ethanol-related Fos-IR alterations. However, genotype differences were observed in the DG in saline treated animals and may contribute to the ethanol-induced Fos-IR alterations.

3.2.2 C-Fos Levels After Repeated Ethanol Exposure.

Next, Fos-IR was assessed in mice after repeated ethanol exposure. Initial analysis in the PVN indicated that only one out of four mice in each genotype displayed Fos-IR (not shown). To verify that Fos-IR was reduced in animals repeatedly exposed to ethanol compared to animals acutely exposed to ethanol, sections were processed simultaneously. Fos-IR appeared to be reduced in animals repeatedly administered ethanol compared to acute ethanol (Figure 3.4). But no discernable differences in genotype were observed.

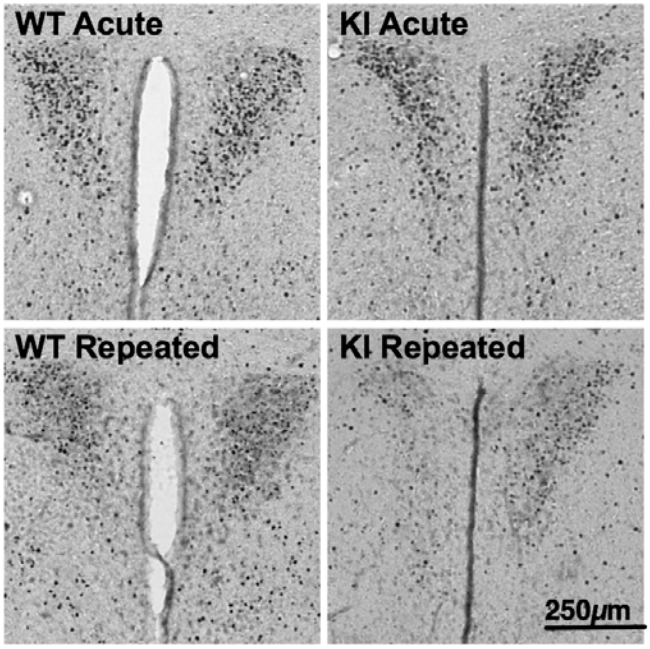

Figure 3.4. PVN C-Fos Levels After Repeated Ethanol Exposure.
Representative images of the PVN from mice given acute ethanol or repeatedly administered ethanol. The number of c-fos immunoreactive neurons appears to be decreased in animals repeatedly administered ethanol compared to those given a single ethanol exposure. No differences in genotype are observed. Repeated ethanol panels represent samples where some reduction was observed. However, it should be mentioned that other samples had little to no Fos-IR in both genotypes, whereas one sample from each genotype displayed Fos-IR similar to acute ethanol-exposed animals. n = 4 per genotype for repeated ethanol.

In the EW, initial analysis indicated that repeated ethanol exposure resulted in induction of large numbers of Fos-IR neurons (Figure 3.5). However, visual comparison of Fos-IR in the EW of mice repeatedly administered ethanol with mice acutely administered ethanol (Figure 3.2) did not appear to be different. Therefore, no further analysis was done.

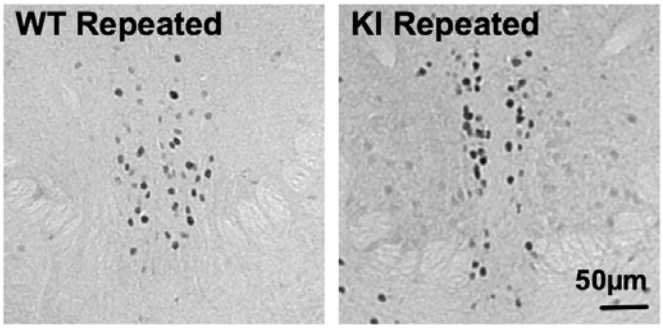

Figure 3.5. EW Fos-IR Levels After Repeated Ethanol Exposure.
Representative images of the EW from repeatedly administered ethanol animals. Repeat ethanol administration appears to cause an increase in increase in c-fos immunoreactive cells. No difference in genotypes is observed. n = 4 per genotype.

In the DG granule cell layer, initial analysis of mice repeatedly administered ethanol appeared to have trace amounts of Fos-IR (not shown). Sections from mice repeatedly administered ethanol also appeared to be reduced compared to Fos-IR observed in saline and acute ethanol treated mice in Figure 3.3. To further verify this reduction, sections from mice acutely and repeatedly administered ethanol were processed simultaneously. Repeatedly exposing mice to ethanol resulted in decreases in Fos-IR in both genotypes compared to acutely exposed animals (Figure 3.6). Knockin mice repeatedly administered ethanol appeared to have a greater decrease in Fos-IR compared to wildtype mice, with little to no Fos-IR being observed in knockin samples. This greater reduction in Fos-IR neurons of knockin mice may be due to the reduced levels of Fos-IR initially observed in knockin mice. However, in one of the four knockin mice, the entire granule cell layer of the DG displayed high amounts of Fos-IR (not shown). Overall, while repeated ethanol exposure decreased Fos-IR in the DG of both genotypes, knockins appeared to be more affected.

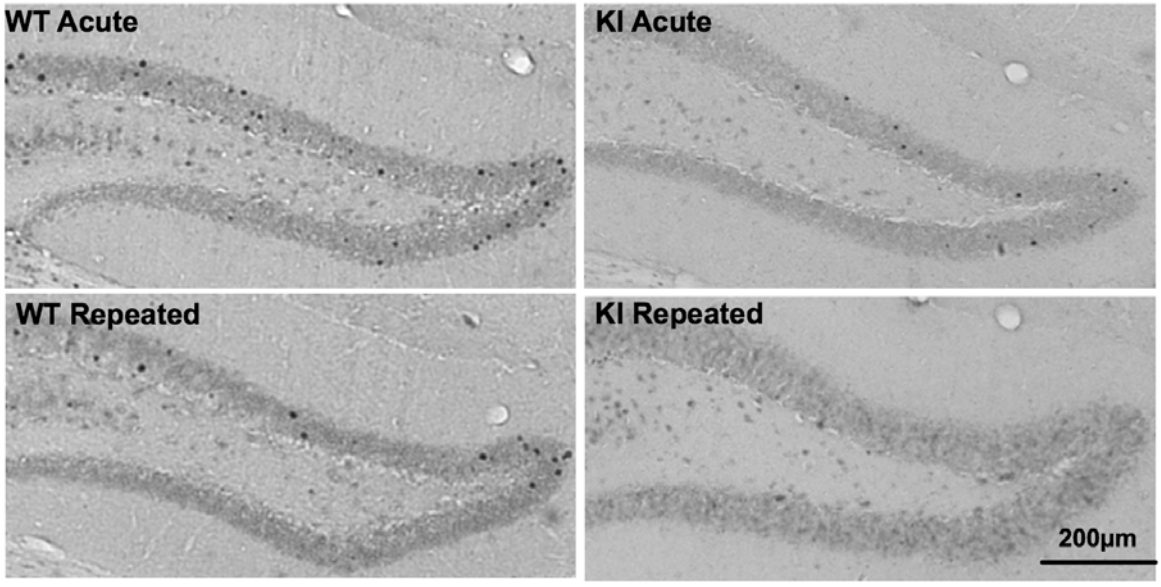

Figure 3.6. DG Fos-IR Levels After Repeatedly Administered Ethanol.
Representative images of the DG from acute and repeatedly administered ethanol animals. Repeated ethanol appears to reduce the number of c-fos immunoreactive cells compared to acute ethanol exposure. The level of c-fos immunoreactive cells appears to be reduced (acute) and/or ablated (repeated) compared to controls. n = 4 per genotype for repeated ethanol.

Thus, repeated ethanol exposure resulted in a concomitant decrease in Fos-IR in the PVN compared to acute ethanol exposed animals. In the DG, Fos-IR was also decreased following repeated ethanol compared to acute ethanol, with knockin mice being more affected than wildtypes. Conversely, Fos-IR in the EW did not change compared to acutely exposed animals. Overall, these results suggest that chronic ethanol exposure results in differential changes in Fos-IR depending on the brain region. Additionally, knockin mice were possibly more affected in the DG than wildtypes.

3.2.3 C-Fos Levels During Ethanol Withdrawal

Lastly, both genotypes were assessed to determine whether altered changes in Fos-IR developed during the withdrawal phase after chronic ethanol exposure. Interestingly, initial analysis indicated little to no Fos-IR was observed in the PVN eight hours following the last ethanol exposure in either genotype (Figure 3.7, left panels). However, Fos-IR was observed elsewhere in the sections such as the cortex, thereby indicating that the absence of Fos-IR in the PVN was not due to nonfunctional immunoreactivity. Conversely, Fos-IR was observed in the EW during the withdrawal phase (Figure 3.7, middle panels), but there appeared to be no genotype differences. This appeared to be reduced compared to acute and chronically treated mice (Figures 3.2 and 3.5, respectfully), although direct comparisons were not made. In the DG granule cell layer (Figure 3.7, right panels) low amounts of Fos-IR were present in wildtype sections that appeared to be comparable to wildtype mice repeatedly administered ethanol (Figure 3.6). Strikingly, no Fos-IR was observed in the DG granule cell layer in knockin mice. This also appeared to be similar to be similar to knockins repeatedly administered ethanol, although a few mice from the later group did display trace amounts of Fos-IR.

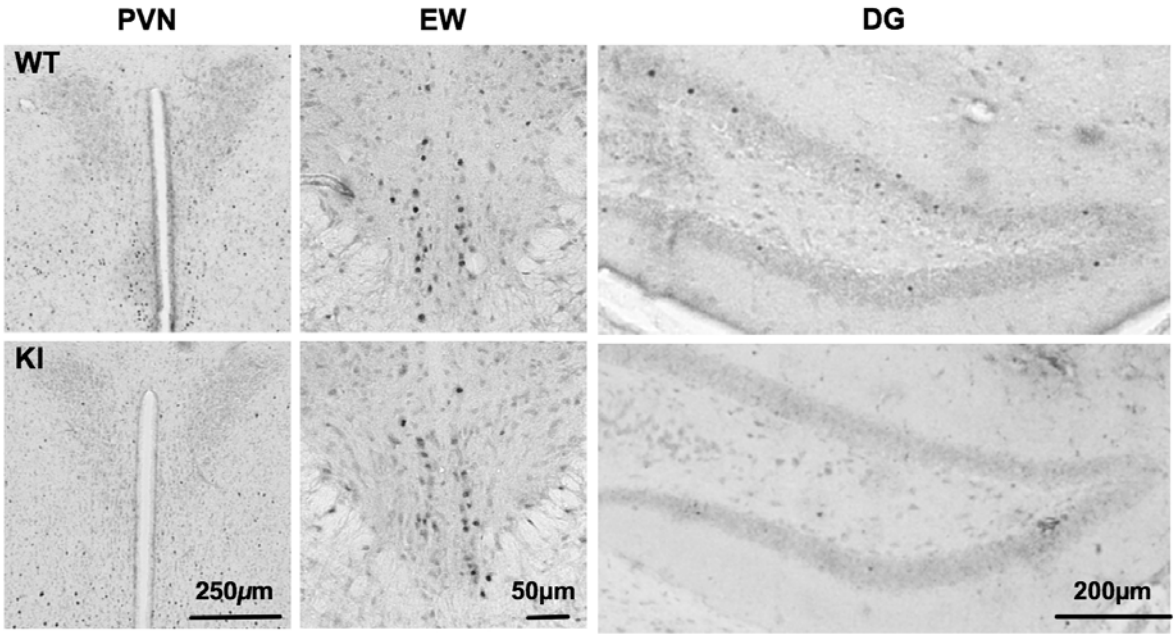

Figure 3.7. C-Fos Levels During Ethanol Withdrawal.
Representative images of wildtype (WT, top) and knockin (KI, bottom) mice eight hours following the last ethanol treatment. No Fos-IR was observed in the PVN of either genotype (left panels). Fos-IR was observed in the EW in both genotypes (middle panels). In the DG, low amounts Fos-IR was seen in wildtype mice, but not in knockins. n = 3 per genotype.

Thus, results from animals during ethanol withdrawal suggest that little Fos-IR was observed in the PVN and DG in both genotypes with knockin mice being more affected in the DG; whereas some Fos-IR was observed in the EW in both genotypes. As with chronic ethanol, ethanol withdrawal causes differential changes in Fos-IR depending on the neuroanatomic region.

3.3 DISCUSSION

Although Fos-IR has been used to dissect regions of importance in ethanol action, the use of this technique in assessing the role that specific genes play in ethanol action has been limited. To date, determining genes involved in ethanol-related Fos-IR has been done with inbred- and selectively-bred lines of mice, and one gene targeted model [241, 244, 249, 252]. In the present report, the PVN, EW, and DG in wildtype and ethanol-insensitive α1 $GABA_A$-R knockin mice

were assessed in response to three ethanol exposure paradigms. These results (summarized in Table 1) indicate that ethanol caused bidirectional changes in Fos –IR, depending on region and time of ethanol exposure and that differences in genotype were observed.

In the PVN, no genotypic differences were observed. Acute ethanol robustly increased Fos-IR, which appeared to decrease in some instances during repeated ethanol exposure, and none was observed during withdrawal. These results are consistent with previously published reports. Numerous studies have indicated that ethanol at various doses can increase Fos-IR in this region [e.g., 231, 235, 392], whereas ablation of Fos-IR occurs during repeated ethanol exposure [e.g., 235]. Interestingly, the latter can occur in as short a period as three days and have long lasting effects [246].

Table 3.1. Summary of Ethanol-Related Fos-IR.
Fos-IR in the various brain regions of each genotype after a single acute ethanol exposure, repeated ethanol exposure, or 8 hours following the last repeated ethanol exposure. All arrows are compared to saline controls. No differences were found in controls between genotypes except in the DG. ↑↑ - substantial increase; ↑ - moderate increase; ↑= - slight increase/no change; = - no change; ↓= - slight decrease/no change; ↓ - moderate decrease; ↓↓ - substantial decrease; n.d. - not detectable. Red (control) and blue (knockin) represent genotype differences.

	Wildtype			**Knockin**		
	PVN	EW	DG	PVN	EW	DG
Acute Ethanol	↑↑	↑↑	↓=	↑↑	↑↑	↓
Repeated Ethanol	↑=	↑↑	↓	↑=	↑↑	↓↓
Withdrawal	=	↑	↓	=	↑	n.d.

One caveat is that environmental factors should be taken into consideration when interpreting PVN Fos-IR after chronic ethanol treatment. Animals given an acute treatment of ethanol in their homecage environment did not exhibit increased Fos-IR whereas those given an acute injection paired with a novel environment had increased Fos-IR in the PVN [231]. In support of this, animals given ethanol in a different environment after chronic ethanol administration developed less tolerance to Fos-IR in the PVN than animals treated in the same environment [250]. These studies are in agreement in that mice treated in a novel environment with acute ethanol had robust increases in Fos-IR. The current work did not address Fos-IR in

the PVN while in the home-cage environment, all mice were treated in a novel environment. Additionally, although mice repeatedly administered ethanol here did show decreased Fos-IR compared to acute ethanol mice, one out of four mice in each genotype remained sensitive to ethanol-induced Fos-IR. Because mice were moved to an adjacent room for their last ethanol exposure (novel environment), this agrees with environmental factors playing a role and may be why ablated Fos-IR was not observed in this region in all mice as in others studies [235, 246].

The PVN is well known to be part of the hypothalamic-pituitary-adrenal axis (HPA), which can regulate many physiologic processes including stress/anxiety-related responses. Because withdrawal-related effects of ethanol include heightened anxiety states [393] and increased susceptibility to stress[248], it was predicted that increased Fos-IR in the PVN would be observed during withdrawal states. However, this was not the case. One possibility could be that the doses employed and or the length of exposure was not sufficient to cause withdrawal-related responses or that the mice were not assessed at an appropriate time point following the last ethanol exposure. However, elsewhere increased Fos-IR has not been observed during withdrawal from high concentrations of ethanol exposure [233, 253] in control mice. However, PKC-epsilon knockout mice did display robust increases in the PVN during ethanol withdrawal; but the same report also had reduced withdrawal-related hyperexcitability.[249] It is also possible that Fos-IR is not necessarily a major immediate early gene induced during ethanol withdrawal states. Other ethanol withdrawal-related studies have taken advantage of other immediate early transcription factors such as zif268 [253].

Alternatively, the absence of Fos-IR in the PVN could be due to the fact that the PVN is not active during the ethanol withdrawal phase unless triggered by exogenous events that increase stress/anxiety. While other studies have shown that withdrawal-related Fos-IR was not observed in the PVN until an external stressor, Fos-IR was dramatically increased in this region [233]. The induction of Fos-IR in the PVN after a stress/anxiety-related event may be related to corticosterone (CORT) and/or adrenocorticotropic hormone (ACTH) levels. Interestingly, some studies have reported increased CORT and/or ACTH levels during ethanol withdrawal phases whereas others have not [253, 394-397]. Although CORT and ACTH levels were not assessed here, it would be interesting to assess them in these mice especially during ethanol withdrawal in the presence or absence of stress/anxiety.

Ethanol-induced Fos-IR after single and repeated ethanol exposures was observed in the EW. Some Fos-IR was observed during the withdrawal-related period. This was not unexpected in wildtype mice as Fos-IR was readily observed in this region after acute ethanol exposure as well repeated ethanol exposure [231]. Additionally, studies have shown that although novel environments will slightly increase Fos-IR in the EW, increased Fos-IR was seen regardless of environment [231]. Also, although Fos-IR was seen during acute withdrawal [252], to my knowledge Fos-IR in the EW during withdrawal from chronic ethanol has not been reported elsewhere. Nonetheless, the withdrawal following repeated ethanol results are in agreement with acute withdrawal results. Interestingly, although α1 appears to be the most abundant α subunit in the EW based on immunohistochemical analysis, no differences were detected in knockin mice in any of the ethanol-related measures. Again, it is possible that the dose of ethanol employed may not have allowed us to detect any differences. Ryabinin et al. [228] have posited that while low doses may primarily involve GABAergic processes, higher doses may involve the interplay of GABAergic as well as non-GABAergic effects. In which case, any involvement of α1 may possibly be masked by other molecular mechanisms. Indeed, studies have demonstrated that hypothermic effects of ethanol may possibly be related to this region [238]. As adenosine receptors have been implicated in ethanol-induced hypothermia [398], it is possible that they may also contribute to Fos-IR induction in the EW. However, further experiments would be necessary to substantiate this relationship.

Genotypic differences were detected in Fos-IR in the DG of the hippocampus in saline treated animals as well as all ethanol-treatment paradigms. Saline treated knockin mice had lower Fos-IR than wildtype mice. Therefore basal behavioral differences (discussed below) may possibly be attributed to the DG. Knockin mice were also more sensitive to ethanol-related Fos-IR reductions after acute alcohol exposure whereas wildtype mice did not appear to be affected. However, quantitative analysis would have to be carried out to determine if reductions were truly present/absent in wildtype mice. Although repeated ethanol exposure decreased Fos-IR in the DG in both genotypes, knockins were more severely affected in that most Fos-IR was ablated in the knockins. Similarly, during ethanol withdrawal, wildtype mice had decreased Fos-IR, but knockin mice were more affected in that no Fos-IR was observed. Overall, results in wildtype mice agree with previously published results in which acute ethanol treated animals did not display decreased Fos-IR [231], whereas repeated ethanol exposure did decrease Fos-IR [399].

It is possible that decreased Fos-IR in the DG may be related to increased cell death, but further experiments would be needed to clarify this possibility.

Unlike wildtype mice, knockins displayed enhance sensitivity to ethanol-related Fos-IR reductions in the DG granule cell layer. However, it is not clear to what extent basal differences in genotype contribute to the differences in DG ethanol-related Fos-IR. However, quantitative assessment with statistical analysis may better address this issue. However, an in depth analysis would involve performing stereology on each brain region, which was beyond the scope of the current experiments. The possibility also exists that specific populations of neurons within the DG may mediate in part the observed alterations in Fos-IR. Populations of interneurons in the DG, specifically parvalbumin-positive cells, contain α1-$GABA_A$-Rs [377, 400]. Further studies would need to be done to determine if Fos-IR colocalizes with parvalbumin-positive cells.

The DG has been implicated in various behaviors, and it is tempting to speculate whether the observed differences in Fos-IR in the brain region correlate with any basal or ethanol-related behaviors. Of importance, as a well-known region in memory, basal and ethanol-impaired cognition may be affected. Studies assessing cognitive function have indicated a memory deficit in this knockin mouse model [369]. Nonetheless, other behaviors attributed to this region include anxiety-related behavior [18, 401, 402] and seizures/convulsions [403]. With the latter, it is interesting to note that ~10% of knockin mice may experience spontaneous seizures. Incidentally, in knockins used here, ~11% had substantial Fos-IR in the DG granule cell layer – remarkably similar to models of spontaneously-induced seizures observed elsewhere [403]. Another possibility may be that the increased seizure susceptibility may result from the knockin mice being in a 'pre-kindled' state. Indeed, the genotype differences in Fos-IR in the DG supports this possibility.

It is not understood why this increased sensitivity to Fos-IR is observed in knockins. If ethanol acts via $GABA_A$-Rs by enhancing the effects of GABA thereby increasing hyperpolarization and decreasing cell activity, then it would be predicted that ethanol would decrease Fos-IR. Along this rationale, wildtype mice would be more susceptible to ethanol-related reductions in Fos-IR than knockins – but these observations in the DG suggest otherwise. Similarly, it is not understood why increases in Fos-IR were observed in other areas. However, it has been suggested that blocking 'feed-forward' inhibition may result in activation of other pathways [245]; in other words, other neuronal circuits that are normally silent become active.

While the current work has yielded some valuable insight into α1 $GABA_A$-R involvement in Fos-IR and potentially which neuroanatomic regions may be involved in α1 $GABA_A$-R mediated ethanol-induced behaviors, some caveats exist. The current work only qualitatively assessed gross observations. Quantification of Fos-IR in each region would allow for more definitive comparisons. Secondly, this analysis was limited to only three brain regions. Although beyond the scope of the current study, valuable insight into the involvement of other brain regions would allow for a more comprehensive understanding of altered regions with respect to α1. Lastly, not all immunoreactivity (i.e., controls, acute, chronic, withdrawal) occurred simultaneously. Therefore cross comparisons between some treatment groups, such as control vs. chronic ethanol, control vs. withdrawal, or acute vs. withdrawal, should be cautiously interpreted. Future experiments should be done by processing sections simultaneously so as to permit more valid comparisons.

In conclusion, α1-$GABA_A$-Rs are involved in Fos-IR in the DG, but not in the PVN or EW. Furthermore, results herein support the hypothesis that α1 is involved in Fos-IR and also points to α1 involvement in specific brain regions, but not others. Overall, Fos-IR and other immediate-early genes remains a powerful tool to dissect genetic involvement in region specific areas. Further implementation of this technique with other genetically modified mouse models can give better insight as to which genes are involved in specific brain regions and potentially correlate altered Fos-IR in brain regions to specific ethanol-related behaviors.

4.0 DETERMINING ROLE OF α1 $GABA_A$-RS IN CHRONIC ETHANOL-INDUCED TOLERANCE AND DEPENDENCE

The driving force that propels individuals toward alcoholism is not known. While environmental factors play a role in alcoholism, much work suggests there is also a significant genetic component [11, 12] with numerous genes involved [404, 405]. Although the elicited effects of ethanol on behavior are well known, the molecular mechanism of ethanol action remains unclear. While many genes have been studied in acute ethanol-related behaviors, fewer studies have assessed tolerance and withdrawal-related effects after chronic ethanol exposure. This is surprising, given the correlation between tolerance, dependence and alcoholism [5, 75, 76]. As no concrete relationship exists between sensitivity, tolerance and withdrawal, different genes may mediate different aspects of the spectra of alcohol-related behaviors.

$GABA_A$-Rs have been highly implicated in alcoholism [293, 294, 303, 406, 407] and in the molecular mechanism of ethanol action [351, 408]. Because of the numerous $GABA_A$-R subunits that exist, the role of specific $GABA_A$-R subtypes in alcohol action and alcoholism has been elusive. Nonetheless, the role of some candidate genes with respect to tolerance and dependence have been assessed in a limited fashion. For instance, in rodents, gene knockouts of α6 and δ $GABA_A$-Rs have been assessed on acute functional tolerance, protracted tolerance to loss of righting and handling-induced convulsions [331, 335]. α1-containing $GABA_A$-Rs have been suggested to play a role in the development of alcoholism. Genetic analysis of drinking behavior in alcoholics has suggested α1 as a candidate gene for alcoholism [292, 306, 409]. Similarly, quantitative trait locus studies in rodents have also reported chromosome 11 (which contains α1) in ethanol withdrawal behavior [291, 410]. Additionally, studies also report decreases in α1 protein in the brain following chronic ethanol exposure [e.g., 363]. Therefore, it is hypothesized that α1 $GABA_A$-Rs mediate in part ethanol-related tolerance and dependence following chronic ethanol exposure.

In this report, the contribution of α1-containing $GABA_A$-Rs to tolerance and dependence from chronic ethanol was examined on numerous ethanol-related behaviors in wildtype control mice and in genetically engineered mice with ethanol-insensitive α1 $GABA_A$-Rs [355]. Additionally, because α1 levels decrease following chronic ethanol exposure [e.g., 363], α1 levels were also examined to determine whether this reduction was dependent on ethanol action through α1 $GABA_A$-Rs.

4.1 MATERIALS AND METHODS

4.1.1 Mouse Production

Wildtype and knockin mice were produced as described in chapter 2. Briefly, wildtype and knockin littermates were derived from heterozygous matings. All mice were of a mixed C57BL/6J × Strain 129SvJ background of the F7 – F11 generations. Mice were between 8 – 14 weeks of age. For all experiments, male and female mice were used, and all mice were ethanol and experimentally naïve. Minimal handling of the animals occurred durng all experimental procedures. Gender was initially analyzed for all experiments. If no overall effect of gender was observed, data were collapsed for further analysis. All treatment and experimental procedures were conducted between the hours of 9:00 and 13:00 with the exception of the final nociception measurement, which was performed 12 h later.

4.1.2 Loss of Righting Reflex (LORR)

Mice were repeatedly administered either saline or ethanol (2.0 g/kg, i.p., Pharmco, Brookfield, CT) once daily for 10 days. On day 11, all mice were given ethanol (3.5 g/kg, i.p.) and tested for the sedative/hypnotic effects of ethanol. Immediately following injection, mice were observed for LORR. When this occurred, mice were placed on their backs in v-shaped troughs and monitored until they were able to right themselves three times in a 30 sec period. LORR was determined as the length of time from when the mouse was placed in a supine position until it

was able to right itself. All mice were included in the analysis. Data were analyzed using 2-way ANOVA.

4.1.3 Rotarod and Hypothermia

Mice were repeatedly administered ethanol (2.0 g/kg, i.p.) once daily for 11 days, and motor ataxia and hypothermia were assessed on day 1, day 6 and day 11. To assess the motor ataxic effects of ethanol, mice were trained to stay on a fixed speed (6.0 rpm) rotarod (Ugo Basile Rota-Rd, model 7650; rod size = 6cm, Stoelting Co., Wood Dale, IL) for 60 sec immediately prior to ethanol treatment on each test day. 15 min following ethanol injection (2.0 g/kg, i.p.), each mouse was placed back on the rotarod and the elapsed time that they remained on the rotating rod was measured up to 60 sec. Mice were retested every 15 min for 75 min and area under the curve was calculated.

To assess hypothermic effects of ethanol, rectal body temperatures were measured via a digital thermometer (Thermalert Model TH-8 with probe RET-3; Physiotemp Instruments, Clifton, NJ). On each test day, basal body temperatures were recorded prior to testing (on days 1, 6 and 11) as well as at each time point immediately following ataxic measurements.

Data for both ataxia and hypothermia were assessed using repeated measures ANOVA with time post-injection as the repeated measure factor and genotype as the between-group factor. Student's t-test was used for further analysis if necessary.

4.1.4 Radiant Tail-Flick

The radiant tail-flick assay was used as described [57]. Mice were lightly restrained in a soft denim cloth pouch which they would enter after a few acclimating trials. The mice would enter the pouch 'head first' so that the tail was exposed. A focused beam of light from the tail-flick apparatus (IITC Life Sciences, Woodland Hills, CA) was directed at a spot ~1 cm from the tip of the tail and the latency to remove tail was recorded via a sensor triggered upon removing the tail. Light intensity was set to a low-moderate intensity that yielded average cutoff times of ~10 sec in preliminary experiments. A maximum cutoff time of 30 sec was used to prevent tissue damage. A minimum of 2 readings was taken for each mouse at each test and the means

calculated. Mice were assessed for basal responses 1 day prior to ethanol exposure. Mice were then given ethanol (2.0 g/kg, i.p.) once daily for 11 days. Ethanol-related effects were assessed 30 min after ethanol injection on day 1 and day 11, as well as 10-12 hours following the last ethanol exposure. Data were analyzed by repeated-measures ANOVA.

4.1.5 Elevated Plus Maze and Light-Dark Box

The elevate plus-maze and the light-dark box were used to assess the anxiety-related effects of ethanol and locomotor activity. Mice were transported to the procedure room one day prior to the start of the experiment. On the following day, mice were treated with ethanol (2.0 g/kg, i.p.) or saline once daily for 10 days. On the eleventh day, mice from both treatment groups were split: mice previously given saline were either given saline (control group) or ethanol (0.75 g/kg, i.p.; acute ethanol group) while mice previously given ethanol were given either ethanol (chronic ethanol group) or saline (withdrawal group).

For the elevated plus maze, on day 11, 10 min following injection mice were placed on the center platform of the maze facing an open arm and allowed to freely explore the maze for 5 min. Open and closed arm entries were recorded as well as time spent in both. A mouse was considered to be on the center platform or to have entered an arm when all four paws were within its perimeter. If a mouse fell off the platform, it was immediately placed back on where it had fallen off.

For the light-dark box, a Coulbourn Truscan arena (25.4 x 25.4 x 40.64 cm) without the photobeam sensors was used with the accompanying light/dark box insert. Mice were placed in the apparatus on the light side facing away from the entry connecting the two sides. The time spent on the light and dark sides, latency to enter the dark side, time to emerge from the dark side and the number of transitions between sides were recorded. A mouse was considered to be on a side when all four paws were within its perimeter.

The acute, chronic, and withdrawal groups were individually compared to the control group on both assays. Data were analyzed using ANOVA with post hoc (Student's t-test) where necessary. All control groups were assessed using one-way ANOVA.

4.1.6 Ethanol Withdrawal-Related Hyperexcitability

To elicit withdrawal from chronic exposure to high concentrations of ethanol, ethanol vapor chambers similar to those described by Goldstein [157, 168] were used coupled with the multiple withdrawal paradigm developed by Becker and Hale [171]. Mice were treated with the alcohol dehydrogenase inhibitor pyrazole (68 mg/kg, Sigma, St. Louis, MO) and a booster dose of ethanol (1.5 g/kg, i.p.) and placed in chambers for a 16 h ethanol vapor exposure period followed by 8 h of recovery. Ethanol was dripped into a 55°C flask at a flow rate of ~120-140 µl/min by a peristaltic pump (Model 75 23-30; Cole Parmer Instruments, Chicago, IL) where it was vaporized and was delivered to a sealed Plexiglas chamber with air at 8 L/min. This procedure was repeated for a total of 3 cycles. Control mice were treated in parallel but were injected with saline instead of ethanol and were placed in chambers with normal air. All injection volumes for pyrazole, ethanol and saline were 0.01 ml/g body weight. At the end of the third 16 h ethanol exposure, blood samples (~50µm) were taken and assessed for blood ethanol concentrations (BECs) using a standard enzymatic spectrophotometric method described in Chapter 2. Ethanol-treated mice that had BECs <50 mg/dl or >300 mg/dl were excluded.

Mice were scored for handling-induced convulsions (HIC) starting 1 h following termination of the last cycle of ethanol exposure. The mice were scored using the scale described elsewhere [171]. Briefly, the mice were scored was as follows: 0 – No response after pick up or gentle 360° spin; 1 – facial grimace after gentle 360° spin; 1.5 – facial grimace on pick up; 2 – tonic convulsion after gentle 360° spin; 3 – tonic/clonic convulsion after gentle 360° spin; 4 – tonic convulsion on pick up; 5- tonic/clonic convulsion on pick up, may be delayed by a few sec; 6 – severe tonic/clonic convulsion on pick up, no delay and often lasting after mouse was released, and; 7 – severe tonic/clonic convulsion prior to pick up, may result in death. Scoring was done hourly for 10 hours and then again the following day at 24 h. Scoring was done by two observers blind to genotype and treatment. If scores differed between observers, the mean of the two scores was used. Area under the curve (AUC) was calculated and data were analyzed by ANOVA. Kaplan-Meier (JMP; SAS, Cary NC) was used to assess survival. Because many mice unexpectedly died during the assay, a modified scoring procedure was implemented. If mice died during the scoring period, they were assigned a HIC score of 7. If mice died during the assay prior to the scoring period, the mice were assigned a score of 8.

Otherwise, peak HIC withdrawal scores from the 24h scoring period were used. Data were analyzed using the nonparametric Mann-Whitney U-test (Statview; Abacus, Berkeley, NC).

4.1.7 α1-GABA$_A$-R Immunoblotting Analysis

Mice were repeatedly administered ethanol (2.0 g/kg, i.p.) or saline once per day for 11 days. Mice were euthanized with an overdose of isoflurane and brains were rapidly dissected and flash-frozen on dry ice, and stored at -80°C. P2 membrane fractions from cortex tissue were processed and protein was quantified using a bicinchoninic acid method as in Chapter 2. Aliquots of 25 µg of protein from each sample were separated by electrophoresis on 10%-SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Wildtype and knockin samples were run on separate gels with investigator blinded to treatment. Detection of α1 was with a polyclonal specific rabbit anti-GABA$_A$-R α1 antibody (1:10,000; 06-868, Upstate, Lake Placid, NY; now with Millipore, Billerica, MA) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (1:5000; NB-730H, Novus, Littleton, CO). Bands were visualized by enhanced chemiluminescence (Western Lightning; PerkinElmer Life and Analytical Sciences, Boston, MA). For normalization, blots were stripped using Re-blot (Chemicon International, Temecula, CA) and reprobed with a rabbit anti-β-actin polyclonal antibody (1:10,000, ab8227-50; Abcam). Multiple exposures of each membrane were done to make sure signal was within the linear range of the film. Band intensity was measured densitometrically (Kodak 1D software; Eastman Kodak, Rochester, NY). Data were analyzed using Student's t-test.

4.2 RESULTS

4.2.1 Repeated Ethanol on Loss of Righting Reflex

LORR was assessed to determine α1-GABA$_A$-R involvement in tolerance to ethanol's hypnotic effects after chronic ethanol exposure. Mice were repeatedly treated with moderate doses of

ethanol or saline for 10 days and LORR to a hypnotic dose of ethanol was assessed on day 11. Repeated ethanol exposure resulted in tolerance to the ethanol's hypnotic effects (Figure 4.1) as evidenced by a reduced duration of LORR in ethanol treated mice compared to saline treated mice ($F_{1, 82} = 33.17$, $p < 0.0001$). No significant effect of genotype, or genotype by treatment interaction was found. Additionally, the number of repeated ethanol treated mice failing to respond to the hypnotic effects of ethanol (i.e., no sleeptime) did not appear to differ between

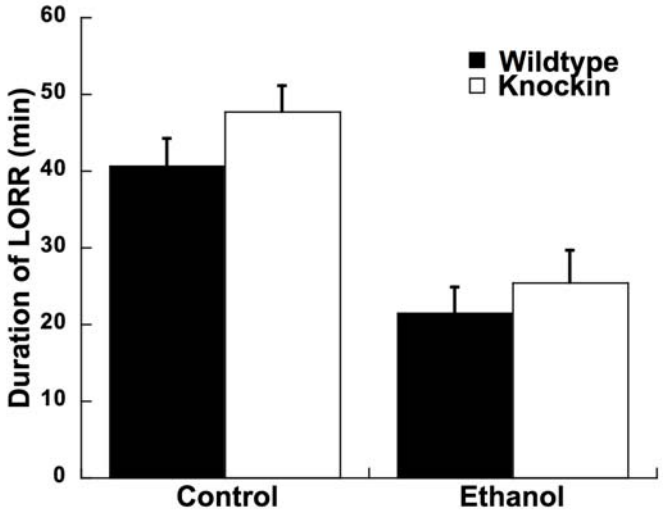

Figure 4.1. Hypnotic Effects of Ethanol Following Chronic Ethanol Exposure.
Mice were treated with saline or ethanol (2.0 g/kg) once daily for 10 days and LORR in response to a hypnotic dose of ethanol (3.5 g/kg) was assessed on day 11. Tolerance to the hypnotic effects of ethanol was observed after repeated ethanol exposure compared to saline as evidenced by the decreased duration of LORR. A significant main effect of treatment was observed ($p<0.0001$), but genotype or genotype x treatment interaction was not significant. Data are presented as mean ± SEM. n = 18 -23 per group.

genotypes (5/23 control and 3/22 knockins). Because it was unclear if the mice failed to respond because of extreme tolerance or because of misplaced ethanol injection, all mice were included in the analysis. The total number of mice not responding to ethanol's hypnotic effects was much higher in all ethanol treated animals (8/45) than saline treated animals (1/37), thereby also suggesting that tolerance was observed. Thus, although tolerance to the hypnotic effects of ethanol was observed, $\alpha 1$ does not affect the development of tolerance to this behavior.

4.2.2 Repeated Ethanol Effects on Ataxia and Hypothermia

To determine if α1-containing $GABA_A$-R are involved in tolerance to the motor ataxic effects of ethanol, mice were tested using the fixed speed rotarod. Mice were treated every day with ethanol (2.0 g/kg, i.p.) for 11 days and tested on days 1, 6 and 11 for motor ataxia. Area under the curve (AUC) was used to compare recovery from ethanol-induced ataxia and was calculated as the area between baseline at 60 sec and recovery curves from ethanol insult (see Figure 4.2 for time-course of motor recovery on days 1, 6, and 11). This analysis revealed a significant main effect of time ($F_{2, 76} = 36.9$, $p < 0.0001$) and the interaction of time x genotype ($F_{2, 76} = 4.7$, $p < 0.05$), but no effect of genotype (Figure 4.3A). Further analysis to determine where the interaction occurred revealed that genotypes differed in recovery from motor ataxia on day1 and day 11. Knockin mice recovered more quickly than controls on day 1, but recovered less quickly than controls on day 11. Because genotypes initially differed on recovery from ethanol-induced ataxia (day1), to better compare the effects of ethanol within and between genotypes, all mice were normalized to the ethanol-ataxia observed on day 1 (i.e., Recovery on day1 was set to 100%; Figure 4.3B). Analysis of AUC expressed as a percent of day1 revealed a significant main-effect of time ($F_{2, 76} = 31.1$, $p < 0.0001$). Further analysis of day6 and day11 revealed that AUC's were decreased in comparison to day 1 ($p < 0.01$), again suggesting the development of tolerance. This analysis also indicated there was a trend toward significance with respect to a main effect of genotype ($F_{1,38} = 3.6$, $p = 0.066$). The suggestive difference for genotype hints that knockin mice developed less tolerance than wildtype mice. To further explore this possibility, genotypes were compared. Knockins were more impaired on day11 as evidenced by the greater AUC compared to controls ($p < 0.01$). Overall, these data suggest that knockin mice had decreased sensitivity to ethanol's motor ataxic effects on day 1 and displayed decreased tolerance after 11 days of chronic ethanol exposure.

Additional experiments using mice injected with saline for 10 days and challenged with ethanol were carried out to assess if training prior to ethanol intoxication had a significant effect on performance while in an intoxicated state. Motor performance of saline treated mice given ethanol on day 11 did not differ when compared to mice given ethanol on day 1 (data not shown).

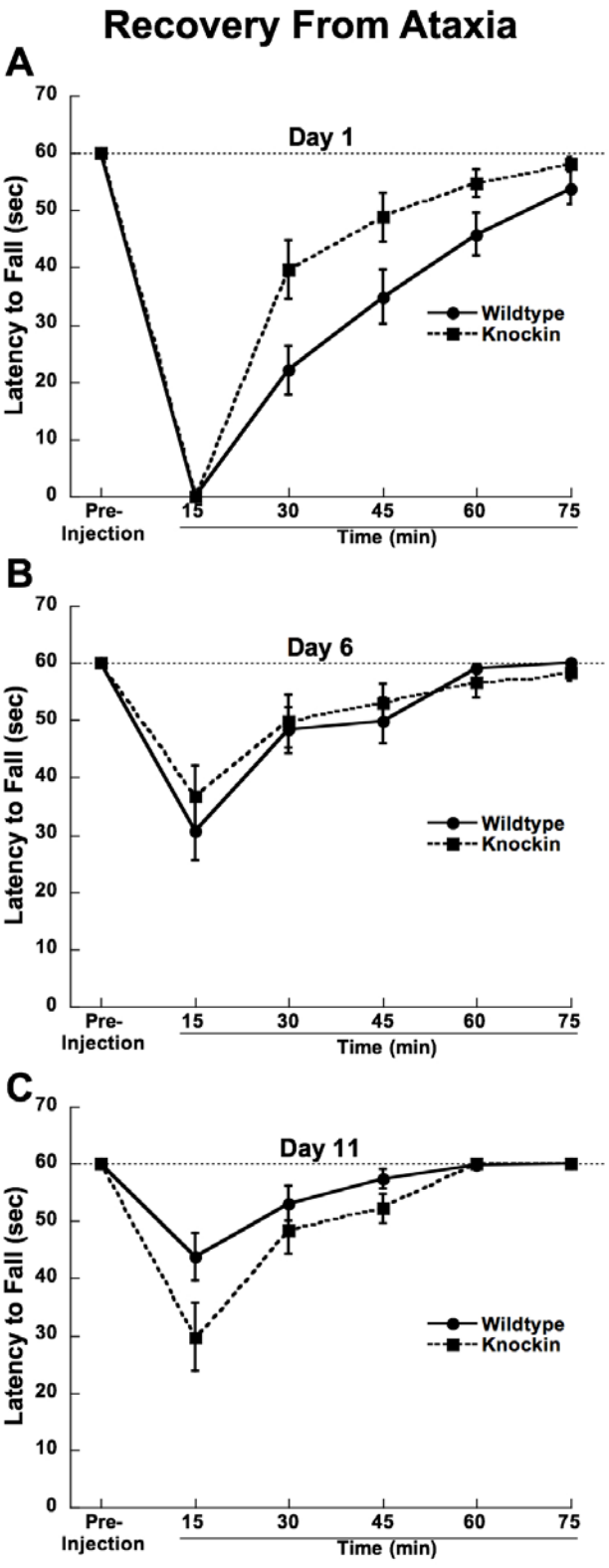

Figure 4.2. Recovery From Ethanol Motor Ataxia.
Recovery from ethanol-induced motor ataxia on day 1(A), day 6 (B) and day 11 (C). Note that before injection mice remained on the rotarod for the 60 sec criteria. After ethanol injection (2.0 g/kg) mice were impaired, but were able

to stay on the rotarod longer as time elapsed. Note that as daily treatments progressed, mice were less impaired following ethanol exposure and recovered more quickly. The area under curves (AUCs, area between curve and line at 60 sec) were used to compare recovery. Data represent mean ± SEM.

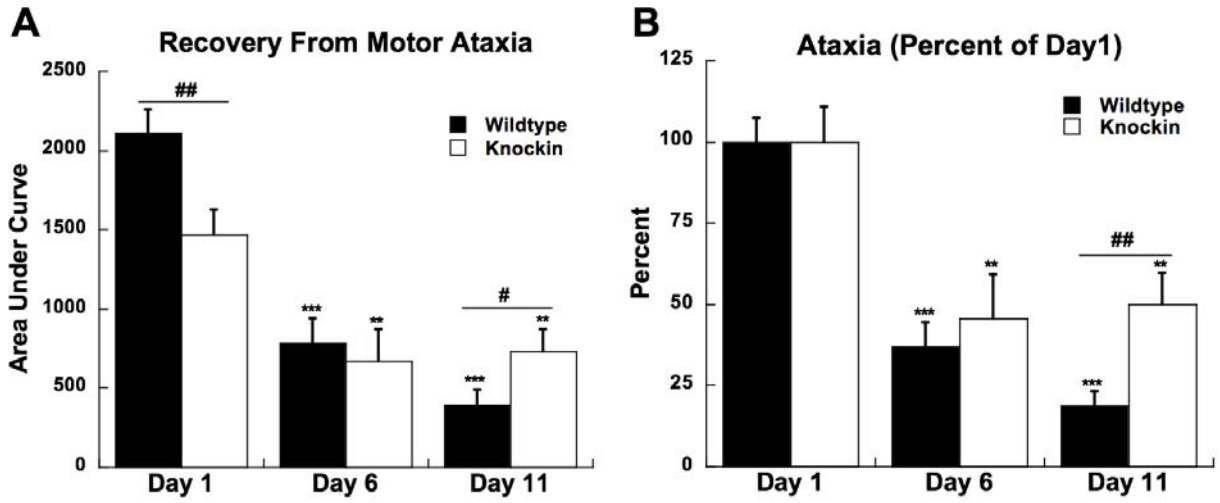

Figure 4.3. Ethanol-Induced Motor Ataxia AUCs
Area under the curve of recovery from ethanol-induced motor ataxia (A). Knockin mice recovered more quickly from motor ataxia on day 1 (##, $p < 0.01$) of an 11 day repeated ethanol exposure compared to wildtype controls. Both genotypes have reduced AUCs on day 6 and day 11 compared to day 1 (**, $p < 0.01$; ***, $p < 0.001$), but, knockin mice were more impaired than wildtypes on day 11 (#, $p = 0.05$). AUC's normalized to percent ataxia based on day 1 (B). Both genotypes recovered more quickly from ethanol-induced motor ataxia on day 6 and day 11 than day 1 (*, $p < 0.01$; **, $p < 0.001$). Knockin mice are more impaired than wildtype controls on day 11 (##, $p < 0.01$). Data represent mean ± SEM. n = 20-21.

The same mice were also tested for ethanol-induced hypothermia concurrently with rotarod. Preliminary analysis of the data revealed that ethanol produced a maximum decrease in body temperature at 30 min post ethanol injection (not shown). Therefore, this time point was selected for detailed analysis. Compared to basal body temperatures ethanol-induced hypothermia on day 1, but no genotype differences were detected ($p < 0.001$). A significant main effect was also detected for day after repeated ethanol exposure ($F_{2, 76} = 70.1$, $p < 0.0001$), but no significant effect of genotype or interaction was observed (Figure 4.4). Thus, tolerance developed to the hypothermic effects of ethanol, but ethanol did not act through $\alpha 1$ $GABA_A$-Rs to induce tolerance to the hypothermic effects of ethanol.

Basal body temperatures were taken before ethanol treatments on day 6 and day 11. Basal body temperatures appeared to increase by ~1 degree after the 11 day repeated exposure (not shown), but this may be attributed to the stress of receiving injections as saline treated animals also displayed similar increases in body temperature (not shown).

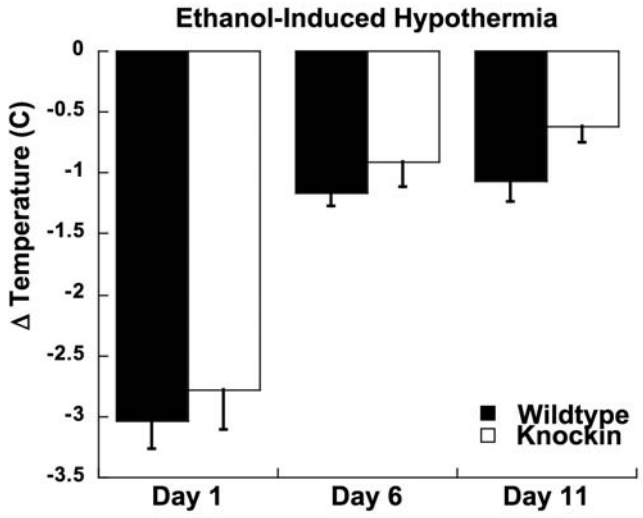

Figure 4.4. Ethanol-Induced Hypothermia.
Mice were tested for ethanol-related hypothermic effects on day 1, day 6 and day 11 of an 11-day repeated-ethanol exposure period. Shown are changes in body temperature 30 min post ethanol injection (2.0 g/kg). Ethanol exposure resulted in a hypothermia (day 1). Tolerance to ethanol's hypothermic effects was seen on day 6 and day 11 ($p < 0.0001$). No differences in genotypes were observed. Data represent mean ± SEM. n = 20-21 per group.

4.2.3 Ethanol-Induced Nociception

To determine if α1-$GABA_A$-Rs are involved in ethanol-related nociceptive effects, mice were assessed using the radiant tail-flick assay. Mice did not differ in basal thermoceptive responses, thereby allowing for valid comparisons between groups (Baseline, Figure 4.5). Mice were then assessed with respect to ethanol after a single exposure, repeated exposure, and during withdrawal. A significant main effect was observed for time ($F_{3, 135} = 50.9$, $p < 0.0001$), but not for genotype or interaction of time and genotype. Further analysis indicated acute ethanol treatment increased latency to tail-flick ($p < 0.001$). Chronic ethanol treatment did not reveal any difference from baseline, but mice had a slightly reduced latency to tail-flick during

withdrawal from chronic ethanol exposure (i.e., day11.5, $p < 0.01$). Thus, acute ethanol resulted in an analgesic effect, repeated ethanol exposure resulted in tolerance to this effect, and an enhanced sensitivity was observed during withdrawal following repeated ethanol exposure. However, these responses did not differ with respect to genotype.

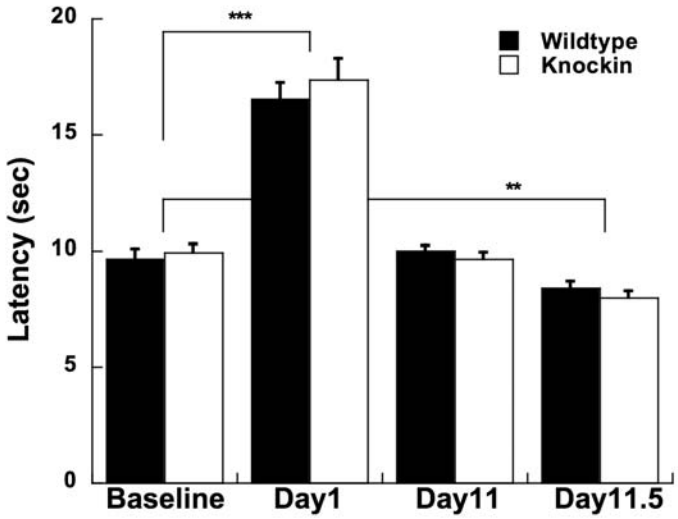

Figure 4.5. Ethanol Effects on Nociception.
Mice were tested for basal and ethanol-related nociceptive responses using the radiant tail-flick. Mice did not differ in basal responses to thermal pain stimuli. Acute ethanol increased tail-flick latencies compared to baseline ($p < 0.001$). Mice tested immediately after the last ethanol exposure (day11) did not differ from basal responses. Mice had decreased latencies during following the last ethanol exposure compared to baseline (day11.5, $p < 0.01$). Data represent mean ± SEM. n = 13-16 per genotype.

4.2.4 Ethanol Anxiety-Related Effects

The effects of ethanol on anxiety-like behavior and locomotor activity were examined using the elevated plus maze. For locomotor activity as assessed by total number of entries (Figure 4.6A), no differences between genotypes were observed in basal activity (one-way ANOVA). Comparing acute ethanol group to controls (two-way ANOVA), a significant main effect of treatment ($F_{1, 40} = 13.0$, $p < 0.001$) was seen. Further analysis indicated acute ethanol increased locomotor activity compared to the saline-saline group ($p < 0.001$). Comparison of chronic ethanol versus control (two-way ANOVA) also had a main effect of treatment ($F_{1, 40} = 18.8$, $p <$

0.0001) resulting in increased locomotor activity compared to the saline-saline group ($p < 0.001$). Conversely, ethanol treated mice that were given saline on the last day (ethanol-saline) did not differ from saline treated animals. In all cases, no effect of genotype or interaction was observed. Overall, acute and chronic ethanol increases locomotor activity.

To assess anxiety-related effects, percent open arm entries (Figure 4.6B) was assessed. No basal differences were observed with respect to genotype (control group). Comparison of the acute ethanol group to the control group by two-way ANOVA revealed a main effect of treatment ($F_{1, 40} = 14.6$, $p < 0001$) and a suggestion of an effect of genotype ($F_{1, 40} = 3.6$, $p = 0.06$), but no interaction. Further analysis indicated ethanol caused an increase in percent open arm entries compared to the saline-saline group. Because of the suggestive genotype effect, genotypes were assessed with respect to treatment. While not significant, there is a trend ($p = 0.07$) for knockins to be more affected after acute ethanol exposure compared to controls. To compare the chronic ethanol group versus the control group, two-way ANOVA indicated a significant main effect of treatment ($F_{1, 40} = 22.3$, $p < 0.0001$), but no genotype or interaction. Chronic ethanol exposure therefore caused an increase in percent number open arm entries compared to the saline-saline group. Comparison of the withdrawal group versus controls by two-way ANOVA indicated no main effect of treatment, but there was a main effect in genotype ($F_{1, 39} = 6.7$, $p < 0.05$). Further analysis was done to determine where the genotype effect occurred. Wildtype mice given saline after repeated ethanol exposure appeared to have decreased percent open arm entries compared to knockin mice ($p < 0.01$). Thus, while acute and chronic ethanol increase anxiolytic-like behavior, there is a trend for knockins to be more sensitive after acute ethanol. There is also a trend for wildtype mice to exhibit withdrawal-related anxiogenic behavior, but not knockins.

Percent time spent in open arms (Figure 4.6C) is also another measure used for anxiety-like behavior. Genotype differences were observed in the control group (one-way ANOVA, $p < 0.05$). To compare the acute ethanol group and controls, two-way ANOVA indicated a main effect of treatment ($F_{1, 40} = 16.7$, $p < 0.001$) and genotype ($F_{1, 40} = 9.6$, $p < 0.01$), but no interaction. Further analysis indicated that acute ethanol increased percent time spent on open arms compared to controls ($p < 0.0001$), and knockins were less anxious than wildtype mice ($p < 0.01$). In comparison of the chronic ethanol and control groups, two-way ANOVA indicated a main effect of treatment ($F_{1, 40} = 20.5$, $p < 0.0001$) and genotype ($F_{1, 40} = 12.6$, $p < 0.005$), but no

interaction. Chronic ethanol increased percent time spent in open arms ($p < 0.0001$) and knockins had increased percent time on open arms than wildtypes ($p < 0.01$). To compare the withdrawal and control groups, two-way ANOVA indicated a main effect of genotype ($F_{1, 39} = 21.8$, $p < 0.0001$), but no effect of treatment or interaction was observed. Knockin mice had increased percent time in open arms compared to wildtype mice. Thus, acute and chronic ethanol increased anxiolytic-like behavior compared to controls, but knockin mice in all groups displayed increased anxiolytic-like behavior compared to wildtypes.

As an additional measure of anxiety-related behavior, percent time in closed arms was also assessed (Figure 4.6D). No genotype differences were seen in control mice. For comparison of the acute ethanol group and controls, a main effect of treatment was observed ($F_{1, 40} = 15.1$, $p < 0.001$). Ethanol caused a decrease in percent time spent in closed arms in the acute group compared to the control group ($p < 0.001$). To compare the chronic ethanol and control groups, two-way ANOVA indicated a significant main effect of treatment ($F_{1, 40} = 7.3$, $p < 0.05$). Chronic ethanol treatment decreased the amount of time spent in closed arms compared to controls ($p < 0.05$). To compare the withdrawal and control groups, two-way ANOVA revealed a main effect of treatment ($F_{1, 39} = 4.9$, $p < 0.05$), an effect of genotype ($F_{1, 39} = 6.1$, $p < 0.05$) as well as a suggestive treatment x genotype interaction ($F_{1, 39} = 3.8$, $p = 0.057$). Because of the suggestive interaction further analysis was carried out. This revealed wildtype mice in the withdrawal group had an increase in percent time spent in closed arms compared to wildtype controls ($p < 0.01$) and compared to knockin mice in the withdrawal group ($p < 0.05$). Thus, acute and chronic ethanol increased anxiolytic-like behavior as seen by decreased percent time spent in closed arms. However, wildtype mice in the withdrawal group displayed increased anxiety-like behavior whereas knockins did not.

Anxiety-related effects of ethanol were also assessed using the light-dark box. For locomotor activity (Figure 4.7A), no differences were seen in basal locomotor activity (transitions) between genotypes. To compare acute and control groups, a main effect of treatment was observed ($F_{1, 30} = 9.9$, $p < 0.01$) by two-way ANOVA . Further analysis revealed acute ethanol increased locomotor activity in the acute group compared to controls ($p < 0.01$). To compare the chronic ethanol group and controls two-way ANOVA a main effect of treatment was observed ($F_{1, 33} = 11.0$, $p < 0.01$). Further analysis indicated chronically ethanol treated mice had increased locomotor activity compared to controls ($p < 0.01$). To compare withdrawal

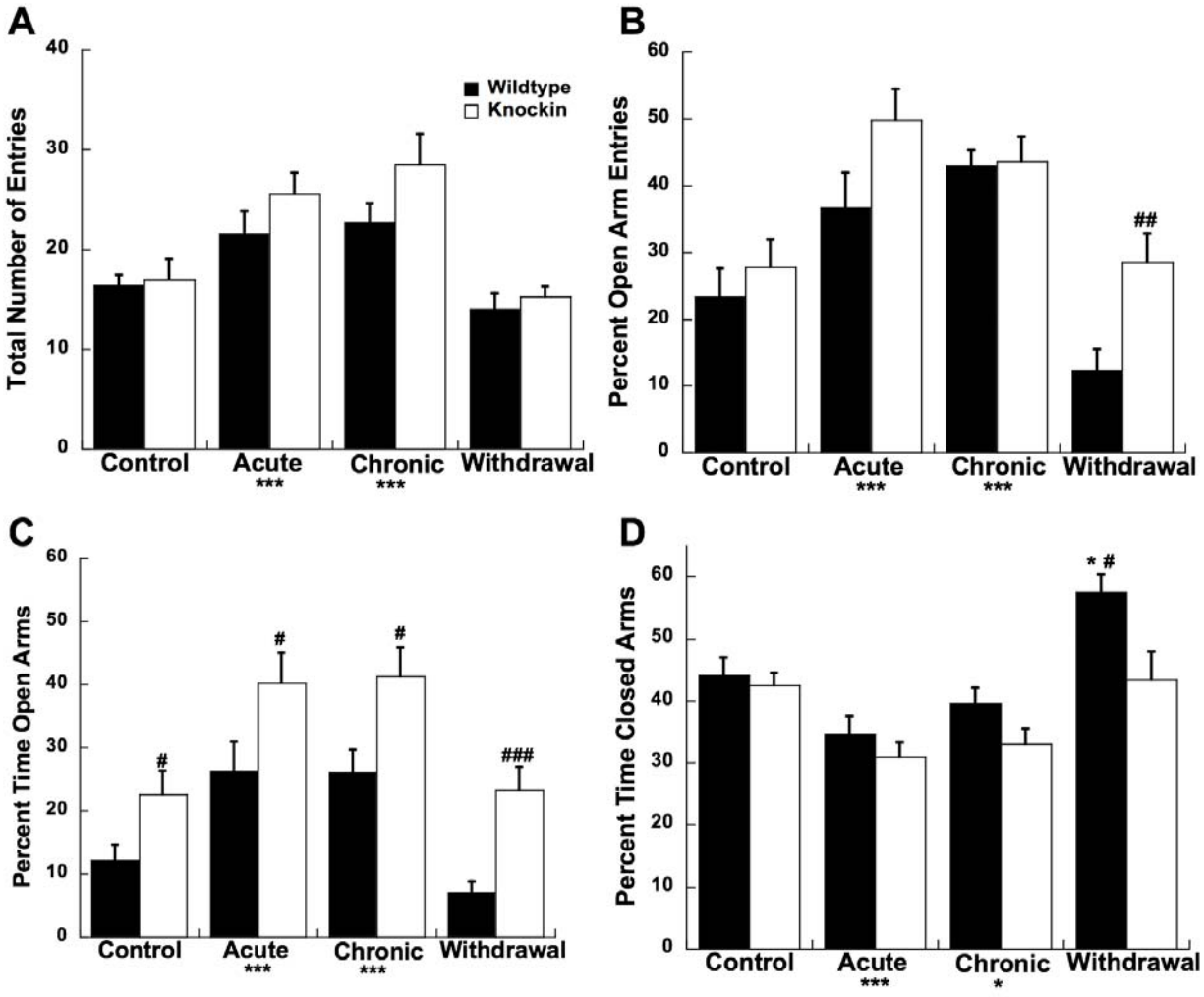

Figure 4.6. Ethanol-Related Anxiety-like Responses and Locomotor Activity With The Elevated Plus Maze. Total arm entries (A), percentage of open arm entries (B), percent time spent in open arms (C), and percent time spent in closed arms (D) measured on the elevated plus maze. Mice were given saline or ethanol once daily for 10 days (First treatment). On the 11th day, mice were given either saline or ethanol (Last treatment). Saline/saline – control group; saline/ethanol – single exposure group; ethanol/ethanol – repeated exposure group; ethanol/saline – withdrawal group. Acute and repeated ethanol exposure increased locomotor activity. Acute and chronic ethanol resulted in anxiolytic effect in both genotypes, but knockin mice may have been more affected after acute ethanol exposure ($p = 0.07$ on % number open arm entries). Knockin mice in the withdrawal group do not appear to display increased anxiogenic-like effects. *, $p < 0.05$; ***, $p < 0.001$ for treatment. #, $p < 0.05$; ###, $p < 0.001$ for genotype. Data represent mean ± SEM. n = 9-12 per group.

and control groups, two-way ANOVA indicated a main effect of treatment was detected ($F_{1,35}$ = 7.9, $p < 0.01$). Further analysis revealed mice in the withdrawal group were less active than controls ($p < 0.01$). No genotype or interaction was detected in any locomotor analysis. Thus, acute and chronic ethanol increase locomotor activity, while withdrawal from ethanol decreases locomotor activity.

To assess anxiety-related behavior, genotypes and treatment groups were assessed with respect to time spent in the light side of the apparatus (Figure 4.7B). No differences were detected with respect to genotype in controls. To compare acute and control groups, two-way ANOVA indicated a main effect was observed for treatment ($F_{1, 30}$ = 16.0, $p < 0.001$), genotype ($F_{1, 30}$ = 7.5, $p < 0.05$), and interaction ($F_{1, 30}$ = 8.3, $p < 0.01$). Due to the interaction, further analysis revealed that knockin mice in the acute ethanol group spent more time on the light side compared to knockin controls ($p < 0.001$) whereas acute wildtype mice did not differ from controls. To compare mice chronically treated with ethanol to controls, two-way ANOVA indicated a main effect of treatment ($F_{1, 33}$ = 15.1, $p < 0.001$), but not genotype or interaction. Further analysis revealed chronic ethanol increased time spent on the light side ($p < 0.001$). To compare mice in the withdrawal group and controls, two-way ANOVA revealed no main effects on any measure. Thus, knockin mice have increased anxiolytic-like behavior after acute ethanol exposure, whereas chronic ethanol results in anxiolytic-like behavior in both genotypes.

A lesser used measure of anxiety-related behavior, latency to re-enter the light compartment (Figure 4.7C) was also assessed. No genotype differences were observed in controls. Comparison of acute and control groups by two-way ANOVA indicated a main effect of treatment ($F_{1, 30}$ = 5.5, $p < 0.05$), but not genotype or interaction. Further analysis indicated mice treated with acute ethanol re-entered the light compartment more quickly than controls ($p < 0.05$). Comparison of either chronic or withdrawal groups to controls did not reveal any significant effects. Thus, acute ethanol decreases latency to re-enter the light compartment.

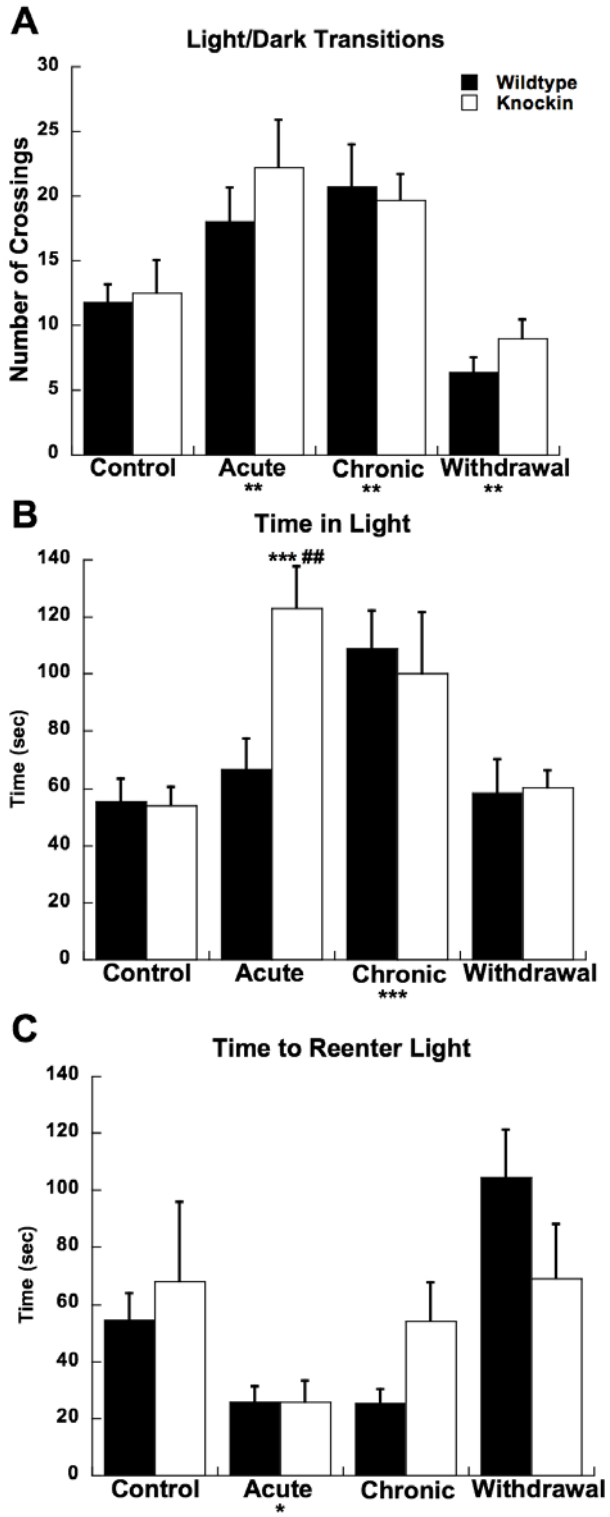

Figure 4.7. Ethanol Related Anxiety and Activity With The Light-Dark Box.
Total transitions (A), time spent in light area (B), and time to reenter light after entering dark (C). Mice were given saline or ethanol once daily for 10 days (First treatment). On the 11th day, mice were administered either saline or ethanol. Mice were given saline or ethanol once daily for 10 days. On the 11th day, mice were given either saline

or ethanol (Last treatment). Saline/saline – control group; saline/ethanol – single exposure group; ethanol/ethanol – repeated exposure group; ethanol/saline – withdrawal group. Knockin mice were more sensitive to ethanol's anxiolytic effects after acute exposure, whereas both genotypes displayed anxiolytic effects following repeated ethanol exposure. Acute and repeated ethanol exposure increased locomotor activity, whereas mice tested 24 hours following repeated ethanol exposure had decreased locomotor activity. No significant effects were detected in latency to reenter. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for treatment. ##, $p < 0.01$ for genotype. Data represent mean ± SEM. n = 7-11 per group.

Overall, based on the results from both anxiety-related behavioral tasks, acute and repeated ethanol administration increased locomotor activity. However, knockin mice appear to be more sensitive to the ethanol's anxiolytic effects after acute ethanol exposure on some anxiety related measures (i.e., trend in percent number open arm entries on plus maze, increased time in light in light/dark assay). Both genotypes displayed anxiolytic-like effects following repeated ethanol exposure. However, only wildtype mice displayed ethanol withdrawal-related phenotypes on some measures of the elevated plus maze, but both genotypes had reductions in locomotor activity in the light-dark box.

4.2.5 Ethanol Withdrawal-Related Hyperexcitability

The role of the α1 subunit in ethanol dependence was tested following multiple exposure and withdrawal from high concentrations of ethanol vapor by assessing for ethanol withdrawal-related hyperexcitability as quantified by the intensity of handling-induced convulsions, (HICs). Blood ethanol concentrations did differ with respect to treatment (Table 4.1, $p < 0.0001$) but did not differ with respect to genotypes at the end of the third cycle of ethanol exposure. BEC's were virtually undetectable in air-exposed mice, but were readily measurable in ethanol vapor exposed groups. Two wildtype female mice were excluded having BECs >300mg/dl. Ethanol administration also resulted in weight loss (Table 4.1, $p < 0.05$), but this also was not significant between genotypes.

Table 4.1 BECs and Percent Weight Loss From HIC Assay.
BECs and percent weight loss are shown for controls and ethanol exposed wildtype and knockin mice. Ethanol exposed mice had higher BECs (ANOVA, effect of treatment, $F_{1, 62} = 173.8$, $p < 0.001$) and percent weight loss (ANOVA, $F_{1, 62} = 6.8$, $p < 0.05$). No effect of genotype or genotype x interaction was observed. For n-values see figure 4.8 legends.

Genotype and Treatment	BEC (mg/dL)	Weight Loss (%)
Wildtype Control	-1.5 ± 5.5	1.7 ± 0.6
Knockin Control	3.2 ± 2.0	1.3 ± 1.2
Wildtype Ethanol	143.7 ± 10.0	2.8 ± 0.5
Knockin Ethanol	165.5 ± 14.8	4.1 ± 0.8

For HICs, a main effect of gender was observed ($F_{1, 55} = 6.8$, $p < 0.05$); therefore males and females were analyzed separately. A time course of ethanol withdrawal hyperexcitability for both genders is shown in Figure 4.8A and 4.8B. Ethanol treated male mice overall have larger time-course curves than female mice, indicating more severe ethanol withdrawal-related hyper-excitability.

For male mice, no differences in genotype were detected in control treated mice (Figure 4.8C). To compare control versus ethanol-treated mice, a two-way ANOVA indicated a main effect of treatment ($F_{1, 23} = 19.3$, $p < 0.001$), and a trend towards an interaction of treatment x genotype ($F_{1, 23} = 4.0$, $p < 0.057$) was observed. Because of the suggestive interaction further analysis was done to determine the observed effect. Although ethanol treated male mice displayed greater AUCs compared to control treated male mice ($p < 0.01$), knockin mice displayed greater AUCs compared to ethanol treated wildtype mice ($p < 0.05$). Interestingly, an ~40% mortality rate (Kaplan-Meier, $p < 0.05$) occurred in ethanol treated male knockin mice whereas none was observed in ethanol treated male wildtype mice (Figure 4.8E). No mortalities were seen in control treated wildtype mice, but it should be noted that one knockin male died (not shown) toward the end of the assay. Of the rest, it should be noted that the observed fatalities were the result of severe tonic-clonic seizures, with the animals often ending in a tonic-extensor position as noted elsewhere [411]. Due to the observed mortalities in knockin mice potentially complicating the analysis of ethanol withdrawal-related hyperexcitability, maximal HIC scores were analyzed using the Mann-Whitney U test. In male mice (Figure 4.8G), there was a trend for ethanol-exposed mice to have higher maximal scores versus air-exposed animals

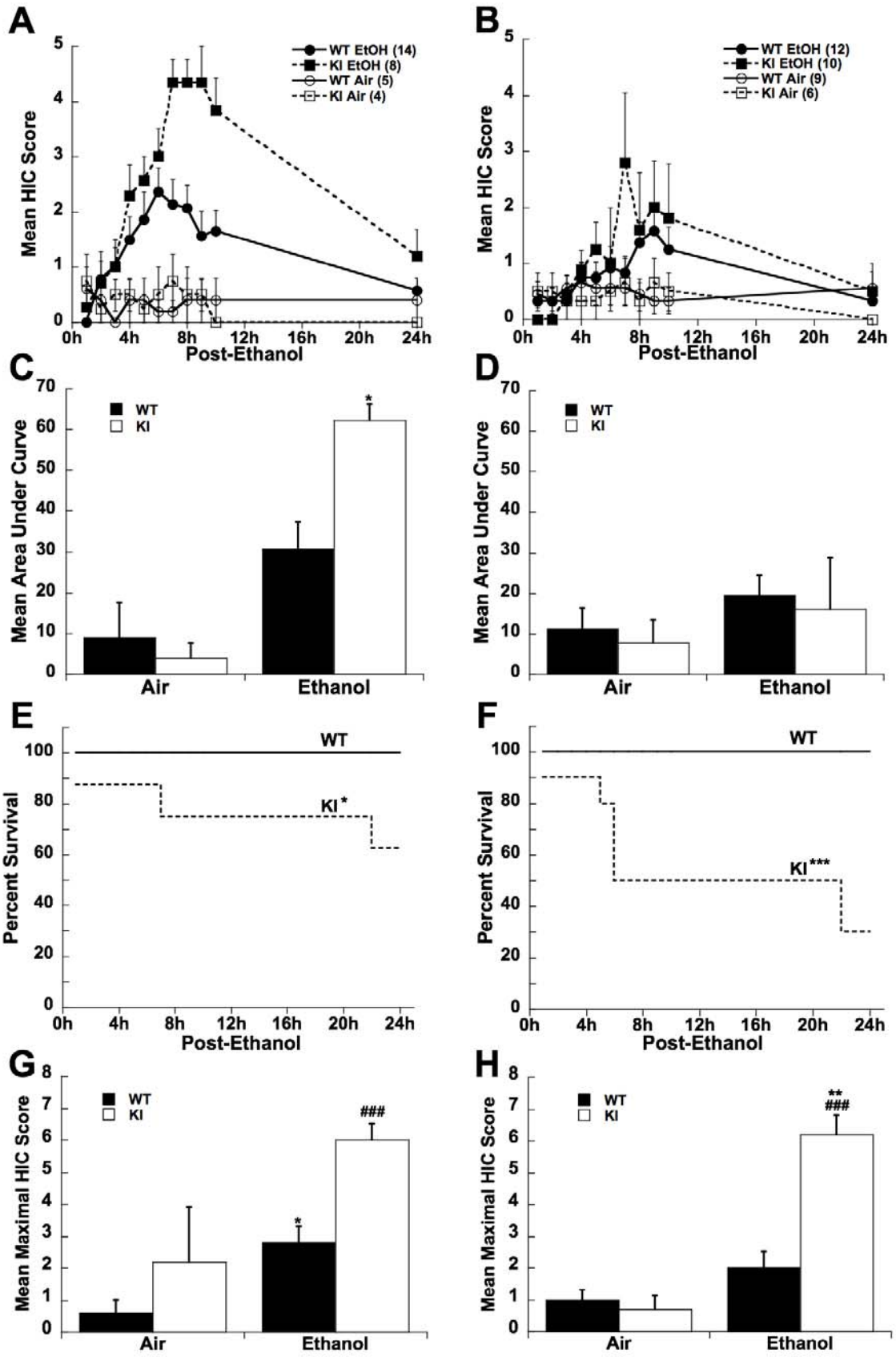
A
Mean HIC Score
Post-Ethanol
WT EtOH (14)
KI EtOH (8)
WT Air (5)
KI Air (4)
B
Mean HIC Score
Post-Ethanol
WT EtOH (12)
KI EtOH (10)
WT Air (9)
KI Air (6)
C
Mean Area Under Curve
WT
KI
*
Air
Ethanol
D
Mean Area Under Curve
WT
KI
Air
Ethanol
E
Percent Survival
WT
KI*
Post-Ethanol
F
Percent Survival
WT
KI***
Post-Ethanol
G
Mean Maximal HIC Score
WT
KI
*
###
Air
Ethanol
H
Mean Maximal HIC Score
WT
KI
**
###
Air
Ethanol

Figure 4.8. Ethanol Withdrawal-Related Hyperexcitability.
Male mice exhibited more severe withdrawal-related hyperexcitabiltiy than females ($F_{1, 55}$ = 6.8, $p < 0.05$). Therefore, male and female mice were analyzed separately. Time-course of mean handling-induced convulsions (HIC) scores in male and female mice, (A and B, respectively) after completion of three cycles of 16h per day exposure to ethanol vapor. Area under the curves for HIC time-course in males and females (C and D, respectively). Male ethanol treated mice displayed more severe withdrawal-related behavior than controls ($p < 0.01$). Knockin mice exhibited more severe withdrawal-related behavior than ethanol treated wildtype mice ($p < 0.05$). No differences were detected in female mice. Survival curves following cessation of ethanol treatment in male and female mice (E and F, respectively). Knockins were prone to withdrawal-related mortality in both sexes. *, $p < 0.05$; ***, $p < 0.001$. Knockin mice had more severe maximal withdrawal-related HIC scores in male and female mice (G and H, respectively). *, $p < 0.05$; **, $p < 0.01$ within genotype. ###, $p < 0.001$ within ethanol.

($U = 12.0$, $p < 0.05$ for wildtype; $U = 6.0$, $p = 0.08$ for knockins). Additionally, ethanol-exposed male knockin mice had higher maximal scores than ethanol-exposed wildtype mice ($U = 6.5$, $p < 0.001$).

For female mice (Figure 4.8D) two-way ANOVA indicated no effect of treatment, genotype or the interaction of treatment and genotype. However, female ethanol-exposed knockin mice had a substantially higher mortality rate that ethanl-exposed wildtypes (Figure 4.8F, ~60%, Kaplan-Meier, $p < 0.001$) due to seizure-like activity whereas no deaths were observed in female wildtype mice. No deaths were observed in air-exposed mice of either genotype (not shown). Ethanol-exposed knockin mice had higher maximal scores than air-exposed knockins ($U = 2.0$, $p < 0.01$) and ethanol-exposed wildtypes ($U = 8.0$, $p < 0.001$). Ethanol-exposed female wildtype mice did not differ from air-exposed wildtype mice. Because of the high mortality rate in knockins, it was rationalized that to continue the experiments would be inhumane. Therefore, experiments were terminated. Overall, results indicated knockin mice are more prone to ethanol withdrawal-related hyperexcitability.

α1 Levels Following Repeated Ethanol Exposure

Lastly, amount of α1 was assessed in the cortex of wildtype and knockin mice following repeated ethanol exposure (2.0 g/kg, i.p. per day for 11 days) or saline. In wildtype mice (Figure 4.9A), chronic ethanol resulted in ~30% reduction of α1 subunit levels compared to saline treated mice (saline 100±9.1%, ethanol 68.8±7.9%; $p < 0.05$). In contrast, knockin mice (Figure 4.9B) repeatedly administered ethanol did not differ from saline treated mice (saline

100.0±11.9%, ethanol 89.2±7.5%). This indicates that while chronic ethanol reduced α1GABA$_A$-R subunit levels in wildtype mice, α1 levels in knockins were unaffected.

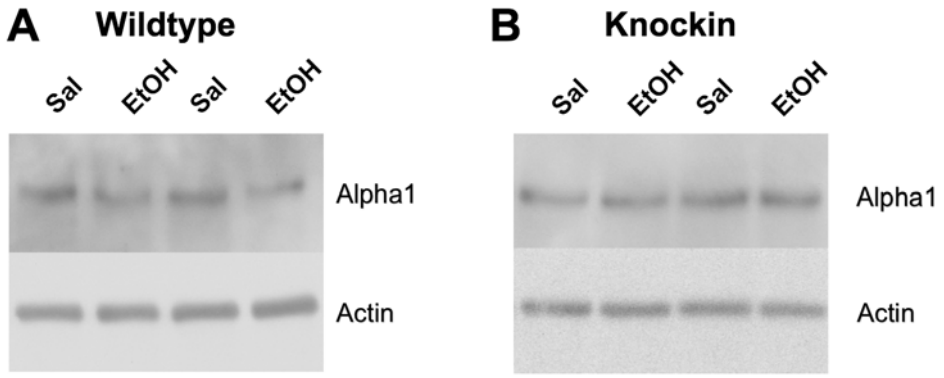

Figure 4.9. α1 Levels Following Repeated Ethanol.
Representative western blots of α1 levels in wildtype (A) and knockin (B) mice following saline or repeated ethanol exposure. Repeated ethanol exposure decreased α1 levels by ~30% in wildtype mice compared to saline treated controls. In contrast, knockin mice did not display any alterations in α1 levels following repeated ethanol exposure. n = 7-9 mice per treatment, per group.

4.3 DISCUSSION

α1-containing GABA$_A$-Rs have been suggested to be involved in the mechanism of action of alcohol and alcoholism. In this report, knockin mice harboring ethanol-insensitive GABA$_A$-R α1 subunits were assessed on a variety of measures of tolerance and dependence – two prognostic behaviors of alcoholism [75, 76]. Knockin mice significantly differed in ethanol-induced motor ataxia, withdrawal related hyperexcitability and anxiety. Additionally, knockin mice did not display ethanol-related decreases in α1 compared to saline controls. In contrast, tolerance and dependence on several other ethanol-related behaviors were not altered in knockins compared to controls, such as righting reflex, hypothermia, locomotor activity and nociception.

Knockin mice were less sensitive to ethanol's acute motor ataxic effects (Figure 4.2 and 4.3). This agrees with our earlier report [369]. Knockins also displayed decreased development of tolerance to ethanol-induced motor ataxia compared to controls. These parallel changes in sensitivity and tolerance are similar to those seen in other experiments. For example, using

genetically selected lines, Radcliffe et al. [95] had shown that mice displaying increased sensitivity to a specific ethanol-related behavior (righting reflex) developed more tolerance, whereas those that were less sensitive did not develop tolerance. Taken together, this suggests that the development of tolerance is directly related to sensitivity. While there are some studies that suggest sensitivity and tolerance are not directly related [218-220] where animals become more sensitized in some instances. The discrepancy between these results and ours may be due to the assay(s) employed and the initial sensitivity of the animals being used.

Interestingly, knockin mice also displayed more severe ethanol withdrawal-related phenotypes. Withdrawal-related hyperexcitability was severe enough to result in fatal seizures in 40-60% of knockin mice. Because the knockin mutation eliminated ethanol action at α1-$GABA_A$-Rs [355], I postulated that knockins would have less withdrawal-related behavior than controls, but the results indicate the opposite. One possibility may be that the knockin mice are in a prekindled state and are therefore pre-disposed to more severe hyperexcitable states. It is possible the changes in α1subunit levels observed in Chapter 2 may relate to this pre-kindled state. Knockins also exhibit ~10% mortality rate where some seizures have been observed (Chapter 2). Interestingly, in all control treated knockin mice in the HIC study, 1 (10%) died whereas no control animals died. This is in line with the observed mortality rate seen in the colony. Alternatively, seizure-protective mechanisms afforded through α1-mediated mechanisms may be eliminated. Because α1 is expressed throughout the brain, it remains to be seen which neuroanatomic region is responsible for ethanol withdrawal-related seizure activity. However, it's tempting to speculate that the hippocampus and/or dentate gyrus which are implicated in spontaneous seizures and temporal lobe epilepsy play a role [403, 412]. Nonetheless, further experiments are warranted to determine if these regions are involved in ethanol withdrawal-related seizure activity. Such experiments may include assessing pentylenetetrazole-induced seizures specifically administered to this region or breeding spatially restricted gene knockins [413].

Knockin mice also displayed increased anxiolytic behavior after acute ethanol compared to controls as seen previously (chapter 2). Interestingly, anxiolytic effects were still observed in both genotypes after repeated ethanol exposure, indicating no tolerance to this effect. This agrees with studies elsewhere [143]. It is possible that this positive effect, which remains following chronic exposure of ethanol may be one reason that individuals continue to seek

ethanol's pleasurable effects. While the anxiolytic effects in chronically treated mice indicate that α1-containing receptors do not appear to be involved in this behavior, investigation into other genes may yield powerful insight into how to potentially curb alcoholism. By antagonizing ethanol's pleasurable effects (e.g., anxiolysis), it is possible that abusers may not as readily seek alcohol. Interestingly, on the elevated plus maze, knockin mice did not appear to display withdrawal-related behavior (e.g., increased time in closed arms); however this was not seen on both anxiety related assays. On the light-dark box, both genotypes had deceased locomotor activity compared to controls, which may or may not be considered as part of the anxiety-related measures. Nonetheless, many studies assessing ethanol withdrawal-related anxiety report reduced activity [reviewed in 191]. Locomotor activity was also increased in both assays after acute and repeated ethanol exposure in both genotypes. This was not unexpected as increases in locomotor activity were seen previously after acute ethanol treatment (chapter 2) and is dependent on dose. Additionally, sensitization to locomotor effects after chronic ethanol is commonly observed [e.g., 92].

Knockin mice did not differ from controls in tolerance to ethanol's hypnotic effects. This is somewhat surprising as α1-$GABA_A$-Rs have been shown to mediate the sedative effects of benzodiazepines [276]. However, recent work has suggested that sedation and hypnosis are mechanistically distinct for intravenous anesthetics [322, 323]. Therefore, it is possible that ethanol-related hypnosis may be mediated by other $GABA_A$-R subtypes. In fact, other studies have suggested that the hypnotic effects of ethanol may be mediated by α2-$GABA_A$-Rs [321]. It would be interesting to assess the hypnotic effects of ethanol as well as tolerance to these effects in mice harboring α2 ethanol-insensitive $GABA_A$-R subunits.

Additionally, knockin mice did not differ from controls in nociceptive responses to ethanol. Acute, chronic and withdrawal-related effects were also observed as reported elsewhere [e.g., 56]. The withdrawal-related responses, while significant, did not have as severe a magnitude as seen elsewhere – although this could be related to the dose of ethanol used and/or length of ethanol exposure. While a moderate concentration of ethanol was employed here, other studies have employed higher doses that may precipitate withdrawal-related seizure effects [56, 149]. However, recent work has suggested that lower doses paradoxically increased ethanol withdrawal hyperalgesia [192]. While $GABA_A$-Rs and cell signaling proteins that regulate $GABA_A$-R function have been suggested to play a role in ethanol-nociceptive effects [150, 200],

these results suggest α1 has little involvement. It is possible that other $GABA_A$-R subunits or non-GABAergic mechanisms mediate this effect (e.g., adenosine receptors [414], calcium channels [415]).

Repeat ethanol exposure decreased α1 subunit levels in the cortex of wildtype mice as observed previously in numerous studies [363, 364]. In stark contrast, α1 levels in knockins were unaffected. This suggests that ethanol acting at α1-$GABA_A$-Rs, results in decreased α1 levels following chronic ethanol exposure. It would be interesting to know what happens to other well known changes in $GABA_A$-R levels following chronic ethanol. Possibly, ethanol acting through α1-containing $GABA_A$-Rs spearheads ethanol-related $GABA_A$-R compensatory changes and that the neuroadaptive processes necessary for tolerance are blunted in these knockins, but this would need to be explored further. Nonetheless, this result is correlative with the decreased tolerance observed in ethanol's motor ataxic effects. Alternatively, although less likely, a floor effect may have occurred. Previous experiments indicate that α1 subunit levels in knockins were decreased by ~50 percent in cortex compared to controls. It is possible, that this may be the minimum physiologically required levels of α1 for 'normal' brain function, although this would need to be further evaluated experimentally.

In summary, the studies in this chapter demonstrated that α1-$GABA_A$-Rs play a role in the development of tolerance to ethanol's ataxic effects as well as ethanol withdrawal-related hyperexcitability, while other behaviors were unaffected. Moreover, they also indicate that ethanol action at α1-$GABA_A$-Rs decreases α1 levels following repeated ethanol exposure. More importantly, the experiments conducted here support the hypothesis that α1 $GABA_A$-Rs mediate in part ethanol tolerance and dependence. Additionally, these studies also lend support to a role of α1 $GABA_A$-Rs in alcoholism.

5.0 SUMMARY AND CONCLUSIONS

This dissertation focused on the role of α1-containing $GABA_A$-Rs in alcohol action. It has been proposed that $GABA_A$-Rs are involved in alcohol action and alcoholism. More specifically, individual subunits of $GABA_A$-Rs such as α1 may exert more influence than others. Gene targeted mice harboring ethanol insensitive α1-$GABA_A$-Rs were characterized and responses to acute ethanol, tolerance and physical dependence were evaluated – the latter two which may precipitate alcoholism. This discussion will first evaluate the use of genetically modified mice, with emphasis on knockins, in research. The second part describes the role of α1 $GABA_A$-Rs in alcohol action. Potential therapeutic interventions for alcohol abuse and alcoholism as well as future directions are discussed. The last part describes the relevancy of this work toward an understanding of the relationship between alcohol sensitivity, tolerance and physical dependence.

5.1 USE OF GENETICALLY MODIFIED MODELS

Identification of the role of specific genes/gene products in biological action and pathophysiology holds tremendous promise in the betterment of health. Often times, assessment of specific gene products is limited due to the availability and specificity of certain pharmacologic agents. In the $GABA_A$-R system, some agents act at all types of $GABA_A$-Rs (e.g., picrotoxin, bicuculline), while a substantial number of others show selectivity (e.g., benzodiazepines) but not complete specificity. As subtype specific agents are not readily available, pharmacologic dissection of specific $GABA_A$-R subtypes with respect to biological processes as well as substance use and abuse (i.e., alcohol) has been hindered. In this regard, genetically modified models have proven to be invaluable tools. Because of limitations (i.e., compensation, lethality) with traditional knockout and transgenic technologies, it is difficult to

definitively establish if the results are due to modification of the gene of interest or compensatory changes in other targets. Because of the lack of limitations, knockin models represent a more ideal system with which to study the interaction (or lack thereof) of ethanol and specific gene products.

In the present studies, the use of knockin models harboring ethanol-insensitive but otherwise near normal receptors, allows for a better representation of the role of the α1 subunit in ethanol action compared to global knockouts. Unlike the global knockouts where α1 is absent and redundancy of other subunits may mask the exact involvement of α1 in ethanol action, functional α1 $GABA_A$-Rs are still present in the knockin mice. Also, the α1 knockin gene is under the control of the endogenous promoter thereby allowing for proper spatial and temporal control. Ethanol-related behavioral differences were observed between knockin and knockout mice (below, Table 5.1). It is possible that redundancy may account for the absence of observations in knockout mice. It should also be noted that specific behaviors (such as ataxia)

Table 5.1. Ethanol Responses in α1 Genetically Modified Mice.
Comparison of ethanol-related responses in α1 knockout and α1 HA/HA knockin mice. Knockout mice and knockin mice differed in ethanol-induced anxiolysis, acute functional tolerance, preference and withdrawal. = - not different; ↓ - decreased; ↑ - increased; n.d. – not determined. 1 – Kralic et al. [327]; 2 – Blednov et al. [328]; 3 – Blednov et al. [329]; Boehm et al. [325]; 5 – Werner et al. [369]; ★ – Current studies.

Ethanol Behavior	**α1 Global Knockout**	**α1 Knockin (HA/HA)**
Acute Hypnosis	=[1] ↓males[2]	=[5,] ★
Acute Anxiolysis	=[1]	↑[5,] ★
Acute Exploratory	=[1] ↑[3]	= (unpublished observations)
Acute Motor Ataxia	=[1] (accelerod)	↓[5,] ★ (recovery)
Acute Functional Tolerance	=[1]	↑★
Acute Analgesia	n.d.	=★
Ethanol Preference	↓ consumption, ↓ preference[3]	↑ consumption, ↑ preference[5]
Acute Withdrawal	↓[4]	=[5]
Chronic Withdrawal	=[3]	↑★

were not directly comparable due to differences in methodology between the α1 knockin and α1 global knockout studies. Conversely, some behaviors such as hypnosis did not differ in either mouse model, thereby further supporting the absence of α1 involvement in ethanol-induced righting reflex.

Unexpectedly, the use of knockin technology in ethanol-insensitive α1 $GABA_A$-R knockin mice did not ablate compensatory effects. Additional work on these mice also suggests that the knockin mutations are not phenotypically silent [355], in that they had increased activity in a novel environment. Additionally, on the cellular level, recordings from miniature IPSC's indicated a faster decay time constant [416]. Therefore, compensatory changes need to be taken into consideration when interpreting results even in knockin studies. Nonetheless, molecular and phenotypic alterations in knockin mouse models are not novel observations. Alterations in knockin gene product levels have been noted elsewhere [350, 417]. Similar to our results, other studies have reported regional differences in knockin gene product levels [417, 418] and other knockin studies have suggested that knockins may not be behaviorally silent [373]. One possible way to circumvent this scenario is to intensively screen the desired mutation in *xenopus* oocytes or HEK293 cells to be more definitive of the mutations before creating an animal model.

The possibility exists that the molecular and phenotypic alterations may be restricted. Indeed, results here indicated no apparent changes were observed in glutamic acid decarboxylase, GABAergic interneurons, or in a subset of pyramidal neurons and glial cells. Thus, any alterations may be isolated to the $GABA_A$-Rs and do not involve other components of the GABAergic system. However, only a limited analysis was done, and further studies such as assessing glycinergic or glutamatergic systems would need to be conducted to address this aspect more definitively.

However, unlike knockouts, knockin models have a limited scope of use. Due to the specificity of their mutations (i.e., ethanol insensitivity herein), knockins may not be used to address other α1-related hypotheses. In spite of these caveats, knockin models still represent a better system for studying specific processes. More importantly, the α1 knockin mice used in the present work were a more ideal model for assessing alcohol-related affects than α1 knockouts due to the mutation rendering them insensitive to ethanol, but still maintaining near normal GABA responses [355].

5.2 α1-GABA$_A$-RECEPTORS AND ETHANOL-RELATED EFFECTS

5.2.1 Ethanol-Related Behaviors

While GABA$_A$-Rs are implicated in ethanol action, the role of specific GABA$_A$-Rs remains unknown. Here, the involvement of α1-GABA$_A$-Rs in ethanol action was evaluated on a variety of ethanol-related measures. These results show that α1 is involved in ethanol-related motor ataxia. Results herein agree with previous studies in which knockin mice have been shown to be less sensitive to motor ataxia by recovering more quickly from ethanol-impaired motor coordination [369]. Conversely, knockins had decreased development of acute/rapid tolerance and chronic tolerance. Motor ataxia is primarily attributed to the cerebellum [59] where normal amounts of α1 were observed. Therefore these results are most likely the result of lack of ethanol action at α1-GABA$_A$-Rs. Since α1 is found in different cell types in the cerebellum (Purkinje cells, granule cells and stellate/basket cells) [313, 419], the possibility exists that specific cell types such (e.g., granule cells) may further mediate ethanol-induced ataxia via α1. This could be further tested using a conditional gene modified model expressing α1 knockin subunits only on these specific cell types [413].

α1 may be involved in anxiety-related effects of ethanol. Knockin mice displayed increased anxiolytic responses following acute ethanol compared to controls. However, other studies have suggested that α2- and α3-containing receptors may be involved in anxiolytic responses [277, 420]. It is possible that increased expression of α3 and possibly α2 GABA$_A$-R subunits that were observed in cortex of the knockin mice compared to controls are responsible for the observed phenotypes. Anxiety-related behaviors are complex and may involve the interplay of numerous brain regions. Although the cortex may mediate aspects of anxiety, other studies indicate structures such as the hippocampus, dentate gyrus, and amygdala may play prominent roles [e.g., 18, 143, 401, 402]. Because subunit levels were only investigated in cortex and cerebella, it is not known whether subunits were altered in other brain regions. It is also possible that rendering α1 insensitive to ethanol may indirectly affect anxiolytic responses. For instance, alterations in the neurocircuitry within these regions or between regions may have resulted in the observed anxiolytic effects.

Interestingly, despite the observed genotype differences in ethanol-induced anxiolysis after acute ethanol, no differences between genotypes were observed in mice chronically treated with ethanol. This suggests that other molecular mechanisms are involved. However, potentially decreased anxiety-related behavior indicates that α1 may be involved in withdrawal-related anxiogenic effects of ethanol, but this result was observed on one of the two behavioral measures and is therefore not conclusive. It should be noted that only the traditional measures of anxiety (e.g., time in open arms, light area) were assessed here. Ethological measures, such as stretch-attend postures or rearings were not recorded, although other reports have employed these to better address anxiety-related measures [141, 178]. Additionally, although not reported elsewhere, it may be interesting to note time spent immobile (freezing) as this may be an indicator of heightened anxiety and/or fear.

Our studies of ethanol withdrawal-related hyperexcitability revealed that α1 is involved in ethanol dependence. Knockin mice exhibited more severe ethanol withdrawal-related hyperexcitability and lethal seizures. It remains to be determined whether this heightened sensitivity to ethanol withdrawal is due to ethanol not being able to act via α1-containing receptors. One possible explanation is that due to altered channel kinetics of α1-containing $GABA_A$-Rs, cells are less hyperpolarized, thereby rendering them more susceptible to potentiation during the withdrawal period. Miniature inhibitory postsynaptic currents in ethanol-insensitive α1 knockin mice display faster rate of decay resulting in decreased hyperpolarization. If less chloride flux is occurring across cell membranes, then potentially decreased energy would be required to cause α1-containing cells to become depolarized. Another possibility may be due to alteration of seizure protective molecular mechanisms (discussed below, section 5.2.2). However, this is only speculative as ethanol withdrawal-induced seizure-related signaling mechanisms remain to be identified.

Apart from the above interpretations, the possibility exists that knockin mice are already in a ‘pre-kindled’ state before even being expose to chronic ethanol. Several lines of evidence from these studies lend support to a pre-kindled state. For instance, the dentate gyrus is a well known region involved in spontaneous seizures and temporal lobe epilepsy [403]. Coincidentally, Fos-IR indicated reduced activity in the dentate gyrus of knockin animals basally and after acute, chronic and withdrawal periods of ethanol exposure. Observed Fos-IR in the dentate gyrus may be correlated with observed α1-related behavioral responses. α1 $GABA_A$-Rs

in the hippocampal areas have even been implicated in withdrawal related effects and seizures. Indeed, dysfunctional regulation of glycolysis-dependent phosphorylation of α1 within the temporal lobe has been linked to epilepsy [412]. While the possibility exists that specific populations of neurons in the dentate may mediate specific ethanol related effects (e.g., interneurons [400]), however, this was beyond the scope of the current study. Additionally, molecular evidence supports a pre-kindled state. Numerous studies have shown that chronic ethanol decreased α1 and increased α4 $GABA_A$-R subunits [e.g., 289, 363, 364, 421, 422] similar to those observed in naïve α1 knockin used here. Behaviorally, approximately 10% of α1 knockin mice died, potentially attributed to seizures. Taken together, it's tempting to speculate that the α1 knockin mice in these studies were indeed more susceptible to hyperexcitable and/or seizure related events, which may be seen in control mice after multiple ethanol withdrawal cycles.

Apart from seizure susceptibility, it is also possible that the aforementioned molecular deviations may correlate to other behavioral effects. For instance, the dentate gyrus has also been implicated in anxiety-related responses [18, 401, 402]. If knockins already exist in a 'predisposed' state, then the possibility exists that behaviors seen after chronic ethanol exposure may be observed earlier in knockin mice. Results herein support the increased/continued anxiolytic state following chronic ethanol exposure. Indeed, this may be another possibility as to why knockin mice are more susceptible to ethanol-induced anxiolysis after an acute exposure, whereas in control mice multiple exposures at lower doses are required to become sensitized to the anxiolytic effect. Again, Fos-IR reported here as well as elsewhere [231, 399] supports this correlation. Decreased Fos-IR was observed after chronic ethanol but not during acute ethanol in wildtype mice, while decreased Fos-IR in knockins occurred after acute and chronic ethanol. Nonetheless, these observations remain correlative and could be completely coincidental. Other regions may be responsible for the anxiolytic effect. The EW is one of few regions known to contain urocortin producing cells, a strong ligand of CRF receptors [423]. The fact that these cells are possibly active as suggested from Fos-IR may be related to the anxiolytic effects seen during chronic ethanol studies. It is also possible that decreases in urocortin during ethanol withdrawal may contribute to anxiogenic effects. It would be of interest to determine whether altered urocortin levels in this region contribute to anxiety-related responses during ethanol-

related events. However, the fact that the EW is not changed in either genotype after acute ethanol limits this possibility.

Apart from ethanol-related motor ataxia, anxiety and withdrawal hyperexcitability, α1-$GABA_A$-Rs are not involved in tolerance or withdrawal effects of ethanol-related hypnosis, hypothermia, or nociception. It is again possible that compensatory effects in specific brain regions could be masking the actual involvement of α1 with respect to these behaviors. Nonetheless, the lack of genotypic effects of these assays suggests that other $GABA_A$-R subtypes or nonGABAergic related targets may be mediating these effects. For instance, α2 $GABA_A$-Rs may mediate ethanol-induced hypnosis [321], whereas adenosine receptors have been implicated in ethanol-related hypothermic effects [398]. Detailed analysis of additional GABAergic and nonGABAergic targets will continue to determine the exact role of specific genes products in ethanol action.

5.2.2 α1-$GABA_A$-Rs and Potential Molecular Mechanisms

While it is widely held that ethanol typically results in decreased excitatory and increased inhibitory actions in the brain [260], ion channels such as $GABA_A$-Rs are not static entities blindly shuffling chloride ions across membranes. Aside from direct interaction of ethanol at α1-$GABA_A$-Rs causing enhanced receptor potentiation, numerous intracellular mechanisms may result in tolerance and withdrawal to ethanol. Indeed, many intracellular processes regulate $GABA_A$-R expression and function after initial ethanol exposure and chronic ethanol (i.e., tolerance mediated mechanisms). For instance, Kumar et al. [364] had shown that α1-containing receptors are internalized following chronic ethanol administration. Additionally, PKC and PKA isoforms can regulate channel function [424, 425]. PKC isoforms may also impart their effects on $GABA_A$-Rs after chronic ethanol [426], and genetic modification of PKC isoforms has suggested their involvement in behaviors related to alcohol sensitivity and tolerance and withdrawal [104, 214, 249]. Although no direct evidence has linked PKA to $GABA_A$-Rs after chronic ethanol, PKA has been implicated in chronic ethanol [427]. Other proteins involved in regulative phosphorylation such as fyn kinase (primarily associated with NMDA-Rs) are suggested to play a role in $GABA_A$-Rs and ethanol action [428]. Behaviorally, fyn knockout

mice exhibited reduced acute functional tolerance and transgenics had decreased ethanol withdrawal-related anxiogenic effects [429, 430].

Apart from direct regulation of $GABA_A$-Rs, intracellular signaling cascades and crosstalk between $GABA_A$-Rs and other neurotransmitter systems may be involved in ethanol effects – specifically chronic ethanol. Studies have shown that ethanol-induced Fos-IR via action at $GABA_A$-Rs involves extracellular signal regulated protein kinases (Erk's) [229]. Erks were also shown to be dysregulated during chronic ethanol using a repeated ethanol binge model. Specifically, Erk activity was reduced from ethanol exposure whereas withdrawal elevated Erk activity [431]. Other factors such as cyclic AMP (cAMP) signaling may play a role in ethanol effects [432], and chronic ethanol has been shown to reduce cAMP activity *in vitro* [433]. $GABA_A$-Rs may also interact with other neurotransmitter systems. $GABA_A$-Rs have been shown to be coupled to dopamine receptors thereby allowing cross-talk between the two systems [434]. As the dopaminergic neurotransmitter system has been implicated in drug seeking effects [174], this crosstalk plays a role in psychological dependence effects of ethanol, such as craving and seeking behaviors. Numerous possibilities exist for a molecular basis for tolerance to ethanol via $GABA_A$-R mediated ethanol action. Because these signaling cascades may overlap with other processes related to addiction, this warrants future detailed analyses. Overall, while our results indicate that ethanol acts through $\alpha 1$ to cause subsequent known neuroadaptations in wildtype mice but not knockins, taken together with the above-mentioned molecular signaling possibilities, this lends support to $\alpha 1$ potentially being a cornerstone for ethanol-related neuroadaptations.

5.2.3 Potential Ethanol-Related Therapies

Because the studies shown herein point to direct involvement of $\alpha 1$-$GABA_A$-Rs in ethanol action, therapeutic intervention may be directed at combating alcohol abuse and alcoholism. While $\alpha 1$-containing receptors were shown to be involved in ethanol-related motor ataxia, $\alpha 1$ specific antagonists could be developed to decrease ethanol-related side effects. Nonetheless, if such an antagonist existed, caution should be exercised with its use. Alcohol is a promiscuous molecule that elicits its affects at a variety of molecular targets. Thus, while such a compound could decrease motor ataxia, it could lead users who take it to have a false sense of security when

driving while impaired. Cognitive impairment may still occur thereby leading to increased traffic-related incidences. Alternatively, because of α1's widespread distribution, inhibiting action at such a vast number of $GABA_A$-Rs may further increase seizure susceptibility. Nonetheless, such a compound if taken at low doses may be able to alleviate the pleasurable anxiolytic effect of ethanol thereby possibly causing alcoholics to not seek alcohol for this effect. Also, based on these results, previously developed α1 selective modulators such as zolpidem (Ambien™) may decrease ethanol withdrawal-related effects.

5.3 RELATION OF SENSITIVITY, TOLERANCE AND WITHDRAWAL

5.3.1 Sensitivity and Tolerance

The results presented here suggest that the development of tolerance is directly related to initial sensitivity. Animals that are less sensitive to the effects of ethanol-induced ataxia appear to be less tolerant. While this is not novel, this and other studies lend support to the hypothesis that the negative attributes of ethanol action are needed to necessitate the development of tolerance [215]. For instance, mice that are more sensitive to the hypnotic effects of ethanol develop more tolerance to this behavior [95]. Gene knockout models have also shown that mice which are less sensitive to the hypnotic and hypothermic effects also develop decreased tolerance [214, 398]. As results herein were conducted at moderate doses, this further extends the hypothesis that sensitivity and tolerance are directly related. From a practical point of view, as alcoholics have been shown to be less sensitive to ethanol's effects [206], screening for ethanol sensitivity may be one possibility to aide in determining susceptibility to alcoholism in the general population. However, sensitivity is not the sole determinant of alcoholism. Another shortcoming of such a screen is the potential to be used to discriminate against individuals in health care.

5.3.2 Acute and Chronic Tolerance

α1 Knockin mice displayed decreased acute functional tolerance as well as decreased chronic tolerance to ethanol's motor-impairing effects compared to controls, which also indicates that acute tolerance is directly proportional to chronic tolerance. This agrees with studies elsewhere that suggest a direct relationship between acute/rapid tolerance and chronic tolerance [96, 112, 223]. However, studies elsewhere suggest that various forms of tolerance are mechanistically distinct [225]; but these assessed the relationship of acute and rapid tolerance. It is possible that whereas acute and rapid tolerance are mechanistically different, rapid and chronic tolerance may exist through similar mechanisms. Further work would have to be done to address this.

5.3.3 Involvement of Withdrawal

While more work has been directed towards a relationship between sensitivity and tolerance, less is known about the relationship between sensitivity and withdrawal. However, one study related initial sensitivity to ethanol withdrawal-related effects. For instance, work has suggested that "withdrawal severity is negatively genetically correlated with sensitivity and magnitude of tolerance to ethanol hypothermia" [221]. While differences were not seen in the present study with respect to hypothermia, this correlation can be related to observed results on motor ataxia. Indeed, α1 knockin mice assessed here displayed decreased sensitivity and decreased tolerance on ethanol-related motor ataxic effects in addition to heightened withdrawal severity, further supporting this negative correlation. However, if hypothermia is taken into consideration as originally noted, then our results would not support this relationship. Clearly, much more work is necessary to further define the relationship between sensitivity and withdrawal. Additionally, further work needs to be done to delineate the relationship be sensitivity and psychological tolerance.

5.4 FUTURE DIRECTIONS

While many of the studies here assessed the role of α1 $GABA_A$-Rs in physical tolerance and dependence, they do not give insight into psychological dependence – such as drug seeking/craving behavior. Understanding alcohol seeking and craving is important because, aside from anxiety-related effects, these two behaviors may underlie what drives people to succumb to this disorder. Considering that crosstalk (discussed above) between neurotransmitter systems can occur, this it is quite possible that $GABA_A$-Rs may play a role in psychological addiction. Additional experiments would need to be done to address this. Other potential behavioral experiments include assessing memory/cognitive effects following chronic ethanol.

Aside from these behaviors, because several ethanol-related effects did not differ here, further investigation into the role of other $GABA_A$-R subunits may indicate the subunits that are involved in these ethanol-related effects. Because α4 subunits are consistently unregulated in response to chronic ethanol [e.g., 363] and are implicated in low dose ethanol effects [59], this would appear to be a candidate target for many of the behaviors assessed here, such as motor ataxia, anxiety, and withdrawal-related hyperexcitability. α2 is also a potential candidate as it has been suggested to be involved in ethanol-related behaviors such as ethanol-induced hypnosis [321] as well as alcoholism [321, 435, 436]. Lastly, because much remains unknown about cellular mechanisms regulating ethanol sensitivity, tolerance and withdrawal, areas such as signaling cascades (discussed above) needs to be explored further.

In summary, this work has identified α1-$GABA_A$-Rs in the mechanism of ethanol action. Although, ethanol is inherently complex in neuronal action further work needs to be done to determine the contribution of other gene products. By chiseling away at the various components of ethanol action we are beginning to elucidate the mechanism of ethanol action. Further elucidation of the mechanism of action of α1 $GABA_A$-Rs in tolerance and dependence could deepen our understanding of the molecular mechanisms behind alcohol abuse and alcoholism. By understanding the molecular mechanisms of ethanol, alcohol abuse may be lessened and alcoholism could potentially be cured.

APPENDIX A

FIGURE REPRINT PERMISSION

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September 7, 2007

Dr. David F. Werner
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