Role of the α4-containing GABA A receptors in anesthetic and ethanol antagonist effects: Insights from a global knockout mouse model

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Despite their widespread use, the precise molecular actions of anesthetics and alcohol are unknown. Although anesthetics have made surgical intervention palatable, anesthetics are not free of alarming side effects such as anesthetic awareness and post-operative cognitive deficits. While alcohol is consumed for positive effects such as anti-anxiety, euphoria, and relaxation, alcohol consumption also results in adverse behavioral effects such as sedation, motor incoordination, and cognitive impairment. Chronic alcohol consumption results in alcohol withdrawal syndrome, tolerance, and dependence. Understanding the mechanisms of action of anesthetics and alcohol are critical for preventing anesthetic side effects and for developing effective treatments for alcohol abuse and alcoholism.

Many putative targets of anesthetics and alcohol have been identified in brain including sodium channels, potassium channels, glutamate receptors, glycine receptors, and especially GABA receptors. Phasic and tonic inhibitory currents mediated by GABA type A receptors are sensitive to modulation by anesthetics and alcohol.

α4 subunit-containing GABA A receptors mediate tonic inhibitory currents, are highly sensitive to GABA, and are strongly implicated in the effects of volatile, intravenous, and neurosteroid anesthetics. Global α4 knockout mice showed reduced tonic current that was not potentiated by the volatile anesthetic isoflurane or the neurosteroid anesthetic, alphaxalone. Specific Aim 1 tested the hypothesis that volatile and intravenous anesthetic effects are mediated via a4-containing receptors by comparing behavioral

responses to these drugs in wild type and α4 KO mice. Results obtained indicate that while α4-containing receptors are required for the amnestic effects of isoflurane, they are not required for mediating the effects of volatile and intravenous anesthetics on other behavioral endpoints. Interestingly, α4-containing receptors are required for low dose alphaxalone-induced locomotor stimulation, but not high dose effects.

α4-containing receptors, when paired with the δ subunit, have been proposed to possess a common binding pocket for ethanol and pharmacologic antagonists of ethanol action. Previous studies in the Homanics lab indicated that the ethanol-reversing effects of Ro15-4513, an imidazobenzodiazepine ethanol-antagonist, were dramatically reduced in α4 KO mice both at the cellular and the behavioral level. Specific Aim 2 tested the hypothesis that a4-containing receptors are required for the ethanol antagonistic effects of RY023, a derivative of Ro15-4513. α4 KO mice showed differential sensitivity to the effects of RY023 in the presence and absence of ethanol on loss of righting reflex and locomotor behavior. We conclude that α4 containing receptors are involved in some intrinsic effects of RY023 but not in the ethanol-antagonistic effects of RY023.

This study of α4-containing receptors has advanced our understanding of anesthetic action and eliminated the theory of a unitary target for ethanol antagonism.

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Preface

Part of Chapter 2 has been published

Rau, V*., Iyer, S.V*., Oh, I., Chandra, D., Eger, E.I.,2nd., Fanselow, M.S., Homanics, G.E., Sonner, J.M., *Gamma-aminobutyric acid type A receptor alpha 4 subunit knockout mice are resistant to the amnestic effect of isoflurane.* Anesth Analg, 2009. **109**(6): p. 1816-22. (* indicates equal contribution)

Parts of Chapter 2 are under preparation for publication
Iyer SV, Chandra D, Homanics GE, 'Intravenous anesthetic responses in $GABA_A$ Receptor α4 Subunit Knockout Mice' *manuscript in process*.

Chapter 3 is also under preparation for publication
Iyer SV, Benavides RA, Chandra D, Homanics GE, 'The ethanol antagonist Ro15-4513 mediates its effects through α4-containing GABA A receptors', *manuscript in process.*

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1.0 Introduction

1.1 Overview

Millions of people all over the world undergo medical procedures that require the use of general anesthetics every day. Each time, these patients are warned about the occurance of unpleasant post-operative side effects and the risks that accompany anesthetic use. Although it has been 150 years since the advent of anesthetics, there is still a dearth of knowledge about the precise mechanism of action of general anesthetics. Gaps in our understanding of anesthetic mechanisms preclude the prediction of incidence of side effects and consequently, patients are exposed to unpleasant experiences such as anesthetic awareness, post-operative cognitive deficits, etc [1, 2]. In addition, our incomplete understanding of the molecular basis of anesthetic action limits our ability to rationally develop newer, safer anesthetics that have limited side effects.

Like anesthetics, the use of alcohol has been prevalent in society for several centuries. Alcohol enjoys a unique position as a beverage whose use is both accepted and criticized in society. Consumption of low to moderate levels of alcohol allows the individual to experience euphoria, anxiolysis and disinhibition. It is for these desirable effects that repeated alcohol consumption is pursued. However, consumption of higher amounts of alcohol causes ill effects such as sedation, impairment of motor abilities, memory deficits, etc. Chronic consumption of alcohol eventually leads to tolerance to alcohol. Because tolerance to alcohol sets in, greater amounts of alcohol are needed each time to achieve the same pleasurable effects of alcohol. In addition, alcohol has great abuse potential and hence regular, repeated consumption of alcohol can often cause drinking behavior to change from a social habit to alcohol dependence and abuse. Unfortunately,

cessation of chronic alcohol consumption produces equally bad effects of alcohol withdrawal such as tremors, irritability, insomnia and seizures [3]. Thus, the cycle of alcohol consumption is notoriously difficult to break.

Although neuroscience research has greatly advanced our understanding of the mechanisms of action of alcohol, we are only now beginning to tease apart the molecular sites of action of alcohol and processes by which alcohol addiction and withdrawal are mediated. A better understanding of these processes will enable the development of agents that can be used to combat the ill effects of alcohol consumption and withdrawal.

While numerous neurotransmitter systems have been implicated in the mechanism of action of both anesthetic and alcohol, one common target is the γ-aminobutyric acid type A ($GABA_A$) receptor system. Although much is known about the role of $GABA_A$ receptors, the vast repository of receptor subunits and the widespread regions of expression in brain and spinal cord have complicated the delineation of functionality. While γ-aminobutryric acid (GABA) is considered the principal ligand for these receptors, it is now known that several endogenous and exogenous ligands allosterically modulate these receptors with varying potencies. Both anesthetics and alcohol are known to modulate $GABA_A$ receptors allosterically. Because the $GABA_A$ receptor system is largely involved in inhibitory neurotransmission, alcohol and anesthetics potentiate inhibitory currents to cause depressive effects. Although our understanding of the complexity of the $GABA_A$ neurotransmitter-receptor system has been advanced over the past several decades, there are still large gaps in our understanding of its functions, especially the role this system plays in alcohol- and anesthetic-induced behavioral responses.

1.2 Introduction to $GABA_A$ receptors

$GABA_A$ receptors are responsible for conducting inhibitory neurotransmission in the brain. These receptors are pentameric, ligand gated chloride channels. Native functional receptors are assembled from 19 different subunits- (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) [4].

$GABA_A$ receptors are of two kinds- synaptic and extrasynaptic, primarily based on location, and conduct two kinds of current that are subject to modulation by ligands. Synaptic and extrasynaptic receptors show differences in their rates of desensitization and affinities to GABA with the latter having slower rates of desensitization and higher affinity for GABA (see review [5]). Phasic current is mediated by synaptic $GABA_A$ receptors, that are typically composed of α1-3, β1-3, γ1-3 subunits [6] and respond to transient high concentrations of GABA (500uM) [7, 8]. These receptors control fast transient inhibitory postsynaptic currents. A persistent tonic current is mediated by extrasynaptic receptors that are usually composed on α4/α6 with β and δ subunits [6] In addition, α5, β and γ2 containing receptors also mediate tonic current [9]. These receptors respond to lower concentrations of GABA (0.1-3uM) [10] such as that encountered during spillover from a synaptic event, or due to leakage from GABA transporters. This tonic form of inhibition is thought to regulate both neuronal excitability and processing of information [11, 12].

$GABA_A$ receptor mediated currents are subject to modulation by anesthetics, alcohol, benzodiazepines, neurosteroids and other central nervous system (CNS) drugs. These drugs modify GABAergic current by influencing affinity of GABA for the receptor and by changing channel kinetics (increased burst frequency, prolongation of decay times, etc.). Anesthetics and alcohol allosterically modulate $GABA_A$ receptors and ultimately cause a combination of behavioral effects - amnesia, anxiolysis, sedation, motor-incoordination, hypnosis, immobilization and analgesia. It is thought that the spectrum of

effects of GABAergic drugs is a result of action on different subunit-containing $GABA_A$ receptors. Hence, the underlying hypothesis of the field is that specific subtype-containing receptors influence specific behavioral effects [13]. This hypothesis has in turn led to the search and development of newer ligands that have specificity and affinity for specific subtypes. Indeed, studies with both isolated receptor systems and genetically engineered mouse models have revealed that certain drugs show a high degree of specificity for certain GABA receptor subtypes [14-17]. Absence of certain subtypes of $GABA_A$ receptors often results in changes in native phenotype as well as drug-induced behavior as observed in genetically engineered models. Experiences such as these add to our understanding about the involvement of GABA in different functions.

1.3 Diversity of $GABA_A$ receptors

The complexity of the $GABA_A$ receptor system is directly related to the different subtypes of $GABA_A$ receptors possible due to subunit combinations. The $GABA_A$ receptor family consists of 19 related subunits in mammals that are encoded by different genes – α(1-6), β(1-4), γ (1-3), δ (1), ε (1), π (1), ρ (1-3) [4]. Although a large number of subunit combinations are theoretically possible, only specific subtypes are encountered *in vivo*. All subunits are polypeptides, approximately 50kd in size each and have four hydrophobic transmembrane domains each. In addition to the 19 distinct subunits, alternate splicing of pre-mRNA of certain subunits (γ2, α5, β2, β4, β3, α6) also contributes to the complex *in vivo* receptor architecture [18-24]. Of these, α, β, γ, δ, ε are encountered in the CNS whereas ρ and π are encountered peripherally [25-27]. It is thought that five subunits participate in the formation of a functional receptor. The most frequently encountered receptor stoichimetry consists of 2α, 2β and 1γ subunit combination [28, 29]. While most

receptors contain a single α isoform within a receptor, a small minority are composed of two different α subunits in combination (α1α2, α1α3, α1α5, α2α3 and α3α5) [30-33]. Similarly with the β subunit, two identical β subunits or two different β subunits can be combined (β1β3, β2β3) within a receptor [34]. The δ subunit replaces γ in a small percentage of receptors in the brain [35]. Immunohistochemical studies indicate that α1, β1, β2, β3 and γ2 subunits are found throughout the brain [29] while other subunits are confined to specific brain regions - α2 (hippocampus, olfactory bulbs, cortex), α3 (raphe nuclei), α4 (dentate gyrus, thalamus, cortex, striatum), α5 (hippocampus), α6 (cerebellum), γ1(cental and medial amygdaloid nuclei, pallidal areas, substantia nigra pars reticulata, inferior olive) and δ (paired with α4 in thalamus, dentate gyrus of hippocampus, cortex, striatum and paired with α6 in the cerebellum) [36-38]. As mentioned earlier, synaptic receptors are typically composed of α1-3, β1-3 and γ1-3 subunits, whereas extrasynaptic receptors are typically composed of α4/6, β2/3 and δ subunit or α5, β2/3 and γ2 subunits.

The actions of anesthetics and alcohol are the result of interactions with numerous neurotransmitter-receptor systems that are beyond the scope of this study. Because $GABA_A$ receptors are the major focus of this research study, discussions of the mechanism of action of anesthetics, alcohol and associated drugs will focus mainly on $GABA_A$ receptors.

1.4 Anesthetics

1.4.1 History of anesthetic research:

The first documented use of an anesthetic (ether) in a surgical procedure occured in 1846 by William Morton. Since then, numerous chemical compounds have been discovered that are used to produce anesthetic effects. Although these compounds show considerable diversity in structure, the spectrum of effects produced by each of them includes sedation, amnesia, hypnosis and immobilization. Fig. 1.1 (reproduced with permission from Wolters Kluwer Health, [39]) depicts the structures of commonly used anesthetics.

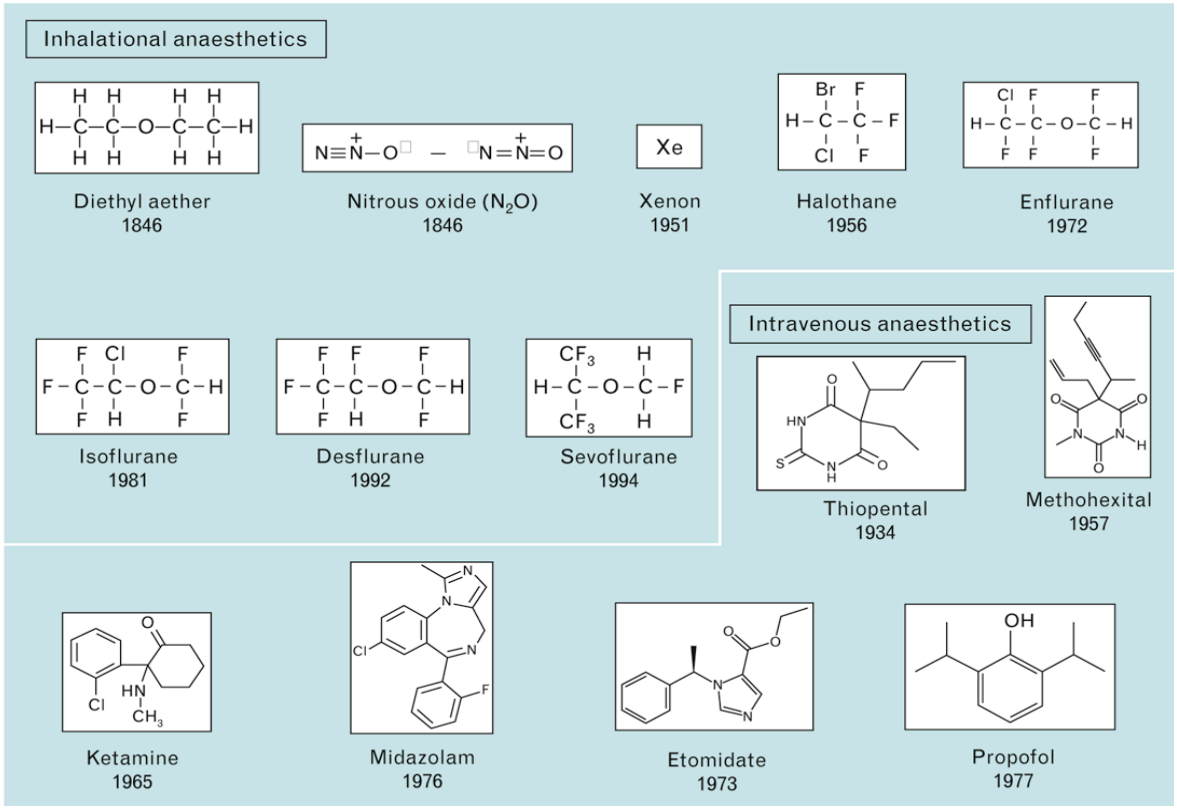

Figure 1.1 Structures of commonly used anesthetics and the year of introduction.

Reproduced with permission from Wolters Kluwer Health. Kopp Lugli *et al*, 'Anaesthetic mechanisms: update on the challenge of unraveling the mystery of anaesthesia'. European Journal of Anesthesiology, 2009, 26 (10): pg 807-20

It has now been more than a century since the formal use of the first anesthetic, however, the mechanism of action of anesthetics continues to remain elusive. In the early 19th century, research into anesthetic targets by Meyer and Overton independently suggested that anesthetics act nonspecifically upon the lipid bilayer of cells to disrupt them leading to anesthesic effects [40, 41]. This theory was put forth following the observation that anesthetics were lipid soluble and that anesthetics of greater potency had higher solubility in lipid-based solvents. According to this theory, anesthetics distribute into the lipid bilayer thus producing changes in the structure of the membrane. This theory predominated into the second half of the 20th century.

However, subsequent research by several groups pointed out flaws in the lipid theory. Seminal work by Franks and Lieb showed that anesthetics were capable of direct interaction with proteins. This was demonstrated on purified luciferase protein which in the absence of a lipid bilayer was dose-dependently inhibited by anesthetics [42]. In addition, Eger and colleagues discovered chemical compunds that were very highly lipophilic but had no anesthetic effect, or only partial anesthetic effects (non-immobilizers) in contrast to that predicted by the Meyer-Overton correlation [43, 44]. Other groups showed that anesthetic action was maintained only with specific stereoisomers of compounds. The (+) isomer of isoflurane was more effective than the (-) isomer in eliciting isoflurane-induced current in molluscan neurons although both isomers were equally effective at disrupting lipid bilayers [45]. This stereospecific activity of isoflurane was confirmed *in vivo* in rats independently by another group [46]. The stereospecifity of anesthetic compounds indicated that far from a non-specific effect, anesthetics bring about discrete behavioral changes owing to other mechanisms [44, 45]. This marked the beginning of a new era – that of protein targets for pharmacological effects. Modulation of inhibitory and excitatory

neurotransmission garnered interest and with it, the study of GABA, glycine, potassium channels, on the one hand, and glutamate, neuronal acetylcholine, adenosine, serotonin receptors, on the other, gained momentum.

Inhibitory neurotransmission is mainly mediated by chloride channels – ligand gated GABA and glycine receptors. Clinical concentrations of anesthetics were found to act on recombinant receptors, they increased chloride currents and caused hyperpolarization [47]. Further, the current potentiation of $GABA_A$ receptors correlated with the clinical potencies of anesthetics [48]. Thus, most anesthetics are capable of acting as $GABA_A$ receptor allosteric modulators. Advances in the study of $GABA_A$ receptors have revealed a vast heterogeneity in $GABA_A$ receptors with differing sensitivity to anesthetics. It is thought that different combinations of these receptor components serve as targets of anesthetics and control discrete anesthetic effects.

1.4.2 Anesthetic effects:

Up to the mid 1800's, patients requiring surgical intervention suffered a horrible ordeal. With the advent of anesthetics, this situation changed. Anesthetics are used clinically for their beneficial effects that render surgical procedures possible. The desired profile of anesthetic effect includes amnesia, hypnosis, immobility and analgesia.

Current anesthetics produce two or more of these effects to varying extents. Hence, surgical procedures often require a combination of anesthetics to bring about a desirable combination of these effects. For example intravaenous anesthetics, etomidate and propofol are used for induction of the anesthetic state. While these agents are good amnestic and hypnotic agents, their immobilizing and analgesic potencies are limited. Hence, muscle relaxing agents (succinylcholine, pancuronium) and immobilizing agents (isoflurane,

sevoflurane) are also used. Thus, multiple anesthetics are often used in combination to obtain a balance of the above attributes desired for surgical procedures. Today, almost 30 million people in the US alone, annually undergo procedures involving anesthetics [49].

The use of anesthetics has to be carefully regulated due to the steep dose response and narrow therapeutic indices of most anesthetics. In spite of their many desirable effects, anesthetics cause side effects that are variable in their intensity in different people. These side effects range from minor discomforts such as nausea, vomiting, headaches to more serious effects such as autonomic instability, respiratory depression, cardiac dysrhythmias and persistant cognitive deficits. The incidence of post-operative nausea and vomiting is about 30% in subjects undergoing anesthetic procedures [50]. Hypothermia and post-operative shivering are observed in 50-60% of patients after volatile anesthetic-induced general anesthesia and in 13% of patients after propofol anesthesia. [51, 52]. The more disturbing effects involve cognitive dysfunctions. One disturbing side effect is 'anesthetic awareness' which is the unexpected, explicit recall of events occuring while under the influence of an anesthetic. About 1-2 patients in every 1000 experience this effect and evidence indicates that the incidence may be higher in children [2, 53, 54]. These episodes are not limited to simple recall but also include reports of mild discomfort to severe pain. This effect has been linked to later psychological effects of nightmares, anxiety and flashbacks [55].

Rodent studies also imply that pediatric and geriatric populations may be at higher risk for anesthetic-induced side effects. Deleterious effects of anesthetics are observed at both the behavioral and the cellular level. Exposure to isoflurane and nitrous oxide decreased memory performance in aged rats [56, 57]. In addition, anesthetic exposure in rodents within the first postnatal week caused neurodegeneration in the hippocampus [58].

This period coincides with the switch of $GABA_A$ receptors from an excitatory to an inhibitory role [59]. Similarly, in humans, pediatric and geriatric populations are particularly at risk for anesthetic-induced side effects. In children, two or more episodes of anesthetic exposure within the first two years of life, was shown to impact learning abilities later in life [60]. Postoperative cognitive dysfunction has been observed in about 25% of geriatric patients a week after anesthetic intervention and in 10% of geriatric patients at 3 months following prolonged exposure to general anesthetics [61, 62].

Although newer anesthetics that are considered safer than their predecessors have been discovered, the lack of knowledge about the precise mode of action of these drugs makes it difficult to predict responses in people and precludes the development of intelligent drug discovery in this field. The seriousness of the side effects summarized above and the possibility of lasting cognitive effects, especially in vulnerable populations such as infants and the elderly, make understanding the mechanism of action of anesthetics a very important goal.

Because anesthetic outcomes involve amnesia, unconsciousness and immobilization among other effects, the process of understanding the mechanisms of action began from the study of brain regions that were likely to be involved in these effects. Several different techniques have been used to study anesthetic effects and results obtained from them have contributed to our current understanding of anesthetic mechanisms. The major effects of anesthetics and our current understanding about the brain regions involved in these effects are discussed below.

1.4.2A Hypnosis and sedation:

Although these terms are used interchangeably, hypnosis is considered distinct from the sedating effect of an anesthetic. Hypnosis, which occurs at high concentrations of anesthetics, refers to the state of unconsciousness wherein response to verbal stimuli is lost. Sedation, which occurs at lower doses of anesthetics, is considered as the step preceding hypnosis where cerebral activity starts to decrease. Cerebral blood flow studies with sedating and hypnotic concentrations of the anesthetic have indicated that the former involves reduction of cortical activity whereas the latter involves depression of sub-cortical structures such as the thalamus, reticular formation and the hypothalamus [63-65].

The first step of hypnosis is depression of ascending corticothalamic pathways. This facilitates depression of the thalamocortical circuitry. At sedative concentrations of the volatile anesthetic sevoflurane and the intravenous anesthetic propofol, a decrease in α waves was observed concomitant with an increase in β waves [66]. This is consistent with transition from wakefulness to drowsiness. As higher hypnotic concentrations were achieved, β oscillations were reduced and δ oscillations predominated. This indicated a transition of anesthetic effect from cortical towards sub-cortical regions. This correlates with transition from drowsiness into deep sleep or unconsciousness. The decrease in activity in brain regions was coroborrated by decreases in glucose metabolism [67].

Imaging studies have noted similarities between the brain during non-rapid eye movement (NREM) sleep and the anesthetized brain [68-70]. One important brain region implicated in sleep processes is the thalamus. Thalamacortical cells that are thought to generate cortical δ rhythms towards normal sleep are thought to be involved in anesthetic-induced δ waves as well [64]. During anesthesia, thalamic neurons have been shown to

enter a low frequency burst mode giving rise to δ oscillations that are characteristic of deep sleep [71]. Thus, anesthetic-induced hypnotic effects overlap with natural sleep pathways. The transition from sedation to hypnosis by higher doses of anesthetics is thought to be coincident with a disconnect in information processing [72-74].

GABAergic circuits among other discrete nuclei are also thought to be involved in pathways of sleep and arousal. These include ventrolateral perioptic nucleus, median preoptic nucleus, magnocellular regions of the basal forebrain and the tuberomammillary nucleus [75-79]. Because each of these brain regions bears efferent and afferant nerve circuits that encompass histaminergic, cholinergic, serotonergic, dopaminergic and GABAergic systems, it is likely that anesthetic effects are the result of cumulative actions on these neurotransmitter-receptors systems.

At the molecular level, specific GABAergic receptors have been identified that are thought to be involved in the hypnosis and sedation caused by general anesthetics. Genetically engineered mouse models have provided information about the role of specific subtypes of $GABA_A$ receptors in anesthetic actions. Through the use of genetically engineered mice, β3 and β2 subunits have been implicated in the actions of some general anesthetics. A β3 global knockout mouse model provided the first report of evidence of general anesthetic actions being altered due to deletion of a GABAergic subunit [80]. However, this result was confounded by developmental compensation [81]. Because global deletion of a gene product can result in compensatory changes by way of upregulation of other gene products that restore function, a knockin approach is sometimes used to circumvent the problem of compensation. A knockin mutation allows normal response to endogenous modulators but selectively alters response to the drug or ligand in question.

Different behavioral assays have been developed to assess clinical endpoints of anesthetics and other drugs in rodent models. For example, hypnotic effects of drugs are tested in rodents by the loss of righting reflex assay (LORR). This assay involves placing mice supine on V-shaped troughs following administration of drugs and recording time until they right themselves. Similarly, sedative effects of drugs are assessed by measurement of reduced motor abilities of rodents studied in an open field arena or other assay involving some motor activity. In this manner, a subsequent β3 knockin mouse study implicated β3-containing GABAergic receptors in the hypnotic actions of etomidate and propofol [15]. These mice bore mutations in the transmembrane region of the β3 subunit (N265M) which rendered them insensitive to etomidate actions. An analogous mutation in the β2 subunit resulted in mice that had reduced sedative responses to etomidate but not propofol [82]. More recently, mice bearing a double knockin mutation in the α1 subunit showed altered sensitivity to sedative-hypnotic effects of volatile anesthetics implicating the α1 subunit-containing receptors in anesthetic action. These double knockin mice bore an isoflurane- and ethanol-insensitive mutation in their α1 subunits that rendered them resistant to the loss of righting reflex (LORR) effects of isoflurane and enflurane but not halothane [83]. The LORR parameter is used as a surrogate for anesthetic-induced unconsciousness in mouse behavioral assays. Interestingly, a double mutation in the α2 subunit that conferred insensitivity to isoflurane-induced current potentiation *in vitro* paradoxically increased isoflurane-induced recovery of righting reflex (RORR) in the mutant mice [84].

Thus, the creation of genetically engineered murine models has provided much information to the understanding of molecular mechanisms of anesthetics. Hence, it is thought that this approach will continue to fill gaps in our knowledge of anesthetic effects.

1.4.2B Immobilization:

Immobilization is a critical effect of an anesthetic, absolutely required for surgical procedures. This attribute of a general anesthesia is best described by the minimum alveolar concentration (MAC). MAC is defined as that concentration of an anesthetic at which half the subjects have lost their response to a noxious stimulus, i.e., surgical incision [85, 86]. It is only in the last 20 years that the primary site of action for this effect was identified as the spinal cord. The observation that cortical electrical activity did not correlate with the immobility produced by an anesthetic sparked a spate of studies with the spinal cord as their focus [87, 88]. Selective delivery of anesthetic to the brain of goats revealed that nearly three times the concentration of anesthetic was required in supraspinal regions to depress movement compared to anesthetic requirement of the whole body [89]. Precollicular decerebration of rats did not affect the immobilizing effect of isoflurane [90]. These studies suggested that the immobilizing effect of an anesthetic depends largely on the spinal cord and is minimally affected by higher brain structures.

Within the spinal cord, specific neuronal circuits are thought be affected by anesthetics. Anesthetics are thought to perturb descending and ascending neuronal influences. *In vivo* electrophysiologic measurements of spinal cord indicated that anesthetics depressed both motor [91] and sensory neurotransmission [91, 92]. Studies with intact isolated spinal cord showed that anesthetics depress motor neuron output arising from dorsal root stimulation to a small extent (~10-20%) and that the effect on dorsal root neurons was not critical to the immobilizing effect [92, 93]. Recent work suggests that inhibition of neurons in the ventral spinal cord by anesthetics is more critical to producing immobility [94]. Specifically, inhibition of the mesencephalic locomotor region may be key to achieving immobility [95]. However, although these studies yielded information about

the circuitry involved in immobilizing effects, the search for a molecular target of anesthetics continued.

Specific study of receptors in the spinal cord suggested that anesthetics potentiated inhibitory chloride channels and also depressed excitatory channels thus spelling a role for GABA and glycine on the one hand and AMPA and NMDA receptors on the other [48, 96-99]. Sodium, potassium and other channels that regulate membrane potentials were also thought to contribute to the effects of anesthetics in the spinal cord.

Pharmacologic studies of different receptor subtypes indicated that they contributed to the effects of anesthetics differentially. For example, the GABA A antagonist picrotoxin increased the MAC of propofol by almost 400% but not of isoflurane [100]. With isoflurane and with ketamine, MAC was increased to a lesser extent with picrotoxin and with the competitive antagonist, gabazine [100]. This smaller increase in MAC for isoflurane and ketamine was attributed to an indirect antagonism of naturally ocurring GABA release. These and other studies showed that GABAergic receptors were likely not involved in the immobilizing effects of inhaled anesthetics [101, 102].

With glycinergic receptors, greater increases in MAC were observed upon the administration of the glycine antagonist, strychnine [103, 104]. Studies with different anesthetics, isoflurane and halothane, on glycinergic receptors indicated that the increase in MAC with strychnine administration correlated with the magnitude of action of different anesthetics upon glycine receptors [101, 104]. This argued for a direct action of these anesthetics on glycinergic receptors as opposed to an indirect effect as observed on GABAergic receptors.

Blockade of NMDA receptors decreased MAC for inhaled anesthetics by approximately 60%. However, the blockade of NMDA receptor activity did not correlate with the extent of functional blockade that anesthetics produce at MAC [105, 106]. Hence, it is thought that NMDA receptors contribute only in part to the actions of anesthetics.

In parallel to pharmacologic studies, genetically engineered murine models were studied for anesthetic sensitivity with regard to immobilization. In GABA receptor β3 knockouts, enflurane and halothane MAC were increased by ~10-25% [80]. Similarly, β3 knockin mice showed reduced sensitivity to enflurane and halothane by ~15-25% even though current potentiation studies showed that normal sensitivity to these anesthetics was maintained *in vitro* [15]. In contrast, the spinal reflexes in β3 knockin mice were not depressed at very high concentrations of etomidate and propofol, mirroring the resistance observed *in vitro* to these anesthetics [15]. These and other studies suggest that GABA A receptors are only moderately involved in modulating the effects of inhalational anesthetics but are involved to a greater extent in the actions of injectible anesthetics. Mice bearing mutations in the glycine receptor system have met with better success. Spastic mice, which have reduced glycine receptor expression due to abnormal splicing of glycine β subunit (Kingsmore 1994), showed a 30% increase in enflurane MAC but no changes with halothane MAC [107]. In contrast, spasmodic mice which bear a knockin mutation in the glycine α1 subunit had decreased responses to glycine but normal responses to enflurane and halothane [108]. Overall, data indicate that glycine receptors may be involved in the effects of inhaled anesthetics. The NMDA receptor ε1 subunit knockout mice did not show changes in isoflurane- or sevoflurane-induced loss of righting reflex. Hence, a role for NMDA receptor subtypes in anesthetic action is still being evaluated. Further studies are

required to understand the involvement of different NMDA receptor subtypes in anesthetic activity.

Previously, the holy grail for dissection of anesthetic action was dictated by the principle 'discrete subunit = discrete effect'. However, at least with immobilization, this view is challenged. An overview of research conducted by independent groups regarding the spinal cord sites of action of anesthetics suggests that anesthetic actions in the spinal cord are not mediated by any one type of receptor but rather by a multitude of receptors acting in concert to produce immobility. Studies that looked at the immobilizing effects of anesthetics lead us closer to the possibility that no single target can explain inhaled anesthetic-induced immobility. Rather a multitude of low affinity targets likely act in concert to bring about anesthetic-induced immobilization. This argument against a single site of action is discussed extensively in a recent review by Eger and colleagues [109].

In contrast to inhaled anesthetics, the intravenous anesthetics, etomidate and propofol were found to mediate their immobilizing effects through GABA receptors. GABA A receptors play a major role in the immobilizing effects of these drugs, given the fact that β3 knockin mice are insensitive to the immobilizing effects of etomidate and propofol [15]. Thus, GABA A receptors may be critical for the effects of intravenous anesthetics such as etomidate and propofol whereas their role in the actions of inhaled anesthetics is limited.

1.4.2C Memory:

Anesthetic impairment of memory is a critical effect of all anesthetics. Amnestic effects of anesthetics are observed at doses well below those causing immobility, typically around 0.1-0.4 MAC. In some cases, however, patients do not experience amnestic effects

of the anesthetic leading to recall of unpleasant details of surgical procedures. This is termed as anesthetic awareness and occurs in 0.1% of patients [2, 53, 54]. At the other end of the spectrum, geriatric patients experience post-operative memory deficits attributable to anesthetic intervention [61]. The reasons behind these effects is unknown.

Memory encoding is a complex process that involves several parts of the brain. Memories are built in a stepwise manner involving acquisition, consolidation and retrieval. The process of programming memory is dependent on sensory cues parsed by cortical and sub-cortical structures followed by transmission to the hippocampus. Exposure to anesthetics disrupts working memory and the transfer of information into long term memory, indicating that it acts on more than one brain area [110]. Different areas of the brain such as the prefrontal cortex, sensory cortex, entorhinal cortex, amygdala, cerebellum and hippocampus play a role in these processes [111, 112]. In fact, lesions of the hippocampus and amygdala have been known to result in memory deficits [113, 114]. Due to the role of the hippocampus in memory processes, GABAergic, cholinergic and glutamatergic receptors expressed in this region are thought to be involved in regulation of memory.

Various GABA A receptor subtypes are expressed in the hippocampus. Studies with genetically engineered mice and anesthetics have revealed that several subtypes play a role in the amnestic responses of anesthetics. Subtypes strongly implicated in this process include α1 and α5 -containing receptors. Global deletion of α1 subunits resulted in reduced amnestic effects of isoflurane [115]. A forebrain specific α1 knockout mouse (that included the hippocampus) also had reduced responses to the amnestic effects of isoflurane [115]. However, a subsequent study with a knockin mutation in the α1 subunit engineered to

impart isoflurane insensitivity showed that mutant mice responded normally to the amnestic effects of isoflurane [83]. This suggested that α1 subunit-containing receptors likely play a marginal role in amnesic effects of isoflurane and that the effects observed in the global knockout may have arisen due to compensation. The amnestic effects of etomidate and enflurane were dramtically reduced in α5 knockout mice [116]. Additionally, these mice had better learning and memory than their wildtype counterparts in the absence of any anesthetic [117]. A knockin mutation in the α5 subunit also produced similar effects in the absence of anesthetics [118]. These studies suggest that deletion of major subunits led to resistance to amnestic effects of anesthetics. In addition, reduction in specific GABAergic receptor subtypes led to enhanced memory [117, 118]. Hence, it stands to reason that GABA A subtypes may play an important role in regulating memory.

Understanding the mechanisms by which anesthetics exert their amnesic effects and relating them to the incidences of post-operative memory deficits such as retrograde and anterograde amnesia will help in the development of safer anesthetics.

Although significant advances in understanding the mechanisms of anesthetics have been made, we are still in the dark about the roles of different receptor systems in anesthetic actions. The hope that consensus sites may be identified for the actions of anesthetics fuels the field of anesthetic research forward. In this regard, the focus of this dissertation is to study the role of a single GABAergic target (the α4 subunit) that has been implicated in anesthetic actions. It is expected that such studies will advance our knowledge about the involvement of different molecular targets in anesthetic actions.

1.5 GABA A receptor mutant models:

Genetically engineered mice have been used to elucidate the physiological and pharmacological significance of particular receptor containing subunits in the actions of anesthetics. These studies have provided a wealth of information about the involvement of different subunits in anesthetic effects. The following table (Table 1.1) summarizes the findings from different mutant mouse screens.

Table 1.1 Anesthetic responses in genetically engineered murine models
Numerous studies have assessed the contribution of different GABA A receptor subunits to anesthetics actions.
KO : Knockout; KI : Knockin; CKO: Conditional Knockout
= no change with respect to genotype; ↓ decreased in comparison to controls ; ↑ increased in comparison to controls

Subunit	Genetic Alteration	Behavioral Response	References
α1	KO	↓ pentobarbital (LORR) ↓ etomidate (LORR) ↓ midazolam (LORR) ↑ ketamine (LORR) = propofol (LORR) ↓ isoflurane (amnesia) = isoflurane (MAC, LORR) ↓ halothane (LORR)	[115, 119]
α5	KO	↓ etomidate (amnesia) = etomidate (sedation, hypnosis, motor ataxia)	[116]
α6	KO	= halothane (LORR) = enflurane (LORR, immobilization) = midazolam (LORR) = pentobarbital (LORR)	[120]
β3	KO	↓ etomidate (LORR) ↓ midazolam (LORR) = pentobarbital (LORR) ↓ enflurane (immobilization) ↓ halothane (immobilization) = enflurane (LORR) = halothane (LORR)	[80]
β2	KO	↓ etomidate (LORR) = pentobarbital (LORR)	[121]
γ2L	KO	↑ midazolam (LORR) = etomidate (LORR) = pentobarbital (LORR)	[122]
δ	KO	= etomidate (LORR)	[17]

		= pentobarbital (LORR) = midazolam (LORR) = propofol (LORR) = ketamine (LORR) ↓ alphaxalone (LORR) = halothane (LORR, immobilization) = enflurane (LORR, immobilization)	
α1 (S270H, L277A)	KI	= isoflurane (immobilization, amnesia) ↓ isoflurane (LORR) ↓ enflurane (LORR) = halothane (LORR, immobilization) = desflurane (immobilization)	[123]
α2 (S270H, L277A)	KI	= isoflurane (LORR, immobilization) = halothane (LORR, immobilization) ↑ isoflurane (RORR)	[84]
β2 (N265S)	KI	= etomidate (LORR, immobilization) = pentobarbital (LORR, immobilization)) = propofol (LORR, immobilization) ↓ etomidate (sedation, motor ataxia)	[82]
β3 (N265M)	KI	↓ enflurane (immobilization) ↓ halothane (immobilization) ↓ isoflurane (immoblization) = isoflurane (amnesia) ↓ etomidate (LORR, immobilization) ↓ propofol (immobilization) = alphaxalone (LORR, immobilization)	[15, 124]
α1	CKO (forebrain specific)	↓ isoflurane (amnesia, immobilization) = isoflurane (LORR) = desflurane (immobilization) = halothane (immobilization, LORR) = pentobarbital (LORR)	[115]
β3	CKO (forebrain specific	↓ etomidate (LORR)	[125]
β3	CKO (paneuronal)	↓ etomidate (LORR)	[125]

1.6. Alcohol

Another area of research that shares the problems of 'multifarious effects through multiple targets' is alcohol (ethanol). The gaps in our understanding of how alcohol mediates its effects of motor incoordination, unconsciousness, alcohol addiction and withdrawal limit our ability to develop effective therapies for treatment of alcohol abuse.

Sadly, the effects of alcohol abuse are far-reaching and pose both an economic and social problem. The following section addresses the need to study the effects of alcohol, advances in alcohol research and potential therapeutic targets for combating alcohol effects.

1.6.1 Alcohol in society:

Ethanol consumption has been a part of social history from as early as 200 BC [126, 127]. In small quantities, alcohol produces a sense of well being, relaxation, loss of inhibiton and emotional arousal. It is for these effects that repeated indulgence of alcohol usually occurs. At slightly higher doses, alcohol produces changes in mood (such as anger, aggression, depression, stupor), memory deficits and motor incoordination (changes in gait and balance). At much higher doses, ethanol finally produces unconsciousness, respiratory depression and even death.

Alcoholism is a major disease that places significant economic burden on society. The indirect cost associated with alcoholism easily surpasses 180 million dollars annually in the US [128]. Nearly 40% of all traffic crash fatalities are linked to alcohol [129]. In 2005, nearly 13,000 people died due to alcohol-related cirrhosis in the United States alone [130]. A strong link has been found between alcohol and aggresive or violent behavior, with alcohol being a significant factor in >50% murders and violent crimes [131]. In addition, a strong correlation between alcohol abuse and incidence of psychiatric problems has also been suggested [132]. As if this was not enough, problems such as alcohol related crime incidence and domestic violence make this drug of abuse a major social hazard.

Thus, both acute and chronic alcohol consumption have serious consequences not only for the individual but also for society. Understanding how these effects of alcohol are mediated would be a definite step towards resolving the negative effects of alcohol. Such

information would give us the ability to design agents that could be used to combat the effects of alcohol. In the following sections, the effects of acute and chronic alcohol and the involvement of neurotransmitter-receptor systems in these actions will be discussed.

1.6.1A Effects and consequences of acute and chronic ethanol consumption:

Acute consumption of large amounts of ethanol cause hangover effects on the following day such as headache, nausea, vomiting and dehydration. Binge drinking also causes mood changes and memory deficits. In addition, binge drinking is often the cause of acute alcohol poisoning, the symptoms of which include mental confusion or stupor, vomiting, seizures, slow or irregular breathing and hypothermia. Acute ethanol toxicity presents an emergency situation warranting the need of therapeutic agents that can reverse the ill effects of ethanol. Commonly used therapeutic agents in the management of alcohol toxicity include benzodiazepine antagonists such as flumazenil [127, 133, 134]. However, these agents are only moderately effective in the treatment of acute ethanol toxicity. Hence, there is a need to discover new agents that may be more effective in antagonizing these effects of acute ethanol.

How does chronic alcohol addiction set in? It is thought that repeated exposure to alcohol produces changes in the brain that reinforce the addicting aspects of alcohol. Inability to self-regulate consumption results in psychological changes such as irritability, anxiety, preoccupation or anticipation of the next exposure to alcohol and withdrawal-related negative effects. Particularly, the negative withdrawal effects are hypothesized to be instrumental in providing the major motivational impetus for compulsive ethanol intake [135]. Brain regions associated with addictive mechanisms include the extended amygdala comprising of stria terminalis, nucleus accumbens shell, sublenticular substantia innominata and the lateral hypothalamus (see review, [136]). Changes in neurotransmitter

functions accompany the reinforcing effects of alcohol (see review [137]). GABAergic, opioid peptidergic, dopaminergic, glutamatergic and serotonergic systems have been implicated in the neurobiology of addiction (see review, [138]). These changes persuade the individual to seek alcohol repeatedly. This leads to addiction and tolerance. Tolerance to alcohol is said to occur when after continued drinking, greater amounts of alcohol are required to achieve the same effect [139]. Due to these effects, the individual requires greater amounts of alcohol each time to experience its pleasurable effects. Failure to imbibe alcohol produces withdrawal changes that are severe and debilitating. These include hyperexcitability, propensity to seizures, irritability, increased anxiety, insomnia and tolerance to typical sleep-aids [3].

Although, a lot more is known about the effects of alcohol and its addictive properties now compared to two decades ago, we are far from deciphering the full spectrum of its complex effects. Due to all these reasons, alcohol and its effects are the focus of much research. As with anesthetics, the molecular mechanisms of action of alcohol are not well elucidated. GABA A, glycine, glutamate and dopaminergic receptors systems are all though to contribute to the actions of ethanol. The involvement of GABA A receptors with alcohol is discussed below.

1.7 Alcohol and GABA:

A plethora of evidence has linked the GABA A receptor system to ethanol effects. Behavioral effects of alcohol were potentiated by GABAergic agonists and reduced by GABAergic antagonists [140]. Differences in GABA A receptor function are observed in mice that show differential sensitivity to ethanol (long sleep and short sleep mice) [141]. GABA A receptor subunit mRNA also are changed in alcohol withdrawal prone and

alcohol withdrawal resistant mice [142]. Further, these mice show changes in upregulation and downregulation of GABA A mRNA levels following chronic ethanol treatment [143]. Studies on alcohol-prefering (P) and non-prefering (NP) rats have implicated the GABAergic system in the nucleus accumbens as critical for initiation of ethanol drinking behavior [144, 145]. Additionally, quantitative trait loci studies have suggested that 20% of candidate genes related to ethanol withdrawal severity are linked to GABA neurotransmission [146].

Studies of direct interactions of alcohol with GABAergic receptors revealed that alcohol potentiated chloride uptake via GABA receptors [147]. When studied with muscimol, 20-60 mM ethanol potentiated chloride uptake in cultured spinal neurons [148], mouse cerebellar microsacs [149] and cerebral cortical synaptoneurosomes [150, 151]. Antagonists of GABA A receptor function such as bicuculline and picrotoxin blocked such effects [150, 152]. Thus, it is now a well known fact that ethanol acts on GABAergic receptors to enhance inhibitory chloride neurotransmission. However, GABAergic receptors differ in their sensitivity to ethanol depending upon receptor composition.

A study by Mihic *et al.*, identified a 45- amino acid spanning region within the second and third transmembrane domains of $\alpha 1/2$ subunits, that was required for direct enhancement by ethanol and anesthetics [14]. Mutation of specific residues in this region ablated sensitivity to ethanol in *in vitro* cell systems [153].

Several GABA receptor subtypes have been shown to be responsive to ethanol. *In vitro* studies showed that synaptic GABAergic receptors were activated by ethanol [14, 154, 155]. However, this potentiation was observed at doses of ethanol ≥ 50 mM. This was later attributed to the fact that these studies focused on synaptic receptors alone. Recent studies by independent groups have revealed a population of receptors uniquely sensitive to

low doses of ethanol [156, 157]. Ethanol was shown to enhance the inhibition of extrasynaptic α4/6βδ-GABA A receptors at concentrations that are physiologically relevant (3-30 mM), although this finding is controversial [157-160]. (This aspect is discussed in greater detail in Section 1.10.2)

The legal limit for alcohol intoxication in the US is 0.08% or 17mM. It is interesting to note that the concentrations at which α4/6βδ- containing receptors are modulated (3-30 mM) is within the range of alcohol intoxication observed in humans. Thus, the doses of ethanol that produce intoxicating effects of sedation and motor ataxia are more likely to be mediated by δ-containing receptors. Human lethal blood alcohol concentrations range from 50 mM-110 mM [161, 162], suggesting that synaptic receptors must be responsible for the high dose effects of ethanol.

In addition to direct actions, ethanol can also mediate post-translational modifications on GABA A receptors [163-165]. GABA A receptor subunits possess phosphorylation sites that are thought to regulate GABA binding in different regions of the brain [166], channel conductance and influence internalization [167]. Deletion of different isoforms of protein kinase C (PKC) produced variable ethanol effects. For example, deletion of PKC γ and δ led to a reduction of some ethanol effects in KO mice [168, 169], while the deletion of the ε isoform caused mice to exhibit increased sensitivity to some effects of ethanol [170]. Other phosphorylating enzymes have also been shown to be involved in modulating the effects of ethanol [171-174]. Finally, ethanol also increases neurosteroid levels and thus increases their actions on GABA A receptors [175]. Thus, ethanol affects GABA A receptor function in a variety of ways.

Distinct changes are observed in GABAergic receptor functions and expression profiles following chronic ethanol treatment. Following chronic ethanol exposure, there is a

decrease in chloride flux in the hippocampus of rats subjected to chronic intermittent ethanol treatment (CIE) [176]. Similar changes have been observed in spinal cord and cortical neurons that were exposed to chronic ethanol treatment [177]. In addition to changes in chloride flux, GABA A receptors undergo changes in expression of subunits. Post chronic ethanol exposure, a downregulation of synaptic α1 subunits occurs [178]. However, α4, α6 and γ2 subunits are increased whereas δ subunits are decreased [178]. Electron microscopy studies revealed that during chronic ethanol withdrawal, concomitant with the decrease in δ, α4 combines with γ2 subunits within the synapse [179]. The α4β2γ2 combination carries less current than the corresponding extrasynaptic tonic current when in combination with δ subunits [178]. The result of this is decreased charge transfer, that possibly contributes to the hyperexcitability seen post-chronic ethanol exposure.

In keeping with the change in subunit combinations, changes in current potentiation by different drugs in hippocampal neurons are observed during withdrawal [180]. During withdrawal, a decrease in mIPSC potentiation by zolpidem [181], decrease in tonic current potentiation by ethanol [179], increased potentiation of mIPSC for gaboxadol [181] [179], increased potentiation of mIPSC by Ro15-4513 [179, 181] was observed.

The changes occuring biochemically are reflected in behavior as well. For example, post-chronic ethanol treatment, the effect of ethanol on LORR was reduced by 92% in CIE-rats compared to control rats. The decrease in δ also contributed to the tolerance seen with ethanol and neurosteroids during chronic ethanol withdrawal [180]. Alphaxalone (neurosteroid anesthetic) -induced LORR, diazepam-induced LORR was reduced dramatically [180]. In addition, the effects of pentobarbital and gaboxadol (THIP) were also reduced [181]. The reduced response to these drugs is a reflection of cross-tolerance or decreased GABAergic function induced by chronic ethanol withdrawal.

Although the molecular mechanisms of ethanol have been studied extensively, there is much that we do not know. For instance, what mechanisms give rise to the changes in receptor expression and combination? Are the changes in the GABA receptor system the cause or effect of withdrawal behavior? If receptor expression changes were prevented during chronic ethanol withdrawal, could withdrawal behavior be alleviated? Research in this area has only scratched the surface of the complex processes taking place in the aftermath of ethanol exposure. The study of ethanol exposure and withdrawal changes *in vivo* involves replicating the biochemical and behavioral effects of chronic ethanol in an appropriate model system. A combination of pharmacologic and genetic engineering approaches are currently being used to understand the many effects of ethanol. Genetically engineered murine models have greatly advanced our ability to study ethanol-behaviors. These studies have yielded some interesting results and are reviewed in [182]. The studies involving GABA A receptor mutant models are summarized in Table 1.2.

Table 1.2: Ethanol effects in genetically engineered murine models
Numerous studies have assessed the contribution of different GABA A receptor subunits to alcohol actions.
KO : Knockout; KI : Knockin; CKO: Conditional Knockout
= no change with respect to genotype; ↓ decreased in comparison to controls ; ↑ increased in comparison to controls

Subunit	Genetic Alteration	Behavioral Response	References
α1	KO	↑ locomotor stimulation ↓ LORR = LORR = motor ataxia = anxiolysis = chronic withdrawal = seizures = tremors	[119, 121, 183, 184]

α2	KO	↓ LORR = anxiolysis	[185]
α4	KO	= motor ataxia = hypnosis = anxiolysis = locomotor stimulation	[186]
α5	KO	↑ locomotor stimulation = LORR = anxiolysis	[185]
α6	KO	= LORR = chronic withdrawal	[120, 187]
β2	KO	= locomotor stimulation ↓ LORR = chronic withdrawal	[121, 183]
β3	KO	= LORR	[80]
γ2 L	KO	= LORR = anxiolysis = motor activity = chronic withdrawal	[188]
δ	KO	= LORR = anxiolysis = hypothermia ↓ chronic withdrawal ↓ drinkng behavior ↓ protracted tolerance	[189]
α1 (S270H, L277A)	KI	↓ motor ataxia ↑ anxiolysis = hypnosis = locomotor stimulation	[190, 191]
α2 (S270H, L277A)	KI	↑ hypnosis ↓ locomotor stimulation	[192]
γ2 S	Tg	= hypnosis	[193]
γ2 L	Tg	= hypnosis	[193]

1.8 Neuroprotectives during Chronic ethanol withdrawal:

Because alcoholism is a widespread disease, with so many far reaching effects, the search is on for agents that can be used to effectively combat the endpoints of withdrawal and dependence. Parameters of chronic ethanol withdrawal behavior have been replicated

and studied in numerous murine models in an effort to better understand the processes underlying withdrawal behavior. The severity of alcohol withdrawal is thought to be another motivating factor in alcoholism relapse. Hence, drugs that are effective at reducing the negative symptoms of ethanol withdrawal may also find use in the treatment of chronic alcohol withdrawal and dependence.

1.8.1 Taurine and ethanol:

The interaction of taurine with ethanol has been the focus of several research groups since the early 1970's and continues to garner interest even today. Taurine derivatives and metabolites were found to interact with ethanol, albeit to varying potencies [194-196]. It has been shown that cysteic acid [197] and taurine [198] reduced circulating alcohol levels while taurocholic acid decreased ethanol preference [197]. Taurine itself combats increases in glutamatergic activity during ethanol withdrawal [199-202]. It is not known if this is due to direct inhibition of glutamatergic receptors or due to an indirect action via GABA or glycine receptors.

Taurine is a sulfonated β amino acid, that is structurally similar to GABA. Not surprisingly, it potentiates GABA and glycinergic neurotransmission [203-207]. It is abundantly found in excitable tissues such as heart and brain [208, 209]. Taurine is synthesized from methionine in the body through a muli-step enzymatic process. It is implicated in calcium homeostasis, osmoregulation, neuroprotection, antioxidation and neurotransmission [208]. Taurine is thought to be in the low millimolar levels in synaptic clefts and taurine immunostaining is observed in presynaptic nerve terminals as well as in dendrites and cell bodies [207, 209]. In binding studies, taurine displaced direct and allosteric agonists of the GABA A receptor [210-214]. Several brain regions such as hippocampus, striatum, cortex and substantia nigra show GABA-A receptor mediated

inhibition when taurine is added exogenously [204, 215-217]. The use of glycine and GABA antagonists produced a reduction in the current potentiation by taurine [205, 206, 218], further solidifying the view that actions of taurine are mediated through GABA A and glycine receptors.

Given the actions of taurine on GABA A receptor, it was intuitive that taurine would have some interactions with ethanol. But what kind of behavioral outcomes would such interactions have? Behavioral experiments with ethanol and taurine indicated that taurine acts as an ethanol antagonist. At low doses of ethanol, taurine inhibited ethanol-induced locomotor stimulation, whereas at higher doses of ethanol the sedative effects of ethanol were diminished [196]. It appears that the route of administration of taurine also differentially affected its mechanisms. For example, taurine administered intracerebroventricularly following a sedative/hypnotic dose of ethanol administered intraperitoneally (i.p.) potentiated the effects of ethanol [219, 220]. However, a peripheral injection of taurine reduced the impairing effects of ethanol [220-222]. Several groups have reported that stimulation of glutamate receptors evoked taurine release into the extracellular spaces [223-225]. Additionally, glutamatergic over-excitation can be countered by exogenous taurine via GABA A and glycine receptors [226-228]. Interestingly, chronic ethanol withdrawal resulted in hyperexcitability of glutamatergic neurotransmission [229-232]. Hence, it was possible that taurine could reduce the excitatory glutamatergic neurotransmission during ethanol withdrawal.

Diaz-Granadoz *et al* [233] tested the effects of taurine administration in C3H/HeJ mice receiving chronic ethanol treatment. Taurine-treated mice showed reduced propensity to handling-induced convulsions (HIC) during withdrawal compared to control mice. This

result indicated that taurine was indeed successful in alleviating withdrawal. But the mechanism by which this effect was mediated is unknown.

Recent work by Jia *et al* showed that contrary to popular notion, extrasynaptic GABA receptors were greatly sensitive to taurine and showed robust tonic current potentiation with taurine [234]. Whereas synaptic receptors required 1-10mM for current potentation [204, 235], extrasynaptic α4-containing receptors responded to only 10-100μM of taurine [234]. In addition, ventrobasal thalamic neurons from α4 KO mice did not show current potentiation with taurine. This demonstrated that α4-containing receptors were required for mediating taurine-induced current potentiation. It is noteworthy that during ethanol withdrawal, α4 levels increase dramatically [178]. Thus, recent evidence indicates that α4-containing GABA receptors may be useful targets for alleviation of ethanol withdrawal symptoms. However, it is not known if α4-containing receptors mediate the protective effects of taurine during ethanol withdrawal.

Understanding the mechanism of action of taurine could provide insight into the mechanism of action of its derivative, acamprosate. Acamprosate, approved by the Food and Drug Administration in 2004, is very successful in treating alcoholism and preventing relapse. However, its mechanism of action is not currently known. In a series of studies, Dahchour and DeWitte demonstrated that acamprosate was capable of reducing several behavioral and biochemical measures of hyperactivity in the aftermath of chronic alcoholism [236-238]. The pharmacological effects of acamprosate have been related to its actions upon central neuromediator systems and both GABA and glutamate pathways have been implicated [239-241]. Acamprosate is remarkably effective in the treatment of chronic alcoholism and several studies have related its effects to local increases in taurine [238]. Therefore, if the mechanism of action of taurine was known, it may aid the understanding

of the mechanism of action of acamprosate as well. This in turn could lead to the development of newer drugs for the treatment of alcohol relapse.

1.9 Ethanol antagonists:

Binge drinking of alcohol produces effects of headache, nausea, dehydration. Consumption of ethanol leads to undesirable behavioral effects but also increases the risk for cancer, alcoholic dementia, liver disease and stroke. Not only this, but alcohol has strong abuse potential. While many generalized GABAergic antagonists such as picrotoxin and bicuculline are effective in antagonizing the effects of ethanol and other GABA agonists, due to their non-specific actions, they also reduce essential inhibitory neurotransmission in the brain leading to undesirable side effects. Currently, the drug of choice for reversing acute ethanol toxicity, is flumazenil. The high incidence of emergency cases of ethanol toxicity, the short half life of flumazenil, coupled with its intravenous route of administration, urgently call for the developement of better, efficacious ethanol antagonists. A more effective ethanol antagonist would be one that is a selective ethanol antagonist. Such a drug should selectively reverse the actions of ethanol alone without producing side effects. If such a drug were to be developed, one can envision a scenario in a social setting where the effects of acute ethanol consumption are reversed prior to driving, or operating machinery, thereby minimizing accidents. In addition, reversal of some effects of ethanol could prevent or slow the advent of a hangover and dehydration. In cases of chronic alcoholism, the use of such a drug during ethanol exposure may prevent the likelihood of development of addiction. In addition, such a drug may find use in the treatment of alcoholism. The possible applications of an ethanol antagonist are endless.

Even if such a drug did not find immediate clinical applications, the drug could be used in research settings to provide additional information about the pharmacology of ethanol.

Current pharmacotherapies for treatment of alcoholism (naltrexone, acamprosate, disulfiram) are few in number and surprisingly, have been discovered serendipitously. Considering the possibilities of a putative ethanol antagonist, it is time systematic drug discovery paradigms were undertaken with vigor to address this problem. In fact, some groups have already taken the lead and have synthesized drugs and developed structure-activity relationships for putative ethanol antagonists.

1.9.1 Atypical benzodiazepines as ethanol antagonists:

Typical benzodiazepines, such as diazepam and flunitrazepam, potentiate GABA A receptor channels. With ethanol, they cause synergistic effects and increase the net chloride current mediated by target GABA A receptors. Behaviorally, they enhance the effects of ethanol - induced anxiolysis, motor incoordination, sedation and hypnosis. Benzodiazepines act primarily on synaptic $GABA_A$ receptors. These are usually composed of $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$ and $\gamma2$ subunits. The benzodiazepine binding site is thought to lie at the interface of an α and $\gamma2$ subunit. Extrasynaptic receptors containing $\alpha4$, $\alpha6$ and δ subunits are thought to be benzodiazepine–insensitive, since drugs belonging to this class do not bind appreciably to or potentiate current through these receptors [242-244].

Ro15-4513 is an imidazobenzodiazepine synthesized in 1984 by Hoffman-LaRoche. It bears structural similarity to flumazenil- a benzodiazepine antagonist. Ro15-4513 antagonizes ethanol-mediated chloride flux [150, 245, 246]. In behavioral studies, Ro15-4513 was more efficaceous than flumazenil, the parent benzodiazepine antagonist in antagonizing the effects of ethanol [247]. Several groups demonstrated antagonism of

ethanol-induced LORR, motor ataxia, and sedation by Ro15-4513 [247-249]. Additionally, at the doses tested, Ro15-4513 did not possess any intrinsic effects [248, 250].

Encouraged by the behavioral antagonism of ethanol by Ro15-4513, several other drugs bearing structural similarity were synthesized in the 1990's [251, 252]. RY023, RY024 and RY080 are structural analogues of Ro15-4513, and as such, all three drugs behave as ethanol antagonists [253-256]. Binding studies on recombinant $GABA_A$ receptor subtypes revealed that the RY series of drugs had higher binding affinity to $\alpha5\gamma2$-containing receptors compared to $\alpha1/\alpha2/\alpha3/\alpha4/\alpha6$-$\gamma2$ containing receptors (Lui 1995, 1996, Cook 2005) [251, 253, 255, 257]. In functional studies, the RY series of drugs negatively modulated all $\alpha\beta3\gamma2$ subtypes, but were most effective at the $\alpha5\beta3\gamma2$ subtype [253, 255]. However, it is important to note that these drugs were not tested against all possible combinations of GABA receptor subunits.

Behavioral assays with RY023 revealed that this drug was effective in reducing ethanol preference as tested by the operant conditioning assay [253]. Ethanol-induced motor ataxia, tested on the oscillating bar test, was also reversed by all tested doses of RY023 [253]. When locomotor effects of ethanol were studied by the open field assay, RY023 was successful in reducing the sedative effect of a 1.25g/kg dose but not of a 0.75g/kg dose of ethanol [253]. However, a 7.5mg/kg dose of RY023, when give alone, produced strong intrinsic effects of sedation [253]. Intrinsic effects of RY023 were not observed on the oscillating bar test for motor ataxia.

An intrinsic effect is defined as a change in behavior caused by a drug compared to a non-active placebo. Since the RY series of drugs produce changes in behavior when given alone [253, 254, 256], they are said to possess intrinsic effects. The relative magnitude of intrinsic effect is often dependent upon the behavioral paradigm under study

[258, 259]. Like with RY023, ethanol antagonism and intrinsic effects were seen with both RY080 and RY024 [254, 256]. Because these drugs are inverse agonists, it was thought that the ethanol antagonism was a result of inverse activity at specific $GABA_A$ receptor subtypes. However, the intrinsic effects of RY drugs on open field showed increased sedation not hyperactivity as would be expected for an inverse agonist. Hence, the actions of RY drugs on ethanol cannot be explained entirely by a subtractive hypothesis.

Based on behavioral studies and binding affinity, it was proposed that the RY series of drugs acted predominantly at α5-containing receptors to bring about ethanol antagonism [251, 253, 255-257, 260]. Actions at α5-containing receptors alone do not explain the differences in intrinsic effects on motor coordination and sedation on the one hand but similar antagonism at ethanol-induced motor ataxia and sedation on the other. Further, the dose and behavior-dependency of the actions of RY drugs suggests that there is more to this story than meets the eye. Thus, our understanding of the actions of these drugs is still incomplete.

Recent work by Wallner, Hanchar and Olsen has challenged the belief that α5-containing receptors may be the predominant site of action for the RY drugs. Evidence towards this was obtained from the observation that Ro15-4513 reversed the effects of ethanol alone at specific doses but not of barbiturates [261]. Thus, its activity was less likely due to inverse agonism and more likely due to a specific site of action that is common to ethanol.

Advances in the study of ethanol actions have shown that certain $GABA_A$ receptor subtypes are more sensitive to ethanol-induced current potentiation than others. As mentioned above, extrasynaptic receptors containing α4/α6-δ subunits have been implicated in low dose ethanol effects [157, 158], although this is controversial [160].

These receptors are more sensitive to GABA and produce tonic current that is modulated potently by ethanol compared to the phasic current produced by synaptic receptor counterparts. Importantly, studies on recombinant α4β3δ receptors showed antagonism of ethanol-induced current by Ro15-4513 [262]. It is important to note that at least one other group did not observe displacement of Ro15-4513 by low doses of ethanol from native δ-containing receptors [263]. However, the reasons for this are currently unknown.

On recombinant α4δ-containing receptors, Ro15-4513 was functionally silent, i.e. it did not potentiate any current. In addition, bound Ro15-4513 was competitively displaced by ethanol as well as other benzodiazepine site ligands such as flumazenil and the RY drugs, RY080 and RY024 [262]. This indicated that Ro15-4513 and the RY drugs shared a common site of action with ethanol on the ethanol sensitive α4/6δ-containing receptors.

Based on these observations, Olsen, Wallner and Hanchar proposed the presence of a common binding pocket for ethanol and these drugs [156, 158, 262, 264]. According to this theory, receptors possess a high affinity and low affinity binding site for ethanol- the former is important for Ro15-4513 reversal. Ro15-4513 and the RY series of drugs possess a core imidazobenzodiazepine ring structure with differing substitutions at positions C7 of the ring. Substitutions at this position are thought to occupy the ethanol binding pocket [265]. It was proposed that the size of the residue at position 7 of the imidazobenzodiazepine ring dictates the ethanol-antagonizing potential of the drug. Ro15-4513 and the RY series of drugs have groups at their C7 positions that likely preclude the binding of ethanol concurrently. Thus, they antagonize the effects of ethanol. In contrast, flumazenil (Ro15-1788) has a fluorine atom at this position - the fluoride residue being small in size does not inhibit the binding of ethanol and allows co-occupancy of the pocket, thus allowing for ethanol effects. In 2006, Hanchar *et al* showed that some of these drugs

also bound with high affinity to α4δ-containing receptors and suggested that binding of Ro15-4513 and its analogs to α5β2γ2 BZ site may be a coincidence, unrelated to their ethanol-antagonist potential [262]. Thus, α4δ-containing receptors are proposed to be the primary target for the ethanol antagonistic actions of Ro15-4513 and RY drugs.

Regardless of their site of action, these drugs have great potential for combating acute ethanol toxicity. In addition, the ability of the RY series of drugs to confer resistance to ethanol-reinforcing effects indicate that they act on sites involved in the addiction pathway. Hence, its possible that these drugs may have anti-addiction potential as well. These ideas hold great promise for the development of a specific ethanol antagonist. Further research in this area has the potential to revolutionize the field of ethanol research and treatment.

1.10 Relevance of GABA A receptor α4-containing receptors:

The architecture and role of receptor isoforms containing the α4 subunit has gained a lot of focus over the past couple years and studies are only beginning to understand the constellation of effects executed by α4-containing receptors. The α4βγ2 subunit combination is encountered both synaptically and extrasynaptically, whereas the α4βδ receptor is exclusively extrasynaptic [16, 179, 181, 266-268]. In addition, an α4β3 binary receptor has been proposed although its presence has not been encountered physiologically [269]. α4-containing receptors are located in the dentate gyrus, thalamus, striatum and cortex, where they conduct tonic and synaptic currents [242].

Tonic current has gained a lot of importance over the last several years as being the target of modulation by several ligands. The role of a persistent tonic current was unclear

until a few years ago. Recent evidence indicates that tonic current is important for homeostasis of neuronal excitability [12]. Neuronal network excitability and information processing is thought to be regulated by tonic current. The effects of tonic current differ depending upon the brain region. In certain regions, tonic currents regulate the noise associated with neuronal firing. An increase in GABA levels leading to increases in tonic current can help decrease neuronal excitability and prevent neuronal saturation [270] This reduces the number of neurons that are simultaneously excited, resulting in sparseness of of neuronal firing [271]. Computational modeling studies have revealed that enhancing the sparseness of coding can help record and store greater information over time [272] Thus, the presence of tonic current in the hippocampus and the cerebellum play an important role in regulating the neuronal firing burden over time.

In regions where both phasic and tonic inhibition occur, changes in the extracellular GABA levels could dictate the predominance of type of neuronal currents [11]. For example, in hippocampal pyramidal cells, tonic GABA currents are small and phasic GABA currents predominate. Addition of picrotoxin which selectively inhibits tonic currents, resulted in an increase in frequency of spontaneous inhibitory postsynaptic currents (sIPSC). This indicated that tonic current in the hippocampus served to regulate the excitability of pyramidal cells [273]. Thus, tonic currents have an important role in regulating neuronal excitability and parsing of information. The modulation of tonic currents by endogenous and exogenous ligands holds vast implications for the physiological processes regulated by them such as cognition, memory, sleep and movement.

1.10.1 α4 and steroid regulation:

Studies show that some GABA A receptors are modulated directly by steroids [274-277] and respond by increases in Cl^- current. This is achieved by increasing open duration and frequency of channel openings [278]. Very high doses of exogenous neurosteroids can cause significant inhibition of GABA A receptors to produce anesthetic-like effects. An important source of circulating neurosteroid precursors are peripheral tissues such as adrenal glands and the ovaries [279]. However, in addition to these, the brain is also capable of synthesizing steroids [280, 281]. Obligatory enzymes and steroid mitochondrial transporters required for the synthesis of pregnane steroids are present in the CNS [280]. A recent investigation utilizing *in situ* hybridization and immunohistochemical techniques revealed that enzymes required for steroid synthesis were expressed in mouse brain in a region specific manner [281]. This study showed the presence of steroid-synthesizing enzymes in Purkinje neurons of cerebellum, neurons of nucleus reticularis of the thalamus and principal neurons of hippocampus and cortex [281]. Based on these studies, it is hypothesized that neurosteroids act in a paracrine as well as an autocrine manner to influence excitability of neurons.

Certain GABA A receptors are particularly sensitive to neurosteroids. Specifically, δ-containing receptors are important for neurosteroid actions [181, 282-285]. It is proposed that changing levels of neurosteroids can modulate the expression of certain δ containing receptors - α4β2δ receptors [267, 286-288]. Depending on the brain region that these receptors are expressed in and the local Cl^- gradient, these receptors show diversity in function which in turn regulates the response to fluctuating levels of steroids. Puberty [289, 290], ovarian cycle associated changes [288], pregnancy [286] and stress are some

conditions during which GABA A receptors play a role in regulation of excitability by neurosteroids.

It is important to note that while fluctuating levels of steroids do influence levels of expression of GABA A δ receptor subtypes, these changes are not always in linear relation to the levels of neurosteroids. It is proposed that during puberty, as levels of the progesterone metabolite 3α-OH-5α[β]-pregnan-20-one (THP) decrease, α4β2δ increases are instrumental in maintaining the level of inhibition due to their polarity dependent response [289, 291]. So, in areas of outward Cl^- current, such as the CA1 hippocampus, THP reduced inhibition [292]. But in areas where Cl^- current is inward, such as dentate gyrus and cortex, neurosteroids enhance inhibition [293-295]. These changed responses likely underlie the modulation of anxiety and stress during puberty. During puberty, mood swings and aversive responses to stress are observed [296-298]. The onset of puberty is marked by a fall in the levels of the endogenous neurosteroid THP [299, 300]. Concurrent with this decline in endogenous neurosteroid, levels of α4β2δ receptors increase in the hippocampus [291]. The increased levels of α4β2δ are thought to be an attempt to normalize neuronal excitability when confronted with reduced levels of neurosteroids.

During the ovarian cycle, levels of endogenous neurosteroids fluctuate so that THP levels are low during the follicular phase, increase during the midluteal phase and finally decline during the late luteal phase [301, 302]. During late diestrous phase of the ovarian cycle, increased δ and decrease in γ2 subunit proteins has been observed in the dentate gyrus granule neurons of the hippocampus of mice [288]. This increase is concurrent with the increases in progesterone and related metabolites [288]. In contrast, during the estrous phase, when progesterone levels are reduced, δ subunits in the dentate gyrus granule neurons are also reduced [288]. Consistent with this, decreased tonic conductance is

observed in these neurons. These changes in GABA A receptor function are associated with decreased seizure susceptibility and anxiety associated with menstrual cycle changes. A corollory to this theory is that dysregulation of GABA A receptor dynamics during menstrual cycle changes could then result in premenstrual dysphoric disorder and other associated syndromes.

When levels of receptor subtypes do not change dynamically with fluctuations in neurosteroids, control of neuronal excitability is disrupted and this in turn is related to disorders associated with fluctuations in neurosteroids such as catamenial epilepsy. Increases in frequency and worsening of seizures across the menstrual cycle have been reported, especially during the mid-menstrual cycle and late luteal phase [303-305]. The increased incidence of seizures during the late luteal phase is attributed to THP withdrawal [306]. Since increases in α4 receptors are observed during THP withdrawal, it was speculated that the increase in α4 could be responsible for excerbation of seizures. Decreasing α4 expression by antisense oligonucleotides in the hippocampus during this period successfuly averted increases in seizure susceptibility [307].

During pregnancy as well, hormonal changes occur with prolonged increases in progesterone levels and its metabolite THP [308, 309]. THP levels increase gradually during the course of pregnancy and are highest around full term pregnancy [310]. During the postpartum period, a rapid decline in THP levels are observed [311]. Levels of α1/2/4 and β1/2/3 did not change during pregnancy in mice [312, 313]. However, decreases in δ and γ2 subunit was observed in the hippocampi of pregnant mice concurrent with the presence of high levels of neurosteroids [311-313]. This corresponded with a decrease in tonic currents and spontaneous inhibitory postsynaptic currents in the hippocampus. Forty-eight hours postpartum, levels of δ and γ2 came back to normal suggesting that rapid

reversal and normalization of inhibition [311]. Thus, GABA A receptors which are sensitive to high levels of neurosteroids are downregulated during pregnancy to prevent excesss inhibition and come back to normal during postpartum once again re-establishing neuronal excitability. However, inability to regulate receptor expression can have adverse behavioral consequences.

It is also important to note that these α4δ-containing receptors undergo a different pattern of regulation in rats [314] suggesting that regulation of GABA A receptor expression during pregnancy maybe more complex and also vary in a species-specific manner. In a rat model, it has been shown that α4 levels do not change during pregnancy when THP levels are high. However, 7 days postpartum, reductions in progesterone occur and this period sees increases in α4 [308]. In a rat model of pseudopregnancy, a reduction in levels of THP was concurrent with increases in levels of α4 expression [315]. Consistent with this, THP withdrawal produced increases in anxiety and insensitivity to benzodiazepines, suggesting a model of postpartum dysphoria. Regardless of the species, it is clear that GABA A receptor α4δ-containing receptors have specific responses to fluctuating levels of neurosteroids that may underlie the behavioral responses observed during this period. Given their role in regulating neuronal excitability, the changes that these receptors undergo need to be better characterized in humans.

Although changes in receptor expression appear to be an attempt to normalize neuronal excitability, dysregulation of such receptor expression can result in premenstrual dysphoric disorder and postpartum depression. Evidence substantiating this claim was obtained from the δ KO mice. Ablation of the δ-containing receptors resulted in resistance to neurosteroid-induced loss of righting reflex and anxiolysis [17]. In addition, the δ KO model showed a propensity for postpartum depression-like symptoms, abnormal maternal

care and reduced offspring survival [311]. During pregnancy, δ receptor levels in the hippocampus, thalamus and striatum were normally reduced [286]. Since endogenous neurosteroid levels are increased during pregnancy, the downregulation of highly sensitive δ-containing receptors is considered a homeostatic mechanism of retaining normal neuronal excitability. However, although an increase in endogenous neurosteroids was observed in δ mice, changes involving the δ subunit could not take place. Hence, neuronal excitability could not be re-established suggesting that changes in neuronal excitability were an outcome of the deletion of δ receptors [286]. The lack of homeostatic regulation of δ is thought to be responsible for neurological and psychiatric disorders associated with pregnancy and postpartum. Given the plasticity of α4 expression during changed hormonal situations, a similar role for α4-containing receptors could be envisioned. In addition, compensatory changes in the δ mice involved reductions in α4 subunit expression, suggesting that deficiencies in the δ KO mice could result from reduced levels of α4 protein as well. Hence, α4 subunit related changes may also be critical to regulation of neuronal excitability during hormonal changes.

The studies summarized above indicate that acute and chronic changes in levels of neurosteroids are critical in modulation of GABA A receptor-mediated neurotransmission. Considering the variety of processes in which α4-δ containing receptors play a role in and their importance in tonic current, further understanding of neurosteroid-α4δ receptor interactions would aid development of pharmacological therapies geared to holistically address hormonal imbalances.

1.10.2 α4 and Ethanol:

While GABA A receptor subtypes respond to ethanol, the sensitivity of different subtypes varies widely. Synaptic GABA A receptors respond to ≥ 50mM of ethanol [14, 154, 316] whereas extrasynaptic receptors are thought to respond to lower concentrations such as 3-30mM of ethanol, although this is controversial [157-159]. The legal limit for alcohol in the US is 0.08% or 17mM. Therefore, receptors that respond to such concentrations of alcohol are more likely be relevant to the physiological symptoms of alcohol intoxication. A concentration of 50mM corresponds to nearly 3 times the legal limit for alcohol intoxication. Therefore, synaptic receptors present targets that are sensitive to very high levels of ethanol and are likely responsible for high dose effects.

Numerous studies have implicated δ-containing receptors in the low dose effects of ethanol. The study of recombinant α4/α6δ-containing receptors in isolated cell systems showed that ethanol-induced tonic current potentiation was observed at concentrations of 3mM-10mM [157] [158, 317]. Independent research groups also provided evidence of tonic current potentiation with ethanol (at concentrations ranging from 3-30mM) in cultured neurons and in neurons from brain slices (from cerebellum- [318, 319] and also from the hippocampus – [320], [159, 179, 321]). Concentrations of 3-30mM are within the range of ethanol amounts that produce low levels of intoxication in humans.

However, an equal number of research groups has failed to observe the effects of low doses of ethanol on α4/α6δ-containing receptors in neuronal cultures or recombinant receptors in isolated cell systems and in neurons from slice preparations [160, 322-324]. The reasons for these discrepancies have not been understood yet. While some of the proposed reasons include difficulties in expression of significant amounts of δ subunit in isolated cell systems, levels of endogenous neurosteroids, posttranslational modifications,

phosphorylation states, etc. However, these reasons alone do not explain the differences in results obtained by different groups.

Although the effects of ethanol on δ-containing receptors have not been resolved unequivocally, a fair amount of evidence suggests that these receptors may be involved in the behavioral effects of ethanol. In fact, the results obtained with Ro15-4513 on α4δ-containing receptors and its ethanol antagonism [156, 262] observed at physiologically relevant concentrations of ethanol lean towards the possibility of α4/6δ-containing receptors as important targets for low dose effects of ethanol.

In addition to potentiation of GABA-mediated current, changes in subunit expression have been observed following chronic ethanol treatment in multiple studies. Based on increased binding of the inverse agonist Ro-15-4513 after chronic ethanol treatment, increases in α4 and α6 subunits were predicted [325, 326]. The chronic ethanol treatment model by Olsen *et al* [178] provided detailed information about the changes occuring during chronic ethanol withdrawal. A reduction in α1 and δ subunits and an increase in α4 and γ2 subunits were reported in the hippocampus [178]. The change in α4 subunit was greatest in the hippocampus even though this subunit is expressed in other brain regions. It is thought that the increase in α4 contributes to the increased seizure susceptibility during ethanol withdrawal based on the correlation between α4 levels and incidence of seizures in several other models [327-329]. Levels of α2 and α5 subunits in the hippocampus were not reduced [178, 330]. A change in localization of α4 subunits was observed from an extrasynaptic location to a synaptic location during ethanol withdrawal [331].

In addition, a reduction in chloride flux was observed in the hippocampus but not in other brain regions such as cortex, striatum or cerebellum [176]. Hippocampal CA1

neurons and dentate gyrus granule neurons from CIE withdrawn rats showed reduced tonic current potentiation in reponse to ethanol, however high doses of ethanol continued to potentiate miniature inhibitory postsynaptic currents (mIPSC) [332]. This difference in sensitivity to ethanol is thought to underlie behavioral tolerance as well. Decrease in the benzodiazepine sensitive subunit α1 and increase in α4γ2-containing receptors in synaptic locations during ethanol withdrawal is thought to be responsible for the reduced sensitivity to classical benzodiazepines as well. The decrease in δ subunit is proposed to be responsible for the decreased response to neurosteroids during withdrawal. Thus, biochemical and functional changes in GABA A receptors during ethanol withdrawal likely mediate the behavioral responses. Thus, the α4 subunit is capable of a great deal of plasticity following ethanol exposure.

A recent sudy demonstrated the role of α4 subunit-containing receptors in ethanol drinking behavior [333]. The nucleus accumbens is implicated in addictive behaviors associated with drugs of abuse such as ethanol [137, 334-336]. α4-δ-containing receptors are expressed robustly in the nucleus accumbens [337, 338] Knockdown of α4 mRNA in the nucleus accumbens shell decreased alcohol intake and preference [333]. Additionally, knockdown of α4 mRNA in the nucleus accumbens core did not influence alcohol preference or intake [333] indicating that α4-containing receptors in the nucleus accumbens shell, but not core, were critical for intitation of ethanol drinking behavior. Since the δ knockout mice also showed reduced alcohol intake [189], together, these studies imply that α4δ- containing receptors could have a role in ethanol drinking behavior.

The studies summarized above suggest a major role for α4-containing receptors in ethanol behavior. While these studies have advanced our understanding of changes during

ethanol withdrawal and their consequences, resolution of the full spectrum of effects of alcohol is yet to be achieved.

1.11 α4 global knockout mouse model:

A global knockout (KO) of the α4 subunit in mice was engineered by Chandra *et al* [339]. These mice showed complete ablation of α4 subunit expression in thalamus, dentate gyrus, cortex and striatum [16]. KO mice were viable, reproduced normally and had no overt phenotypes [16].

Electrophysiological studies revealed that picrotoxin-sensitive tonic conduction in the KO mouse brain was reduced by ~80% in thalamic relay neurons as well as dentate granule neurons compared to the WT [339]. In addition, a small but significant decrease in mIPSC rise and decay time constants was observed indicating a reorganization of synaptic receptors to compensate for the lack of α4-containing receptors. Response to gaboxadol (a novel sedative-hypnotic that is sensitive for extrasynaptic receptors; THIP) was ablated in the ventrobasal thalamic neurons, indicating insensitivity to this drug [339]. Consistent with this observation, the motor ataxic, sedative and analgesic effects of THIP were greatly reduced in the α4 KO mice compared to WT mice. In a pentylenetetrazole-induced seizure susceptibility assay, KO showed higher seizure susceptibility [339]. Studies with ethanol showed that tonic current potentiation by ethanol was reduced greatly in KO dentate granule neurons compared to WT [340].

However, presumably due to reorganization of synaptic networks, ethanol sensitivity of mIPSC's was greatly enhanced in KO compared to WT [340]. Indeed, behavioral responses to acute ethanol-induced loss of righting reflex, motor ataxia and locomotor behavior did not differ between KO and WT [186]. In addition, ethanol metabolism and clearance was

comparable between the genotypes [186]. Considering the proposed role of α4 in low dose ethanol effects, the finding that the KO mice had normal behavioral responses to ethanol was surprising. However, it is possible that the increased synaptic responses to ethanol observed *in vitro* may have masked a role for α4 in these behaviors.

Ro15-4513, an inverse agonist which binds with high affinity at α4-containing receptors [340] was also tested for its effect in the α4 KO. Ro15-4513 has agonist activity at α4β3γ2 receptors but does not modulate α4β3δ receptors [156, 283]. In dentate granule neurons from WT mice, Ro15-4513 potentiated the tonic current but had no effect on synaptic current [340]. This potentiation of tonic current was reduced dramatically in recordings from KO mouse neurons [340]. Synaptic responses to Ro15-4513 in the KO were not different from those in WT. Behavioral studies of ethanol antagonism by Ro15-4513 in α4 KO mice revealed that while WT responded to Ro15-4513, KO mice did not show reversal of ethanol-induced motor ataxia and loss of righting reflex by Ro15-4513 (Fig. 1.2) [341]. This dramatic lack of response to Ro15-4513 in the KO indicates that α4 is critical for modulating the effects of this ethanol antagonist. This result is consistent with the hypothesis proposed by Olsen *et al* that α4-containing receptors may be the main site of action of Ro15-4513 [262]. This evidence forms the basis of experimentation with another ethanol antagonist, RY023, described in Chapter 3.

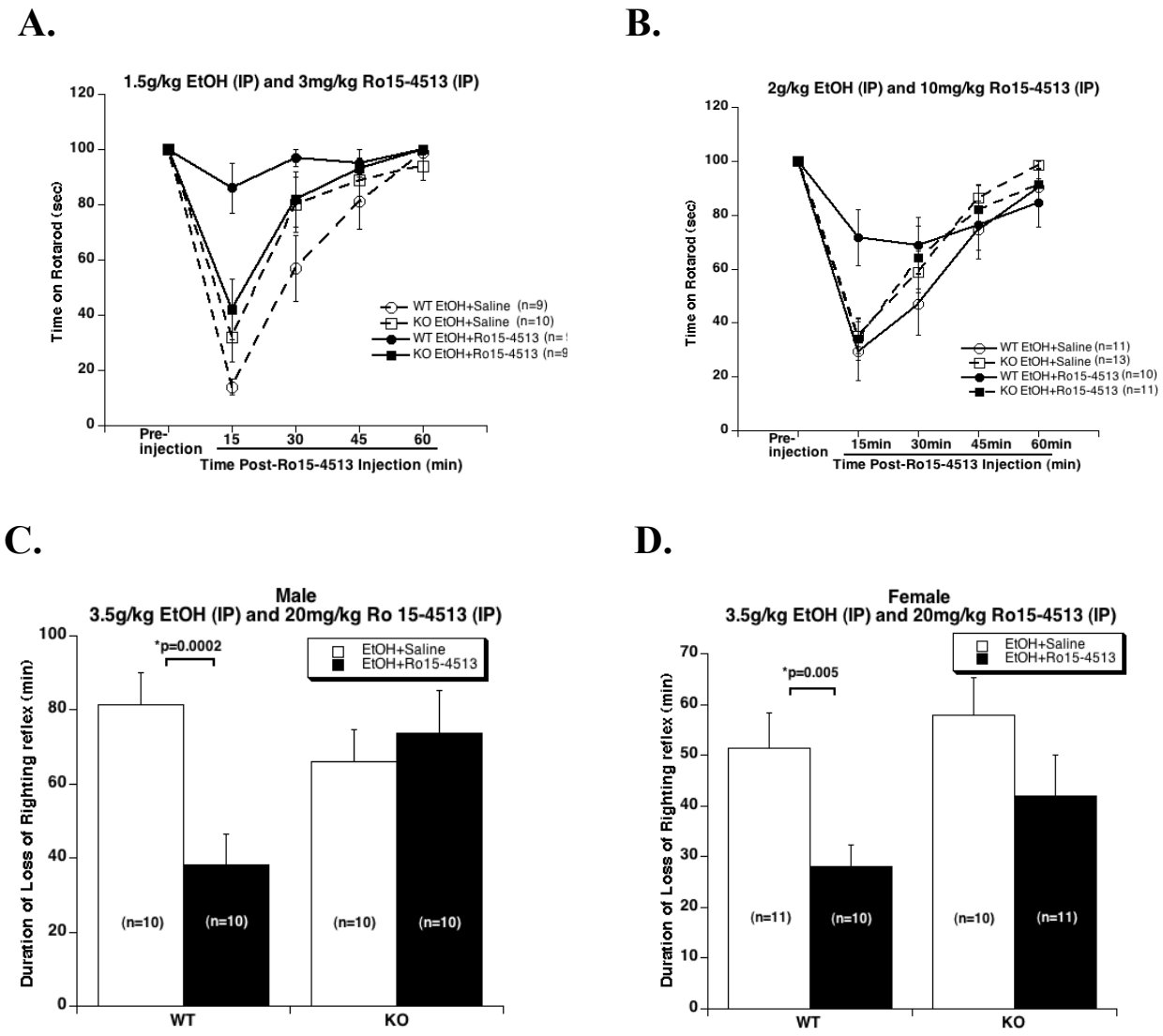

Figure 1.2 Ro15-4513 antagonism of ethanol-induced motor ataxia and loss of righting reflex

α4 KO and WT mice did not differ in responses to ethanol alone at both doses as expected. However, Ro15-4513 antagonism of ethanol-induced motor ataxia at two dose-combinations was greatly reduced in the α4 KO mice compared to WT.

A. 3mg/kg of Ro15-4513 and 1.5 g/kg of ethanol ($p \leq 0.001$)
B. 10mg/kg of Ro15-4513 and 2g/kg of ethanol ($p < 0.05$)

Similarly, the loss of righting reflex responses to ethanol alone did not differ between genotypes. However, Ro15-4513 antagonism of ethanol-induced loss of righting reflex differed significantly between sexes, hence males and females were analyzed separately. While WT mice of both sexes showed significant reductions in their sleep times with Ro15-4513, male and female α4 KO mice were not responsive to the ethanol-antagonizing effect of Ro15-4513.

C. Ro15-4513 antagonism of ethanol-induced LORR in Males
D. Ro15-4513 antagonism of ethanol-induced LORR in Females

Analysis of the universal GABA agonist, muscimol, in α4 KO mice revealed a reduction in binding of muscimol in areas of α4 expression such as caudate putamen, thalamus and hippocampus [342]. In addition, the motor-ataxic effects of muscimol were reduced in the α4 KO implying that at least some effects of muscimol were mediated through α4-containing receptors [342]. Muscimol has long been regarded as a universal agonist of all GABA A receptors. The study with α4 KO mice suggests that rather than being a universal agonist, muscimol acts on specific high affinity sites to mediate its effects, with α4-containing receptors being one of them.

Isoflurane at low concentrations (25-250μM) was found to potentiate tonic currents from ventrobasal thalamic neurons in WT but not in KO [343]. At higher concentrations (250-500μM), synaptic currents (mIPSC's) were potentiated similarly in both WT and KO. Because tonic currents have been implicated in the effects of anesthetics, it is possible that the changes observed at the cellular level may translate behaviorally. Fear conditioning assays evaluating the amnestic effects of isoflurane were conducted by our collaborators (Dr.Vinuta Rau, Dept. of Anesthesiology, University of California, San Francisco, CA) [344]. Amnestic effects of isoflurane were reduced in α4 KO [344] implying that they were resistant to the effects of isoflurane (Fig. 1.3). Thus a reduction in response to isoflurane was observed at the behavioral level. Hence it was likely that other behavioral effects of isoflurane may also be changed in the α4 KO. This

hypothesis was tested in Chapter 2.

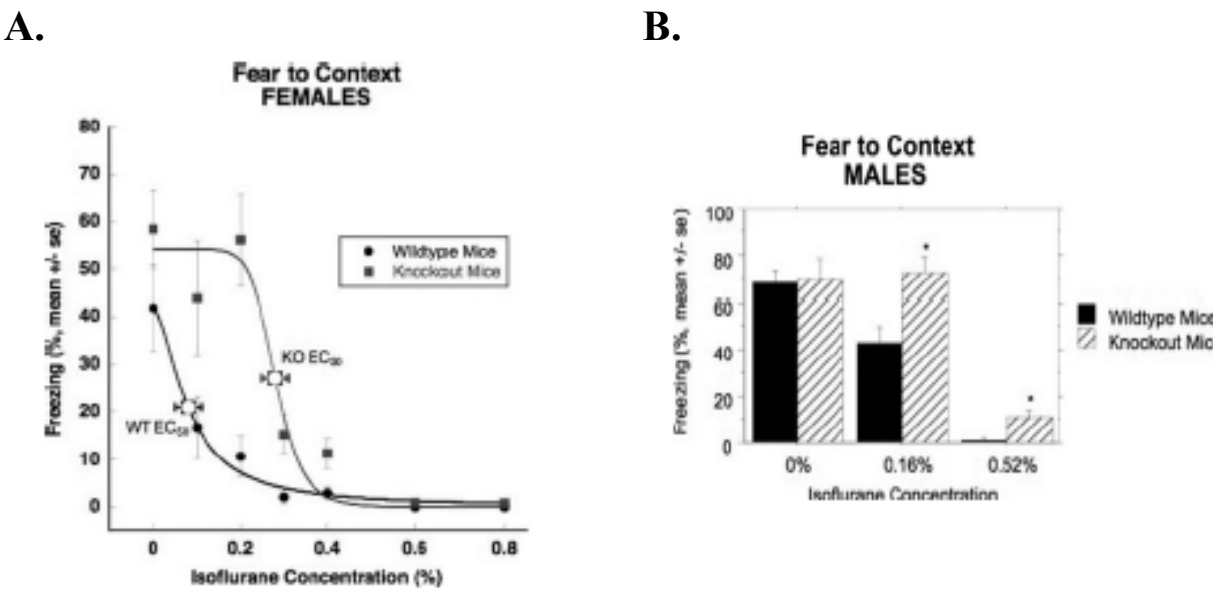

Figure 1.3 Amnesic effects of isoflurane in WT and α4 KO mice

Because a sex difference was observed, males and females were assessed separately.

(A) KO females appear resistant to the amnesic effects of isoflurane (WT EC50 = 0.08±0.03%; KO EC50 = 0.28 ± 0.03%; $p < 0.005$)

(B) Because baseline freezing scores were different between WT and KO males, freezing was equated by changing tha training paradigm for KO. Following exposure to isoflurane at 0.16% and 0.52%, a genotypic difference was noted ($p< 0.05$)
Rau,V.,Iyer,SV.,Oh,I.,Chandra,D.,Harrison.,NL.,Eger,EI.2nd,Fanselow,MS.,Homanics,GE., Sonner,JM, *GABA type A receptor α4 subunit knockout mice are resistant to the amnestic effects of isoflurane*. Anesth Analg, 2009. 109 (6): p.1816-22.

Alphaxalone, a synthetic neurosteroid anesthetic, potentiated the magnitude of tonic current and synaptic current in WT but both tonic and synaptic current potentiation were reduced significantly in the KO [340]. This observation raises the possibility of α4 KO mice being resistant to the effects of neurosteroids. The reduction in behavioral responses to neurosteroids in the δ KO mice [17] also tips the balance in favor of α4 KO mice having reduced reponses to neurosteroids. The possibility of α4 KO mice have reduced behavioral responses to the neurosteroid, anesthetic, alphaxalone has been evaluated in Chapter 2.

Lastly, taurine, an endogenous amino acid that is also a GABA- and glycine-mimetic was studied for its effects on GABAergic current modulation in α4KO and WT.

Previously, it was thought that taurine had greater activity at glycine receptors and was less potent at GABA A receptors. However, only synaptic combinations of GABA A receptor were previously tested. The landmark study by Jia *et al*, showed that contrary to prior belief, taurine acted potently at extrasynaptic GABA A receptors [234]. Also, ventrobasal thalamic neurons from α4 KO mice showed reduced current potentiation by taurine compared to WT mice over a range of taurine concentrations. A recent study by Granadoz *et al* showed that taurine treatment alleviated handling-induced convulsions in mice that received continuous chronic ethanol treatment [233]. Given the plasticity of α4-containing receptors during ethanol withdrawal (see review [345] and the evidence tying the actions of taurine to α4 receptors [234], the withdrawal- alleviating effects of taurine could be mediated through these receptors. This forms the basis for Chapter 4 in which the effects of taurine on ethanol withdrawal behavioral were assessed.

Because α4 has robust expression in the hippocampus, α4 KO and WT mice were tested for differences in learning and memory. An enhancement of hippocampal-dependent learning and memory was observed in α4 KO mice compared to WT mice [346]. Sex differences were also noted with KO females exhibiting enhanced memory compared to KO males. The significance of this sex difference in memory is not well-understood but it is hypothesized to reflect increased defensive behavior in female rodents [347, 348]. A role for differences in endogenous neurosteroid sensitivity is also hypothesized to underlie the enhanced memory in females. This finding is particularly significant in light of the recent study by Shen *et al*, demonstrating a role for α4βδ-containing receptors in learning during puberty [290]. Pubertal δ KO mice were successful in learning a hippocampal-dependent spatial task that WT mice were unable to [290]. Thus, α4δ-containing receptors may be relevant for sex specific differences in memory and learning.

Lastly, deletion of the α4 subunit resulted in compensatory responses in the α4 global knockout mice [349, 350]. An increase in γ2 subunits and a decrease in δ subunit were observed [349, 350]. Immunogold labeling studies of the δ subunit revealed the presence of δ subunits in the cytoplasm, specifically in the endoplasmic reticulum and Golgi complex of thalamic neurons [349, 350]. This indicates that the lack of α4 subunit affects the trafficking of δ subunits to the membrane surface. This implies that the levels of functional δ-containing receptors are reduced in the α4 KO mice. This finding has implications for ethanol and neurosteroid induced behaviors where α4δ-containing receptors play an important role. The reduction in tonic current in the α4 KO mice could thus arise in part by reductions in δ subunit containing receptors. In addition to γ2 subunits, α1 and α2 subunits were also increased [350]. This increase was greatest in those regions with the least amount of change in surface expression of δ subunits. This indicates the possibility of novel α1βδ or α2βδ receptor combinations. In fact, α1δ- containing receptors have been discovered in the hippocampus of both WT and α4 KO mice [320]. These receptors were found to mediate tonic currents that were potentiated by low doses of ethanol [320]. Such changed receptor combinations could also affect behavioral responses in the α4 KO mice.

1.12 Gaps in our knowledge:

Although a lot is known about changes in α4 receptor levels at the biochemical and cellular level, the behavioral implications of these changes are not clearly understood. Because of the regions of expression of α4-containing receptors (thalamus and hippocampus) and the role of tonic currents in anesthetic actions, it would be interesting to determine if anesthetic sensitivity is changed in α4 KO mice. The lack of potentiation by

isoflurane in the α4 KO [343] points towards this possibility. The widespread expression of the α4 subunit and its combination with β3 and β2 subunits, (each of which have been implicated in intravenous anesthetic actions, [15, 82]) gives rise to speculations about the role of α4 subunit-containing receptors in intravenous anesthetic actions. The thalamus has been implicated as an important player in sleep mechanisms [351]. Additionally, tonic current mediated by ventrobasal thalamic neurons are thought to regulate natural sleep pathways [352]. Because sedation and unconsciousness caused by anesthetics are thought to overlap with natural sleep pathways, it is possible that α4-containing receptors could modulate these effects.

The dramatic decrease in response to neurosteroids in the δ KO mice indicated that δ receptors were important modulators of steroid effects [17]. By virtue of their partnership with δ subunits [38, 337], α4 subunits could also have a critical role in neurosteroid modulation. Additionally, fluctuating steroid levels during physiological processes such as ovarian cycle changes and pregnancy have been accompanied by changes in levels of α4 subunit as well [289]. δ KO mice showed a reduction in levels of α4 subunit protein [353]. Therefore, the reduction in behavioral responses to neurosteroids could have been, in part, due to reduced α4 levels as well. Therefore, the relative contributions of α4 subunit and δ subunit remains to be dissected.

Based on this evidence, I hypothesize that α4-containing receptors are a site of action for the effects of anesthetics and neurosteroids. Different behavioral endpoints will be assessed in α4 KO and WT mice with anesthetics and with neurosteroids to investigate the role of α4 containing receptors in anesthetic effects. Understanding the effects of neurosteroids in α4 KO mice and contrasting them against results obtained in the δ KO will allow determination of the relative role of α4 subunit in neurosteroid actions.

Could α4 subunit-containing receptors be a target for ethanol antagonism? Again, α4-containing receptors have been implicated in acute low dose effects of ethanol [156, 158]. As inferred from the reduced effects of Ro15-4513 in α4 KO (Fig. 1.2), these receptors are a site of action for reversal of ethanol effects. Analogs of Ro15-4513 that antagonize the effects of ethanol could function via the same mechanism. Evaluating this possibilty will open up opportunities for development of newer, more specific ethanol antagonists. To understand if α4-containing receptors are a universal target for all known ethanol antagonists, I propose to test another ethanol antagonist, RY023, in WT and α4 KO mice. Both intrinsic effects and ethanol antagonistic effects of RY023 on different behaviors will be assessed in WT and KO mice.

α4 exhibits remarkable plasticity during ethanol withdrawal (see review, [345]). In fact, increases in α4 during chronic ethanol withdrawal are concurrent with the behavioral endpoints of withdrawal such as seizure susceptibility and tolerance to ethanol and classical benzodiazepines. Could the lack of α4-containing receptors affect ethanol withdrawal behavior? Alternately, could the increase in α4 seen during ethanol withdrawal be harnessed to treat withdrawal symptoms better? Because α4-containing receptors have been implicated in seizure susceptibility and are benzodiazepine-insensitive, novel drugs that increase inhibitory neurotransmission through α4-containing receptors could be used to alleviate withdrawal. Recent evidence links taurine to α4 receptors [234] and the fact that taurine alleviates withdrawal seizures [233] suggests that taurine could be acting on increased α4 receptors during ethanol withdrawal.

To assess this possibility, I propose studying chronic ethanol withdrawal responses in α4 KO and WT mice with and without taurine. This will allow us to understand the role

of α4 containing receptors in ethanol withdrawal as well as the role of α4-containing receptors in taurine-induced alleviation of withdrawal.

Thus, a great deal of evidence ties α4-containing receptors to anesthetic, neurosteroid and ethanol effects and determining these effects could resolve conflicts about our understanding of the role of α4 GABA A receptors. Pursuit of such information will advance our knowledge of the mechanism of action of anesthetics, neurosteroids, ethanol, ethanol antagonists and taurine.

1.13 Dissertation:

Specific Aims:

Anesthetics are thought to exert at least some of their effects via the GABA A inhibitory system. Anesthetics bring about a spectrum of effects that consist of sedation, immobilization, analgesia and amnesia. The various components of the anesthetic state are thought to be mediated by discrete targets in the central nervous system [354]. Our understanding of the mechanism of action of currently used anesthetics is far from complete.

Like, anesthetics, alcohol is also thought to mediate some of its effects through GABA A receptors. Alcoholism is a major problem worldwide. 85,000 deaths in the US alone, are attributable to alcohol consumption, annually [355]. Alcohol consumption is associated with a number of social evils such as aggression, accidents, and domestic violence. The acute effects of alcohol include anxiolysis, sedation, ataxia, hypnosis, analgesia and amnesia. Chronic effects of ethanol are more severe, characterized during withdrawal by hyperactivity of the CNS [3]. Our understanding of the mechanism of action of both anesthetics and alcohol is limited.

I am interested in understanding the contribution of a specific GABA receptor subunit, α4, to anesthetic and ethanol action. Extrasynaptic and synaptic $GABA_A$ receptors containing the α4 subunit are widespread in the thalamus and the dentate gyrus, and lower levels are present in the cortex and striatum [339]. The thalamus is considered as a region of major sensory flux in addition to being responsible for states of sleep, consciousness and a motor center, whereas the dentate gyrus is associated with memory. Therefore, it may be inferred that the anatomical localization of the α4 subunit correlates with those areas that

are responsible for mediating the effects of anesthetics and alcohol on behavior such as amnestic effects (hippocampus) and sedative and sensory effects (thalamus).

An α4 KO mouse model was created in the Homanics Lab [16]. Electrophysiological studies on the ventrobasal thalamocortical neurons and dentate granule cells in the α4 KO mice indicated reduced sensitivity to isoflurane and alphaxalone, respectively [186, 356]. In α4 KO mice, tonic current potentiation by Ro15-4513 was ablated. Additonally, behavioral studies revealed that α4 KO mice were resistant to the ethanol-reversing action of Ro15-4513 on ethanol-induced motor ataxia and loss of righting reflex.

In the mid 1990's, the interest in Ro15-4513 as a putative ethanol antagonist resulted in a series of drugs being synthesized that had similar ethanol-antagonist actions [251]. RY023 was one such drug that antagonized the behavioral effects of ethanol [253, 357]. While the drug shows high affinity for α5 containing receptors, its involvement with α4δ-containing receptors has not been evaluated [253]. In light of new knowledge of Ro15-4513's actions on α4-containing receptors, I hypothesize that like Ro15-4513, RY023 also exerts its actions preferentially through α4-containing receptors.

α4 subunit levels undergo upregulation during ethanol withdrawal [178, 181, 358, 359]. Further, the α4 subunit inserts synaptically instead of the α1 subunit [358]. These changes may be responsible for ethanol withdrawal behavior. Ethanol actions in the CNS can also be modulated by concurrent administration of taurine. Taurine is a chloride current modulator and acts on both glycine and GABA receptors. Taurine is believed to exert a protective effect during ethanol withdrawal [360].

Recent evidence indicates that taurine is more potent at extrasynaptic GABA receptors than synaptic GABA or glycine receptors. In fact, studies by Jia *et al*, revealed

that the α4 subunit may be the site of action of taurine and that α4KO did not show tonic current potentiation with taurine [234].

Overall, my aim is to elucidate the contribution of α4 subunit containing GABA receptors to anesthetic, ethanol-antagonistic (RY023), chronic ethanol withdrawal and taurine effects. I propose to do this by studying behavioral effects of anesthetics, RY023 and taurine in mice deficient in α4 GABA A receptors and compared their responses to WT controls.

I hypothesize that the α4 subunit is essential for:

1) **Behavioral response to anesthetics**
2) **ethanol antagonist behavioral responses**
3) **chronic ethanol withdrawal-induced behavioral effects**
4) **taurine-induced suppression of chronic ethanol withdrawal symptoms**

To test these hypotheses I propose the following aims:

Specific Aim 1A: *To elucidate the role of the α4 subunit in the behavioral effects of volatile anesthetics.*

WT and KO mice will be compared for the behavioral effects of inhaled anesthetics, halothane and isoflurane, using the loss of righting reflex and tail clamp/withdrawal assays.

Specific Aim 1B: *To elucidate the role of the a4 subunit in the behavioral effects of the intravenous anesthetics, etomidate, propofol and the neurosteroid anesthetic, alphaxalone.*

The behavioral effects of intravenous anesthetics (alphaxalone, etomidate, propofol) will be evaluated by examining ataxia (rotarod), hypnosis (sleep time), and locomotor effects (open field).

Specific Aim 2: *To determine if a4 is essential for the ethanol antagonist action of RY023*

Antagonistic effects of a RY023 on acute ethanol will be evaluated on two behavioral parameters. Ethanol-induced motor ataxia (rotarod) and ethanol-induced loss of righting reflex will be studied for antagonism by RY023. In addition, the role of α4 GABA A receptors in the intrinsic effects of RY023 (on motor ataxia and sedation) will be studied by comparing the genotypes following injection of RY023 alone.

Specific Aim 3: *To evaluate the role of α4-containing receptors in chronic ethanol withdrawal behaviors and in mediating taurine-induced supression of chronic ethanol withdrawal behaviors.*

α4-containing receptors have been implicated in ethanol actions. These receptors are very sensitive to low physiologically relevant concentrations of ethanol and they undergo changes in expression and relocalization during ethanol withdrawal. The characteristic changes in behavior during withdrawal suggest that changes in α4-containing receptors play a role in ethanol withdrawal effects. In addition to a role for α4 in withdrawal, literary evidence also indicates that taurine has a protective effect during ethanol withdrawal and that taurine modulates current through the α4 subunit.

Hence, to assess these possibilities, α4 KO mice will be assessed for differences in chronic ethanol withdrawal behavior. Seizure susceptibility (handling induced convulsion scoring; HIC), locomotor changes (open field) and protracted tolerance (ethanol-induced loss of righting reflex) will be assayed. In addition, the effect of taurine on these withdrawal parameters will be studied concurrently in these mice.

Chapter 2.0: Role of α4-containing GABA A receptors in behavioral responses to general anesthetics

NOTE: Portions of this chapter have been published [344] and some sections are reproduced here verbatim - these sections have been denoted in italics.

2.1 Introduction

Anesthetics cause a variety of desirable effects such as unconsciousness, immobilization and amnesia. The mechanism underlying these effects is unknown. In addition to their desirable effects, anesthetics also cause severe side effects in a small percentage of the population. The manifestation of these side effects in some but not all people is thought to result from differences in targets of anesthetics. For example, differences in gene expression profiles in different populations may cause the decreased or increased presence of an anesthetic-sensitive target. Hence, understanding how anesthetic effects are mediated will provide some answers as to the molecular targets of these drugs.

α4-containing GABA A receptors are expressed at high levels in dentate gyrus of hippocampus, thalamus, cortex and striatum [337]. They mediate tonic inhibitory current which is thought to play an important role in regulating neuronal excitability [11]. Tonic current has special significance for processes such as memory, sleep, motor coordination, etc. The thalamus in particular is involved in modulation of sleep and consciousness in addition to serving as a sensory gateway along with the cortex [361-364]. The hippocampus is implicated in processes of learning, memory and anxiety [112]. The location of α4-containing receptors and the fact that they conduct tonic currents make them attractive targets for the effects of anesthetics.

It was previously demonstrated that α4-containing extrasynaptic receptors are essential for the cellular and behavioral responses of the sedative/hypnotic, THIP [16, 365]. α4 KO mice showed reduced sensitivity to THIP-induced motor ataxia, loss of righting reflex, and analgesia [16]. Recently, Jia *et al* [343] demonstrated that α4-mediated extrasynaptic inhibition in the ventrobasal thalamic neurons was markedly enhanced by isoflurane, and this enhancement was absent in α4 KO mice [343]. Based on these studies, it was hypothesized that as with gaboxadol, α4-containing receptors may mediate some of the behavioral effects of isoflurane.

α4 subunits are often co-assembled with β3 and/or β2 subunits (and a γ or δ subunit) either in synaptic or extrasynaptic locations. The β3 subunit has been implicated in the effects of etomidate and propofol-induced LORR. A β3 KI (N265M) mouse model revealed that the mutant mice were resistant to the hypnotic and immobilizing actions of etomidate and propofol [15]. The subsequent generation of a β2 KI (N265M) mouse also revealed that the sedative actions of etomidate but not propofol were mediated by β2-containing receptors [82]. A recent study proposed that the binding site for etomidate lies at the interface of an α and a β subunit at transmembrane sites [366]. The identity of the α subunits that pair with β2/β3 subunits to mediate the effects of etomidate are unknown at present. It has been proposed that ~50% of α4 subunits form binary receptors in combination with β1-3 subunits [269]. Additionally, recombinant receptors expressing binary α4β3 receptors were responsive to current modulation by etomidate [367]. Hence, it is possible that α4 subunits are important for the actions of intravenous anesthetics. While the contribution of β subunits to the actions of intravenous anesthetics etomidate and propofol is now known, the contribution of the partnering α subunit is not clearly

delineated. Therefore, I hypothesize that α4-containing receptors are essential for the actions of etomidate and propofol.

α4-containing receptors have also been implicated in neurosteroid effects. α4 subunits combine with δ subunits to form extrasynaptic receptors that are uniquely sensitive to neurosteroids [282, 283, 295, 368] and show modulation by varying levels of endogenous neurosteroids (see reviews [287, 289]. α4δ-containing receptors have special significance in steroid hormone effects (e.g., premenstrual syndrome [288], post-partum depression[311], pregnancy [286], and learning during puberty [290]). Preventing the cycling of δ-containing receptors via antisense RNA techniques resulted in increased excitability during diestrous phase of the ovarian cycle [288]. This was consistent with a role for δ-containing receptors in catamenial epilepsy and anxiety during premenstrual dysphoric disorders. A global knockout of the δ subunit resulted in mice that had dramatically reduced sensitivity to neurosteroids both at the cellular and behavioral level [17, 369]. In addition, the δ KO mice failed to show reductions in neuronal excitability during pregnancy [286], suggesting that the lack of δ-containing receptors led to an inability to regulate homeostatic neuronal control. This in turn was thought to be responsible for psychiatric disturbances during pregnancy and post partum. Consistent with this, δ KO mice showed evidence of postpartum depression and poor maternal behavior [311]. Because the α4 subunit is commonly paired with the δ subunit, it was hypothesized that α4 itself may contribute in some measure to the behavioral effects of neurosteroids.

In this chapter, I report on studies that tested the hypothesis that α4-containing receptors are critical for the behavioral effects of volatile anesthetics (halothane and isoflurane), intravenous anesthetics (etomidate and propofol) and the neurosteroid

anesthetic (alphaxalone). To do this, I compared behavioral responses to these drugs in WT and KO mice.

2.2 Materials and Methods

Homozygous α4 KO and WT littermate controls were created by breeding heterozygous mice that possessed only one copy of the GABA A receptor α4 subunit allele [16]. Mice were of the F3–4 generations, and their genetic background was a mixture of C57BL/6J and Strain 129S1/X1. Mice were genotyped using Southern Blot analysis of tail DNA at weaning, as previously described [16]. Mice were group housed, kept on a 12-h alternating light/dark cycle, and allowed access to food and water ad libitum. For all experiments, both sexes of mice were used for experimentation unless otherwise stated. Due to limited numbers, analysis of results by sex was not performed unless stated. All protocols were approved by the committee on animal care and use at the University of Pittsburgh.

2.2.1 Volatile anesthetic-loss of Righting Reflex (LORR)

Mice of both sexes (10-20 weeks of age) were tested for isoflurane (n=11-13 per genotype) and halothane (n=16 per genotype) -induced loss of righting reflex. For each LORR determination, six to eight unrestrained mice were placed in individual wire-mesh cages mounted on a carousel in a sealed Plexiglas chamber maintained at 33–35°C. Soda lime scattered on the chamber floor maintained the ambient CO_2 tension at <0.05 atmosphere. Controlled concentrations of halothane (Halocarbon Laboratories, River Edge, NJ) or isoflurane (Abbot Laboratories, North Chicago,IL) were delivered to the testing chambers by means of agent-specific vaporizers. A Datex Capnomac Ultima device continuously monitored the concentration of anesthetic gas within the chamber. The mice were equilibrated with anesthetic for 15 minutes and then

scored for the righting reflex while the carousel rotated at 4 rpm. After testing at one concentration of anesthetic, the mice were allowed to recover in anesthetic-free air for 20 min. Anesthetic gas concentrations tested ranged from 0.6%-1.1% atm with an increment of 0.1% in each trial.

2.2.2 Minimum Alveolar Concentration (MAC) of volatile anesthetics:

Mice (10-20 weeks of age) were monitored for response to noxious stimuli in the presence of isoflurane (n= 26-27 per genotype) and halothane (n=16 per genotype) using the tail clamp-withdrawal assay. Briefly, mice were equilibrated with anesthetic agents in a heated, enclosed chamber. The monitoring of chamber temperature, FIO_2 (inspired oxygen concentration), and anesthetic concentrations were similar to procedures used for the LORR assay. Following equilibration with anesthetic, a hemostat was applied to the tail and the mouse was scored for purposeful, gross movement in response to the tail clamp. A positive response was defined as the lack of any movement to the noxious stimulus, i.e., the tail clamp. Mice were allowed to recover for 15 minutes in air before testing at the next concentration. The range of concentrations tested was 1.0%-1.8% with 0.2% increments.

For LORR and MAC assays, half maximal effective concentration values (EC_{50}) were obtained from concentration response curves using the iterative nonlinear least-squares method for quantal responses [370]. The Z statistic was used to compare genotypes on both assays. Males and females were combined for analysis owing to limited number of mice. Previous experiments have shown that sexes do not differ in their responses to these anesthetics.

2.2.3 Recovery of Righting Reflex (RORR) with volatile anesthetics:

Mice (12-20 weeks of age, n=7-11 per genotype) were exposed to 1.4% atm of each anesthetic for one hour inside a sealed plexiglass chamber. Thirty minutes into the

exposure, mice were placed on their backs and exposure resumed for the remainder of the hour. Following this, the gas was turned off, the exhaust fan was turned on and pure oxygen was allowed to flow into the chamber at a flowrate of 5L/min. The time for the mice to right themselves was recorded. During the experiment, normothermia was maintained by a heat lamp. Data were analyzed by one-way ANOVA with genotype as main effect.

2.2.4 Intravenous anesthetic-induced LORR:

Drug Solutions:

Etomidate (Amidate, 2mg/ml, Hospira Inc., Lake Forest, IL), propofol (Diprivan, 10mg/ml, Astra Zeneca, Wilmington, DE), and alphaxalone (Cat. No. P5052, Sigma Aldrich, St.Louis, MO) were used. Alphaxalone was solubilized in 22.5% β-hydroxycyclodextrin (Cat. No. H107, Sigma Aldrich, St.Louis, MO) and sonicated to arrive at a stock concentration of 7mg/ml. All injections were administered intraperitoneally (i.p.) except propofol which was administered by the retro orbital (r.o) route.

Age-matched mice (8-20 weeks) of both genotypes and sexes were injected with propofol (40mg/kg, r.o., 0.004ml/g body weight, n=8-15 per genotype), etomidate (20mg/kg, i.p., 0.01ml/g body weight, n=10-13 per genotype) or alphaxalone (70mg/kg, i.p., 0.01ml/g body weight, n=20 per genotype) for the LORR assay. Immediately after they lost their righting reflex, mice were placed supine on a V-shaped plexiglass trough until recovery. A heat lamp was used to ensure that body temperatures were maintained throughout the experiment. Endpoint of time to return of righting reflex was determined by ensuring that a mouse was able to right itself consecutively three times within 30 seconds. For these assays, mice that had durations of LORR greater or lower than two standard

deviations from the mean were excluded from the analysis. Data were analyzed by one-way ANOVA with genotype as main effect.

2.2.5 Recovery from injectible anesthetic-induced motor ataxia:

Mice were tested for recovery of motor ataxia with etomidate, propofol and alphaxalone. All training and testing was performed on a fixed speed rotarod (Ugo Basile 7650, Varese, Italy) at 8RPM.

A) Etomidate (Amidate)

Only male mice were used for this assay. Mice were trained on the day of the test to a criteria of 120 sec on fixed speed rotarod at 8RPM. Training trails were spaced 20 minutes apart and at least 4 such trials were administered. Mice that did not achieve the criterion were excluded from the assay. Following training, etomidate was injected (i.p., 0.01ml/g body weight) at 20mg/kg. Mice lost their righting reflex briefly. Upon recovery they were tested on the rotarod every 20 minutes until recovery to baseline performance (120 sec) was regained. Area under the curve (AUC) was measured as the area between baseline performance (120sec) and the impairment produced by etomidate over the time course of the experiment. AUC were compared by one-way ANOVA with genotype as main effect.

B) Propofol (Diprivan)

Mice were trained on the previous day to a criterion of 100 sec. Each mouse was given at least 4 trials to ensure that the task was learned. The inter-trial duration was 5 minutes. Once training was complete, mice were housed overnight in the testing room in their home cages. On the test day, mice were subjected to 2-3 trials to confirm compliance to criteria. Subjects that did not achieve the criterion of 100sec consecutively on the rotarod were excluded from the test. Following this, propofol was administered at 25mg/kg, retro-orbitally (0.005ml/g body weight) by diluting the clinical preparation with saline to a

concentration of 5mg/ml. Mice briefly lost their righting reflex. Following recovery of righting reflex, mice were placed on the rotarod at intervals of 5 minutes until recovery to baseline performance (100 sec) was regained. Area under the curve (AUC) was measured as the area between baseline performance (100 sec) and the impairment produced by propofol over the time course of the experiment. AUC were compared by one-way ANOVA with genotype as main effect.

C) Alphaxalone

On the day of the experiment, mice were subjected to training trials on a fixed speed rotarod. At least 4 training trials were administered at 10 minute intervals so that mice achieved a criterion of 180 sec on the rotarod. Mice that did not achieve a performance of 180 sec on the rotarod at least three times consecutively were excluded from the assay. Alphaxalone was solubilized in 22.5% β-hydroxycyclodextrin and injected at 50mg/kg (i.p., 0.01ml/g body weight). Mice were tested every 10 minutes after injection until recovery to baseline performance (180 sec) was achieved. Area under the curve (AUC) was measured as the area between baseline performance (180 sec) and the impairment produced by alphaxalone over the time course of the experiment. Results were analyzed by one way ANOVA with genotype as main effect.

2.2.6 Intravenous anesthetic-induced changes in locomotor behavior:

A) Etomidate was diluted with saline to a concentration of 0.3mg/ml. The sedative effects of etomidate were evaluated at a dose of 3mg/kg for a period of 10 minutes by an open field assay. Mice were placed in the testing room one day prior to the experiment. On the morning of the experiment, mice were weighed and then injected with etomidate (i.p., 0.01ml/g body weight) and placed separately in new cages. Five minutes later, mice were

placed in the center of a plexiglass walled arena each, (43.2 cm X 43.2 cm X 30.5 cm) for automated recording of locomotor behavior. The chambers were located within sound-attenuating cubicles (Med Associates, St.Albans, VT) that were provided with a red light and a fan (top right corner). Distance traveled (cm) over a 10 minute period was recorded by an automated program (Med Associates, St.Albans, VT). Between mice, the chambers were cleaned with 70% ethanol followed by water to remove any evidence of smells and excrements left behind. Because baseline (saline) scores did not differ between genotypes [16] in this assay, only drug-induced behavior was measured between genotypes. Data were analyzed by one way ANOVA. Only males were used in this assay.

B) Alphaxalone was diluted with 22.5% β-hydroxycyclodextrin and sonicated to make a solution of 1.5 mg/ml. The locomotor stimulatory effect of alphaxalone (15mg/kg) or vehicle (22.5% HBC in saline) was tested 10 min after injection in the open field. Activity was recorded for a period of 10 min as described above. Total distance covered over a 10 min period and distance covered in the center zone were measured. The center zone was demarcated as the central portion of the open field (11.25 X 11.25cm). Center zone activity was expressed as percent distance covered in center zone. For this assay, if total distance was greater or lower than two standard deviations from the mean, mice were excluded from the analysis.

2.3 Results:

2.3.1 Effects of Isoflurane and Halothane on LORR:

For isoflurane-induced LORR (Fig. 2.1 A), the EC_{50} for WT (0.73 ± 0.02) and KO (0.74 ± 0.02) mice did not differ. For halothane-induced LORR (Fig.2.1 B), the EC_{50} for KO mice (0.90 ± 0.01) was increased ($p<0.05$) compared with WT controls (0.84 ± 0.02).

2.3.2 Effect of Isoflurane and Halothane on MAC:

WT and KO mice did not differ in their isoflurane MAC (Fig.2.1 A) (WT EC_{50} =1.45 ± 0.03 vs. KO =1.37 ± 0.03). Similarly, genotype did not affect response to halothane (Fig.2.1 B) on this assay (WT EC_{50} = 1.61 ± 0.03 vs. KO EC_{50} = 1.59 ± 0.03).

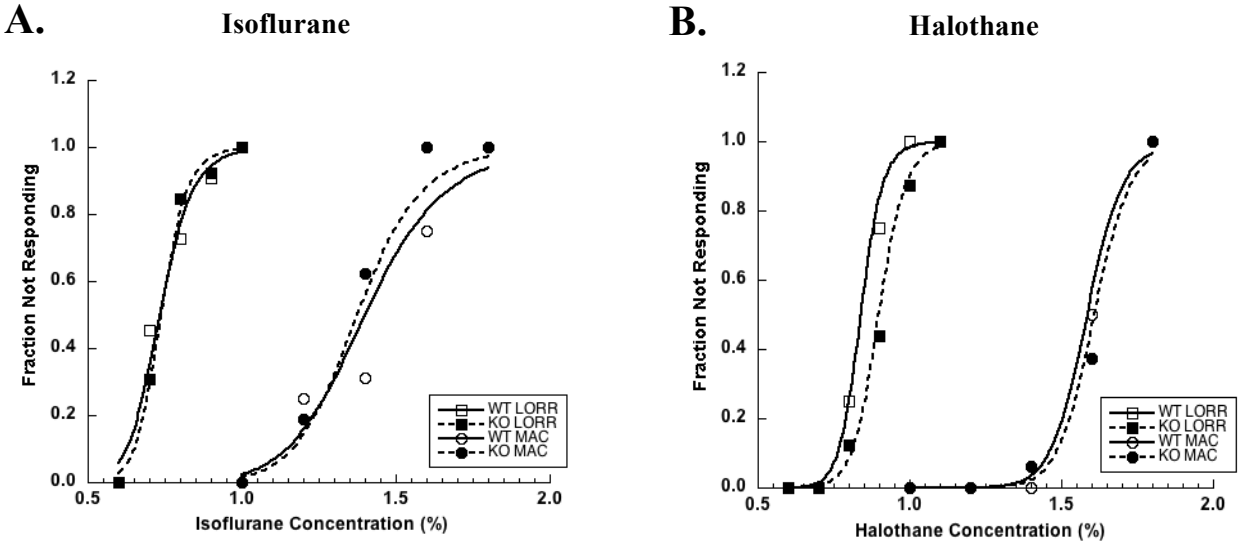

Figure 2.1 Loss of righting reflex (LORR) and minimum alveolar concentration (MAC)

(A) Isoflurane: Concentration response curves did not differ between WT and KO on isoflurane-induced LORR and MAC

(B) Halothane: With halothane, a small but significant difference was observed on the LORR assay. No difference was observed between genotypes on halothane-induced MAC.

2.3.3 Effects of Isoflurane and Halothane on RORR:

A one-way ANOVA showed that WT and KO mice did not differ in their mean recovery time with isoflurane- and halothane-induced loss of righting reflex at 1.4% atm. Time taken by WT to recover from isoflurane was 7.13 ± 1.16 min. KO mice were comparable in time to recovery as well ($KO_{\text{mean recovery time}}$ = 7.02 ± 1.42 min). Similarly, time taken to recover from a 1.4% atm concentration of halothane did not differ between the genotypes ($WT_{\text{mean recovery time}}$ = 8.15 ± 1.07 min vs. $KO_{\text{mean recovery time}}$ = 9.6 ± 0.88 min).

2.3.4 Etomidate- and propofol - induced LORR:

The duration of LORR with etomidate is depicted in Figure 2.2 A. For etomidate-induced LORR, a two way ANOVA by sex and genotype revealed an an effect of sex ($F(1,20) = 5.4$; $p < 0.05$). Hence, males and females were analyzed separately.

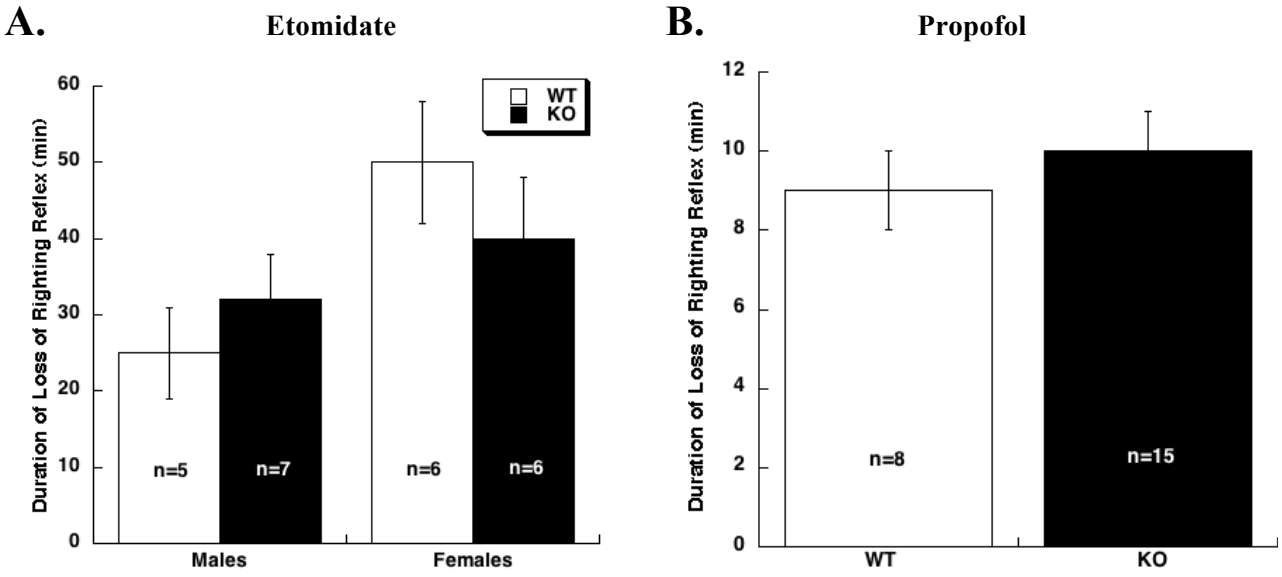

Figure 2.2. Loss of righting reflex with injectible anesthetics - etomidate and propofol

(A) A 20mg/kg dose of etomidate produced loss of righting reflex in both WT and KO. Since an overall ANOVA elicited an effect of sex, the genotypes were analyzed separately. However, no differences were seen between genotypes within either sex.

(B) Propofol, 40mg/kg, produced loss of righting reflex in both WT and KO mice No differences were observed between genotypes.

Data are expressed as mean ± SEM.

Genotypes did not differ in response to etomidate within either sex. Propofol-induced LORR is depicted in Figure 2.2 B. A one-way ANOVA revealed no differences between WT and KO on the duration of LORR with propofol.

2.3.5 Alphaxalone induced-LORR

The LORR effect of the neurosteroid anesthetic, alphaxalone, was compared between WT and KO mice. A one-way ANOVA indicated that alphaxalone-induced LORR did not differ between genotypes. (Fig. 2.3)

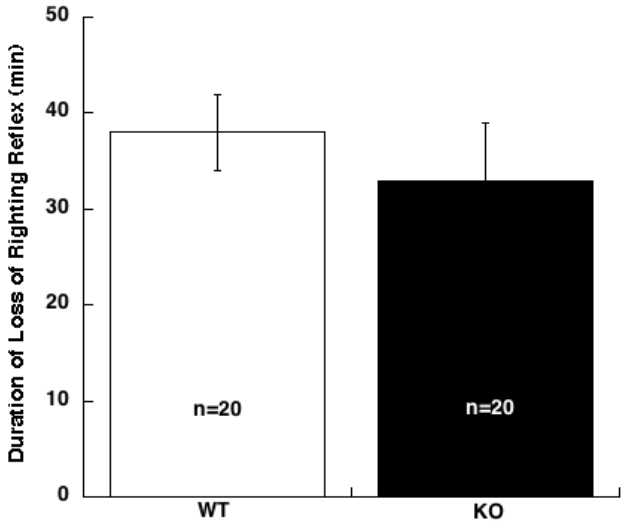

Figure 2.3 Loss of righting reflex with Alphaxalone

Alphaxalone-induced loss of righting reflex at 70mg/kg did not differ between WT and α4KO mice. Data are expressed as mean ± SEM.

2.3.6 Etomidate- and propofol - induced ataxia:

To test if α4 plays a role in motor ataxic effects of intravenous anesthetics, the motor-impairment caused by 20mg/kg of etomidate and 25mg/kg of propofol was tested.

The time course of performance on rotarod after etomidate (Fig. 2.4 A) and propofol (Fig. 2.4 B) administration in both WT and KO are shown. A one-way ANOVA analysis of AUC for performance on rotarod with etomidate showed no differences between the genotypes. Similarly, with propofol, one-way ANOVA did not yield a difference between genotypes.

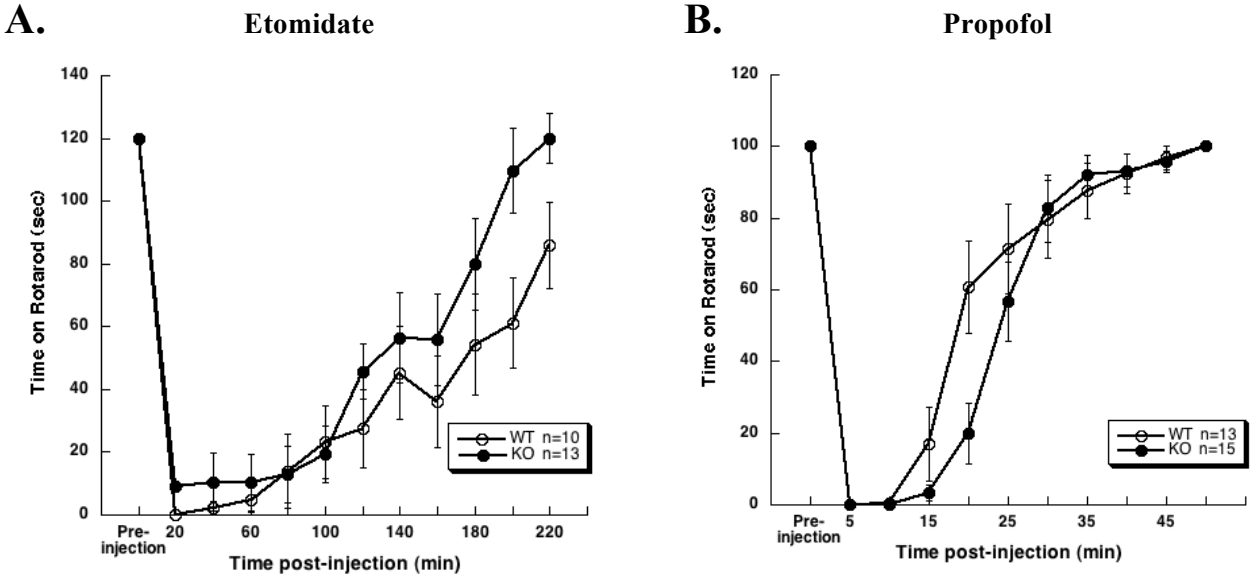

Figure 2.4 Motor ataxic effects of injectible anesthetics, etomidate and propofol

A. Etomidate, 20mg/kg, produced motor ataxia in WT and KO mice. No genotypic differences were noted.
B. Propofol, 25mg/kg, also produced motor ataxia in WT and KO mice. No genotypic differences were observed.

Each time point represents mean performance ± SEM

2.3.7 Alphaxalone-induced ataxia:

The motor-incoordinating effects of alphaxalone on WT and α4 KO were assessed (Fig. 2.5). A one-way ANOVA on the AUC for performance on rotarod did not show significant differences between genotypes.

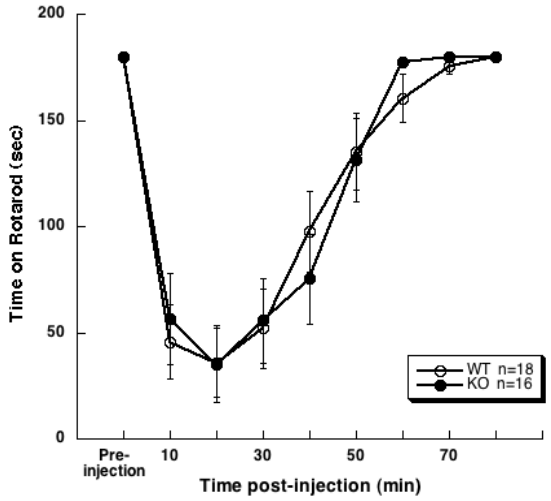

Figure 2.5. Alphaxalone-induced motor ataxia

Alphaxalone, a neurosteroid anesthetic, was assessed for its motor ataxic effects at 50mg/kg in WT and KO mice. No effect of genotype was observed. Each data point represents mean performance at that time point ± SEM.

2.3.8 Etomidate-induced locomotor behavior:

To test the hypothesis that low dose effects of etomidate are mediated by α4-containing receptors, sedative effects of etomidate were assesed by the open field assay (Fig.2.6). Pilot experiments revealed that a 3mg/kg dose of etomidate produced a significant sedating effect (not shown). Because saline-treated mice did not differ by genotype on 'total distance covered' measures, based on previous experiments with this line of mice [16], only sedating effects of etomidate were compared between genotypes. A one-way ANOVA of total distance covered with genotype as between-subjects factor showed no significant differences between WT and KO. Thus, the mean distance covered over a 10 min period under the influence of etomidate was comparable between WT and KO .

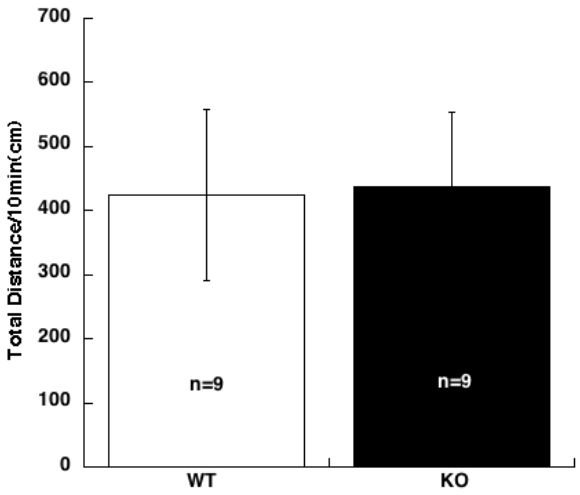

Figure 2.6 Sedative effects of etomidate

(A) A 3mg/kg dose of etomidate produced a sedative effect causing a reduction in the total distance covered over a 10 minute period compared to saline (based on pilot data). No differences were observed between WT and KO mice.
Data are expressed as mean ± SEM.

2.3.9 Alphaxalone-induced locomotor-behavior:

Because high dose experiments (LORR and rotarod) with alphaxalone did not yield any differences between the genotypes, contrary to observations at the cellular level [340], behaviors at lower doses of alphaxalone were subsequently tested.

For total distance covered over the 10 minute period (Fig. 2.7 A), an overall two-way ANOVA revealed a significant main effect of treatment [$F(1,63) = 7$, $p<0.05$] and genotype ($F(1,63) = 4$, $p<0.05$), but no interaction. Subsequent pair-wise comparisons revealed that a treatment effect of alphaxalone was observed in WT ($p< 0.05$) but not in the KO. In addition, WT and KO differed in their response to alphaxalone ($p< 0.05$). Thus, alphaxalone produced a locomotor stimulatory effect in the WT but not KO.

Next, percent distance covered in center zones was compared between WT and KO (Fig. 2.7 B). Percent distance covered in center zones was used as a surrogate for anxiety-like behavior in mice in this assay. However, a two-way ANOVA did not yield an effect of treatment or genotype, or an interaction. Thus, a 15mg/kg dose of alphaxalone did not produce anxiolytic effects on this assay.

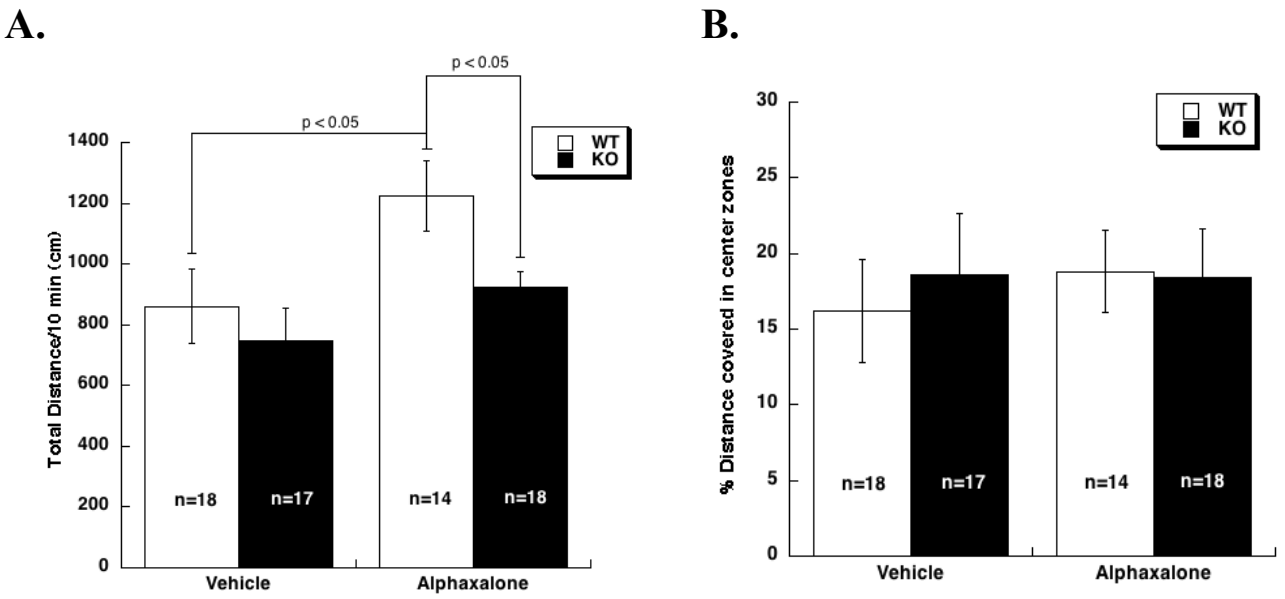

Figure 2.7 Locomotor behavior with the neurosteroid anesthetic, alphaxalone.

(A) Total distance covered by WT and α4 KO mice with a 15mg/kg stimulatory dose of the neurosteroid, alphaxalone, over a 10 minute period was recorded. A significant treatment effect was apparent in the WT ($p<0.05$) that was absent in the KO. Alphaxalone-treated KO were resistant to the stimulatory effects of alphaxalone compared to WT ($p<0.05$)

(C) Percent distance covered in center zone by WT and α4 KO mice with a 15mg/kg stimulatory dose of the neurosteroid, alphaxalone, over a 10 minute period was recorded. No differences were observed between vehicle-treated and alphaxalone-treated WT and α4 KO mice.

Data are expressed as mean ± SEM.

2.4 Discussion:

2.4.1 Role of α4-containing GABA A receptors in effects of volatile anesthetics:

Effects of isoflurane and halothane on LORR and immobilization were tested as part of this study. Halothane-induced LORR differed between WT and KO, with KO displaying some resistance to the effects of halothane on this behavior. However, given the small difference in EC_{50}'s between WT and KO, it is unlikely that α4-containing receptors are a major target for halothane. No differences between WT and KO were noted on halothane-induced immobilization. Surprisingly, WT and α4 KO mice were comparable in their responses to isoflurane-induced LORR and immobilization. Duration of RORR at 1.4% ATM of isoflurane and halothane were also assessed. Time taken by mice to recover did not differ between genotypes indicating that α4-containing receptors are not likely to be involved in this effect of isoflurane and halothane. The results obtained here are in contrast to results reported by Jia *et al* [343] at the cellular level, where isoflurane-induced tonic current potentiation was ablated in the ventrobasal thalamic neurons of α4 KO mice.

In parallel with the above studies, amnestic effects of isoflurane were assessed in WT and α4 KO mice by our collaborators at UCSF (Dr.Vinuta Rau, Dept. of Anesthesiology, University of California, San Francisco, CA). In fear conditioning assays, α4 KO mice showed resistance to the amnestic effects of isoflurane. KO mice required higher concentrations of isoflurane to achieve an amnestic effect. Thus, KO's had higher EC_{50}'s compared to WT. This result corroborates well with the reduced sensitivity to isoflurane in KO noted in cellular studies [343]. Thus, α4-containing receptors appear to be important mediators/modulators of inhaled anesthetic-induced amnesia. Each of these results is discussed in further detail below.

α4 KO mice displayed reduced responsiveness to isoflurane-induced amnestic effects on the fear conditioning assay. In keeping with the results obtained by Moore *et al* [346], only hippocampal dependent memory showed reduced response to isoflurane. This finding is consistent with the observations that GABA A α4-containing receptors are highly expressed in the hippocampus and the hippocampus is selectively involved in fear conditioned to context but not tone. The roles of the amygdala and hippocampus in fear conditioning have been well characterized. Lesions of the amygdala block fear to tone and context, whereas lesions of the hippocampus block only fear to context and leave fear to tone intact [114, 371]. The high levels of expression of α4-containing receptors in the hippocampus and the selective role of the hippocampus in fear conditioned to context reinforce a specific role for the GABA A α4 receptor subtype in inhaled anesthetic-induced impairment of hippocampal-dependent learning.

The α4-containing receptor combination that mediates this effect is of interest. Although the majority of hippocampal GABA A α4 receptors colocalize with the δ subunit extrasynaptically, there is a substantial portion of α4-containing receptors that colocalize synaptically with the γ2 subunit [372]. Therefore, it is possible that isoflurane produces its suppression of learning and memory by extrasynaptic as well as synaptic inhibition. However, synaptic effects of isoflurane are dependent on isoflurane concentration. Previous studies revealed that KO of the GABA A receptor α1 subunit, which is thought to primarily mediate phasic inhibition [6, 373] also produces a resistance to the amnestic effects of isoflurane [115]. The EC_{50} for α1 WT mice (males and females combined) was approximately 0.15% isoflurane, and the EC_{50} for α1 KO mice was approximately 0.37% isoflurane [119]. This is much higher for the KO in comparison to the results in this study: EC_{50} for α4 WT mice (averaged between males and females) was 0.12% isoflurane,

whereas the EC_{50} for α4 KO mice was 0.21% isoflurane. Although the EC_{50} values for both GABA A α1 and α4 WTs are similar, more isoflurane was necessary to suppress freezing by 50% in the GABA A α1 KO than the α4 KO. This is consistent with the finding that isoflurane at a very low concentration (25μM) directly activates GABA A receptors containing the α4 subunit, but only higher concentrations (200μM) are able to directly activate GABA A receptors containing the α1 subunit.

This activation of synaptic receptors with higher concentrations of isoflurane is one possible explanation for the resistance in response to isoflurane-induced amnesia but not on isoflurane-induced LORR or immobilizing effects in KO mice. On LORR assay, there was no significant difference between WT and α4 KO mice with isoflurane. Isoflurane-induced LORR is mediated at higher concentrations compared to amnestic effects of isoflurane. This gives rise to the possibility of activation of synaptic receptors at the concentrations used to assess LORR response of isoflurane. Since synaptic current responses to isoflurane are unchanged between WT and α4 KO mice [343], it is possible that this behavioral effect involves mainly synaptic current potentiation. A modest, but statistically significant, difference on halothane-induced loss of righting reflex was observed between genotypes. The halothane EC_{50} that was required to suppress this response in KO mice was increased by approximately 7% compared with WT mice. However, the biological significance of this modest change in anesthetic response is unclear. The possibility that compensatory responses may lead to overestimation or underestimation of the true contribution of α4-containing receptors to anesthetic-induced loss of righting cannot be excluded. Nonetheless, I conclude that α4-containing $GABA_A$ -Rs are not required for mediating the hypnotic effect of inhaled anesthetics.

No effect of α4 gene ablation was observed on immobility in response to halothane or isoflurane as measured by MAC. α4-containing receptors have restricted expression in the spinal cord and consistent with this [374], α4-containing receptors are likely not involved in mediating the immobilizing effects of volatile anesthetics. This observation agrees with the theory that $GABA_A$ receptors are not key mediators of volatile anesthetic-induced immobilization (see review[109]). Similar results have been obtained with other genetic mutant mice. For example, a global α1 KO did not have changed MAC responses to isoflurane [119]. Although a global KO of the β3 subunit increased enflurane MAC by ~26% and halothane MAC by ~7% [80], such increases were attributed to compensation [81]. Subsequent generation of the β3 KI also showed marginal increases in MAC (~15-20%) for enflurane and halothane [15]. An isoflurane-insensitive mutation in the α1 subunit of $GABA_A$ receptors did not affect the isoflurane- or halothane-induced MAC responses in mice [83, 123]. Similarly, mice bearing an isoflurane-insensitive mutation in the α2 subunit had no changes in MAC for isoflurane and halothane [84]. Apart from genetic screens, pharmacologic studies also do not support a role for $GABA_A$ receptors in immobilizing effects of volatile anesthetics [124]. These studies suggest a lack of correlation between *in vitro* potentiation of $GABA_A$ receptors and the *in vivo* responses for MAC. Adding to this, studies from the global α4 KO model here support the theory that $GABA_A$ receptor involvement in MAC of volatile anesthetics is negligable.

Lastly, if α4 GABAergic receptors were involved in hypnosis induced by isoflurane, they could modulate resistance in two ways: 1) resistance to 'go under' (induction) or 2) shorter duration of effect, i.e., 'coming out' (emergence). The mechanisms by which anesthetic induction versus anesthetic emergence are achieved are not resolved. Because neural circuitry of sleep induction and arousal involve overlapping pathways,

different receptors may play important roles in one process relative to the other. For example, orexigenic agonists and antagonists have the potential to disrupt the stability of the anesthetic state [375, 376]. The ablation of orexigenic neurons delayed the emergence from but not the induction of anesthetic state with isoflurane and sevoflurane [377]. Similarly, I hypothesized that α4-containing receptors may have a role in emergence from anesthetic influence. To assess this, RORR was measured in WT and α4 KO with both isoflurane and halothane. However, the lack of difference between the genotypes suggests that α4-containing receptors are not critical for modulating emergence from either anesthetic. A recent study by Gompf *et al* [378], showed that halothane-induced RORR occured normally in spite of lack of orexigenic neurons in contrast with isoflurane- and enflurane-induced RORR [377]. This supports the theory that different anesthetics act on diverse neuronal circuits to exert their effects. Hence, different wake-promoting systems must modulate emergence from anesthetics.

In conclusion, results obtained from the study of volatile anesthetic effects suggest a role for hippocampal α4 receptors in the low dose amnestic responses of isoflurane. In addition, results from this study suggest that α4-containing receptors are not involved in the LORR, immobilization and emergence from anesthetic state induced by isoflurane and halothane. The present results lend support to the hypothesis that different receptor subtypes likely mediate different anesthetic end points.

2.4.2 Role of α4 containing- GABA A receptors in effects of intravenous anesthetics:

Knockout of the α4-containing receptors did not alter response to the motor impairing effects of etomidate or propofol. No differences were observed between WT and KO on either etomidate or propofol-induced LORR. Low-dose effects of etomidate on

sedation were also found to not differ between WT and KO mice. These results indicate that α4-containing receptors are not required for the motor ataxic and hypnotic actions of etomidate or propofol or the sedative actions of etomidate.

Previous studies have shown that β3 and β2 subunits are important for the hypnotic and sedative effects of etomidate [15, 82]. A point mutation in the β3 subunit ablated sensitivity to the hypnotic and immobilizing effects of etomidate [15]. β2 subunits were responsible for the sedating actions of etomidate and propofol in a similar study [82]. Other lines of evidence have indicated that tonic current is subject to modulation by both etomidate and propofol. A study by Bai *et al*, assessed the sensitivities of synaptic and tonic current to propofol in hippocampal pyramidal neurons over a range of concentrations [379]. Both synaptic and tonic currents were potentiated by propofol. However, the net inhibitory charge mediated by tonic conductance was significantly greater than that mediated by synaptic conductance. Similarly, etomidate produced robust tonic current potentiation in thalamic neurons compared to synaptic currents [352]. Thus, tonic current is sensitive to modulation by both etomidate abd propofol. Both β2 and β3 subunits which are associated with the sedative and hypnotic effects of etomidate are strongly expressed in the ventrobasal thalamic nuclei and the reticular thalamic nuclei respectively [337]. Because α4 subunits can combine with β2 or β3 subunits and γ2 or δ subunits, and because α4-containing receptors have strong expression in the thalamus, I sought to determine if the absence of α4-containing receptors affected the actions of etomidate and propofol. My results indicate that α4 subunit-containing receptors are not critical for the effects of etomidate- or propofol-induced LORR or motor ataxia.

An overview of effects of etomidate-induced LORR in various GABA A subunit mutant models show interesting results. No changes in etomidate-induced LORR were

observed with global KO murine models of α1, α5, γ2, β2 and δ subunits [17, 116, 119, 121, 122]. In contrast, the global β3 KO, conditional β3 KO and β3 KI mice, all showed reduction in the LORR effects of etomidate [15, 80, 125]. This suggested that the β3 subunit of GABA A receptors was critical for effects of etomidate. The lack of effect in other mutant mouse models suggests the possibility that the actions of etomidate may be dictated solely by the β3 subunit while the identity of the α subunit may not be as critical. To confirm this, however, mice bearing deletions of α2, α3 and α6 subunit-containing receptors will have to be studied with etomidate.

As in the case of volatile anesthetics, it is possible that the high dose effects of etomidate are mediated predominantly by synaptic current potentiation. To determine if low dose effects of etomidate depended upon tonic current mediated by α4-containing receptors, sedative effects of etomidate were studied at a 3mg/kg dose. However, no differences were observed between the genotypes implying that α4-containing receptors are not required for this effect.

Thus, at least for the behaviors studied here, α4-containing receptors are not required for the effects of etomidate and propofol. However, the involvement of α4-containing receptors in other behavioral effects of etomidate and propofol cannot be excluded. One behavior worth further study is the amnestic effect of etomidate. Knockout of α5 subunit-containing receptors led to a reduction in the amnestic response to etomidate [116]. Like the α5 KO mice [117], α4 KO mice also have enhanced memory [346]. In addition, α4 KO are resistant to isoflurane-induced amnesia [344]. Since α4-containing receptors were required for the low dose amnestic effect of isoflurane, it can be speculated that α4 KO mice would show a similar reduction in amnestic response to etomidate. Based on the results seen with isoflurane and the increased memory in α4 KO, it is likely that α4

KO may show resistance to the amnestic effects of etomidate as well. Such a result would then reinforce a role for tonic current potentiating receptor subtypes in the hippocampus as being critical for low dose effects of anesthetics such as anesthetic-induced amnesia.

While these results suggest that α4-containing receptors are not essential for the effects of etomidate and propofol, it is possible that compensatory responses in the global α4 KO mask the true role of α4-containing receptors. Increases in α1, α2 and γ2 subunits have been observed in α4 KO mice [350]. This could result in novel combinations of receptors that take over the role of α4 receptors for etomidate and propofol actions. Another possibility arising from novel subunit combinations is 'silent receptors'. Given that many anesthetics act as allosteric modulators of GABA A receptors [380, 381], an otherwise 'silent receptor' may showed increased GABA sensitivity in the presence of anesthetics, thus giving rise to anesthetic-induced tonic GABA inhibition [367]. Such receptors may explain how anesthetic sensitivity is maintained in α4 KO mice in spite of reductions in tonic current in the thalamus and hippocampus.

In conclusion, the results indicate that α4 subunit-containing receptors are not essential for the actions of etomidate and propofol on the behaviors studied here. However, further analysis of compensatory changes and responses to these anesthetics on additional behaviors is required before a comprehensive understanding of the role of the α4 subunit in anesthetic actions is achieved.

2.4.3 Role of α4 containing-receptors in neurosteroid effects:

The lack of α4 subunit-containing receptors affected the locomotor behavior of the KO mice at a low dose of the neurosteroid, alphaxalone. WT mice had increased locomotion in response to alphaxalone whereas KO mice did not. Thus, it appears that α4

containing receptors are critical for the locomotor stimulatory effect of neurosteroids. This is particularly interesting because the locomotor stimulatory effect was studied at a relatively low dose of the neurosteroid alphaxalone. In contrast, experiments with alphaxalone revealed no differences between genotypes on the high dose effects of LORR or motor incoordination. This suggests that α4-subunit containing receptors are not required for these behaviors.

The α4 subunit undergoes extensive changes in expression with changes in levels of endogenous steroids during hormonal cycles (for review, see [382]). When combined with the δ subunit, the resulting receptor is highly sensitive to actions of neurosteroids [282-284]. Knockout of the δ subunit resulted in mice that had reduced sensitivity to neurosteroid-induced anxiolysis and hypnosis implying that the δ subunit was critical for the behavioral effects of neurosteroids [17]. Hippocampal dentate gyrus neurons from δ KO mice showed reduced tonic current potentiation in response to neurosteroids [369]. Characterization of the δ KO mouse brains revealed that expression of α4 was also reduced in critical regions such as thalamic nuclei, dentate gyrus and caudate putamen [353]. Therefore, the reduction in neurosteroid sensitivity in δ KO could, in part, be attributed to reduction in α4 levels as well. In the α4 KO, tonic and synaptic current potentiation due to alphaxalone was reduced in hippocampal dentate gyrus granule neurons [340]. Hence, I hypothesized that α4 subunit-containing receptors were critical for the behavioral effects of neurosteroids.

In contrast to my expectations, high dose effects of alphaxalone on LORR and motor ataxia were unchanged in α4 KO mice. The findings here indicate that α4-containing receptors are not required for the hypnotic and motor ataxic effects of neurosteroids.

Motor behavior is articulated by participation of several brain regions. While the cerebellum is considered critical for motor coordination, the thalamus is involved in relay of information between the cerebellum and the primary motor cortex. Thus, disruption of motor activities could occur anywhere along the cerebello-thalamo-cortical pathway. For example, with α4 KO mice, tonic current potentiation by gaboxadol was reduced in the ventrobasal thalamic neurons of the α4 KO [16]. A concurrent reduction in the motor ataxic effects of gaboxadol was seen [16]. Although cellular responses to alphaxalone were reduced in the α4 KO mice, decreases in motor ataxia with alphaxalone was not observed suggesting that alphaxalone mediates its effects in a manner different from that of gaboxadol. It is possible that the motor ataxic effects of alphaxalone depend upon brain regions that do not express α4 such as the cerebellum [242, 337]. Indeed, α6-δ containing receptors that are strongly expressed in the cerebellum are also sensitive to neurosteroids [295]. Since α4 subunits are absent in the cerebellum, it is unlikely that α6δ-containing receptors in the cerebellum are changed in the α4 KO mice. Hence, it is possible that motor ataxic influence of neurosteroids is mediated by these cerebellar receptors.

A similar explanation can be put forth for the LORR effects of alphaxalone. Like motor ataxic effects of alphaxalone, the hypnotic effects of neurosteroids could similarly be attributed to the effects on brain regions that do not involve α4-containing GABA receptors. This explains the lack of a difference between the WT and KO on the LORR effects of alphaxalone.

However, both motor ataxia and LORR were assessed at relatively high doses of alphaxalone. In the α4 KO, hippocampal dentate gyrus neurons showed reduced current potentiation with alphaxalone [340]. The possibility of α4δ-containing receptors being responsible for low dose effects of alphaxalone was, therefore, tested by assessing

locomotor behavior. Consistent with my hypothesis, α4 KO mice did not respond to a stimulatory effect of alphaxalone in the open field assay. Thus, α4-containing receptors appear important for this effect of neurosteroids. The fact that a change in behavior of α4 KO was observed at a low dose of alphaxalone suggests that like with isoflurane, α4-containing receptors play an important role in the low dose effects of alphaxalone. Coupled with the results obtained with amnestic effects of isoflurane, this finding suggests that tonic current-mediated by α4-containing receptors is sensitive to low doses of anesthetics and neurosteroids.

It is possible that compensatory changes may have masked a role for α4 containing receptors at higher doses. In the α4 KO mice, there is evidence of compensatory response in terms of increased α1, α2 and γ2 [349, 350]. The increased α1 and α2 subunits could combine with δ subunits to form receptors responsive to neurosteroids. α1δ-containing receptors that are sensitive to low doses of ethanol and potentiate tonic current in response to them have been discovered recently [320]. Such receptors may be potentiated by neurosteroids as well. This could explain why high dose effects of neurosteroids are unchanged in the α4 KO mice. Novel combinations of these receptors may be involved in mediating the effects of neurosteroids thus masking the true role of the α4-containing receptors in neurosteroid actions.

2.5 Summary:

In conclusion, the above experiments shed light on the role of α4-containing receptors in anesthetic actions. The involvement of α4-containing receptors in amnestic effects of isoflurane and locomotor stimulatory effects of alphaxalone suggest that α4-containing receptors modulate these anesthetic behaviors. The lack of effects with

etomidate and propofol indicate that α4-containing receptors are less likely to be involved in their effects in spite of being critical modulators of tonic currents.

Contrary to the vast amount of data linking α4 subunit-containing receptors to neurosteroid effects, deletion of α4-containing receptors affected only the response to low doses and not high doses of neurosteroids. It is possible that α4-containing receptors become relevant only in cases of changed levels of endogenous neurosteroids such as premenstrual syndrome and pregnancy. Previous studies with neurosteroids have indicated that changes in behavior were coincident with changes in levels of endogenous neurosteroids and expression of α4 protein (see review [382]). However, α4 homozygous knockouts are known to reproduce normally [16], and have normal maternal behavior although this has not been studied formally. Hence, it appears that α4 subunits have a lesser role than δ in modulation of neurosteroid effects.

An important observation is the common theme of effects observed in the α4 KO with isoflurane and alphaxalone. Differences between WT and KO were observed on the amnestic effects of isoflurane and the locomotor stimulatory effect of alphaxalone. It is interesting that both drugs showed differences on behaviors that were studied at low doses but not of high doses of these drugs.

The inhibitory charge transfer associated with tonic current potentiation is greater than that associated with synaptic current potentiation in presence of anesthetics. This was demonstrated by Bai *et al* while studying the effects of propofol and midazolam on tonic and synaptic current potentiation [379]. Similar results have been shown with effects of etomidate and gaboxadol on tonic and synaptic current potentiation in thalamic neurons [352]. These observations coupled with the differences in behavioral effects of low doses of isoflurane and alphaxalone in the α4 KO compared to WT suggest that the tonic current

mediated by α4-containing receptors may be particularly sensitive to low doses of anesthetics and neurosteroids, i.e. the initial effects of anesthetics may be modulation of tonic current potentiation. This is consistent with the reductions in low dose behaviors of anesthetics observed with deletions of receptors involved in tonic current potentiation such as α5, δ and now α4 [116] [17, 311]. Hence, there is a greater likelihood that low dose anesthetic effects such as cognitive impairment, locomotor behavior and anxiety are modulated by tonic current mediating GABA A receptors.

Interestingly, this result also correlates with the theory that α4δ-containing receptors are sensitive to low doses of ethanol. At present there is a lack of appropriate behavioral assays that can effectively measure the low dose effects of ethanol. Behaviors such as sedation, motor ataxia and hypnosis reflect moderate intoxication induced by ethanol. Recently, Moore *et al* developed an assay to specifically evaluate the low dose cognition-impairing effects of ethanol [383]. With the development of such assays, dissection of low dose effects of ethanol and anesthetics should be easier.

Thus, results from the study of anesthetic effects in α4 KO mice implicate α4-containing receptors in the low dose effects of isoflurane and alphaxalone. In addition, thes results corroborate with the idea that tonic current potentiation is important for low dose anesthetic responses.

Chapter 3: Role of α4-containing GABA A receptors in the intrinsic and ethanol antagonizing effects of RY023

3.1 Introduction:

The negative effects of acute and chronic alcohol consumption place huge economic and social burdens on society. Overconsumption of alcohol leads to sedation, motor-incoordination, memory deficits in addition to severe nausea, headache, dehydration, etc. [3]. Repeated consumption of alcohol also holds the risk of addiction and development of alcohol dependence.

For the above stated reasons, it is enticing to consider the possibility of an ethanol antagonist that would allow one to counter the ill effects of alcohol. The discovery of effective ethanol antagonists will also aid in treatment of acute toxicity caused by ethanol. In addition, a fast acting ethanol antagonist will be useful as a therapeutic agent in reversing the effects of acute ethanol. For example, a drug that can reverse the motor-incoordinating, sedating, and cognition-impairing effects of ethanol before operation of vehicles or machinery will cause fewer accidents and thus, allow for more responsible drinking. Because ethanol has strong addiction potential, it produces effects that increase the urge to drink more frequently and in greater amounts. An ethanol antagonist could conceivably prevent such changes in the brain and thus prevent dependence on ethanol as well.

The development of specific ethanol antagonists has been the focus of much research since the discovery of Ro15-4513 – an imidazobenzodiazepine drug synthesized by Hoffman-La Roche Pharmaceuticals [384]. Ro15-4513 acts as an ethanol antagonist both in behavioral studies [247, 249, 385, 386] and biochemical assays [150, 245, 246].

Being a benzodiazepine-like compound, Ro15-4513 was proposed to function via benzodiazepine-sensitive GABA A receptors. The true mechanism of action of Ro15-4513 was unknown until the discovery of ethanol-sensitive GABA A receptors.

Ethanol-sensitive receptors are thought to contain α4/α6 and δ subunits [158]. These receptor subtypes are involved in mediation of tonic current upon activation by low amounts of GABA which makes them suited to modulation by ethanol as well [156-158, 317, 318]. In studies with recombinant receptors expressed in heterologous systems, these subtypes were potentiated by 3-50mM of ethanol whereas α1, α2, γ2 -containing receptors were potentiated by much higher amounts of ethanol (~100mM) [14, 154, 318, 387]. Initially, it was thought that Ro15-4513 reversed ethanol actions due to its partial inverse agonist activity on synaptic receptor subtypes. However, further study of the specificity of Ro15-4513 for ethanol antagonism and its comparison with other inverse agonists showed that the antagonistic effects of Ro15-4513 was specific for ethanol [156, 262], thus arguing in favor of a unique ethanol - Ro15-4513 binding pocket. A series of studies by Olsen, Wallner and Hanchar provided evidence towards a common binding pocket [156, 158, 262, 264]. In brief, the findings of Wallner, Hanchar and Olsen can be summarized as follows (reviewed in [265]):

1) Ro15-4513 binds with high affinity to α4δ and α6δ -containing receptors.
2) Ro15-4513 has mild partial agonist activity on α4β3γ2, does not modulate α4β3δ receptors and has inverse agonist activity at other α1/2/3/5 subunit-containing receptors.
3) Low concentrations of ethanol displace bound labeled Ro15-4513 from α4δ-containing receptors.

4) Flumazenil and β-carboline derivatives also displace bound Ro15-4513 from α4-δ receptors.

The generation of an α4 KO mouse model provided conclusive evidence towards the role of α4-containing receptors in the actions of Ro15-4513. In electrophysiological studies, tonic current potentiation by Ro15-4513 was reduced in dentate gyrus granule neurons of α4 KO mice [340]. Analysis of the ethanol antagonistic effects of Ro15-4513 revealed that while WT responded with a reversal of ethanol-induced motor ataxia (Fig. 1.2), KO did not respond to Ro15-4513 [341]. At high doses of ethanol, (3.5g/kg), LORR effect was reduced by Ro15-4513 in WT but was unchanged in KO (Fig. 1.2) [341]. This indicated that α4-containing receptors are critical for the ethanol-antagonizing effects of Ro15-4513. Since other ethanol antagonists had been derived from the prototypical Ro15-4513, it was likely that these derivatives also mediate their effects through α4-containing receptors. In fact, binding studies by Hanchar *et al* indicated that compounds that were structurally similar to Ro15-4513 also showed affinity to α4-containing receptors and displaced Ro15-4513 from its binding site [262].

RY023 is one of a series of substituted imidazobenzodiazepines that was originally synthesized from the prototypical ethanol antagonist, Ro15-4513 [251]. Characterization of the binding profile of RY023 revealed that it had both high affinity (Kd= ~2.7nm) and selectivity (~75 fold) for α5β2γ2 GABA A receptors [251]. At recombinant receptors, RY023 produced a small reduction in GABA binding (~25%) [251, 260]. It possesses convulsant properties at doses ≥ 40mg/kg [251, 260]. RY023 was assessed for its functional modulation at α1β3γ2, α2β3γ2, α5β3γ2, α4β3γ2, α6β3γ2 -containing receptors in xenopus oocytes [253, 255]. It was found that RY023 negatively modulated all the above

subtypes, but was more effective at the α5β3γ2 receptors than at the others. The binding affinity and modulation of RY023 at δ-containing receptors was, however, not assessed.

Behavioral characterization of RY023 showed that it was effective in antagonizing ethanol effects on multiple behavioral paradigms. In drinking behavior experiments, RY023 infusions in the CA1 and CA3 regions of the hippocampus produced a reduction in ethanol drinking behavior [255, 357]. In addition to drinking behavior, RY023 was evaluated for its ethanol-antagonistic potential on motor ataxia (oscillating bar test) and locomotor behavior (open field activity) [253]. On both these behaviors, RY023 was successful in antagonizing the effects of ethanol in rats. These experiments revealed that RY023 was effective in antagonizing several behavioral endpoints of ethanol, just like Ro15-4513.

Prior to the findings of Wallner, Hanchar and Olsen, because the RY series of drugs had only been tested against α-γ subunit combinations, the affinities and the efficacies of these drugs at α4δ subunit combinations was not known. Although RY023 itself has not been studied in competition binding assays with Ro15-4513 or in current modulation through α4δ-containing receptors, other compounds of the same family (RY024 and RY080) have been tested [262]. The findings indicate that these drugs may also be potent modulators of ethanol through binding at the α4-δ receptors. To test this hypothesis, α4 KO mice were subjected to several behavioral assays with ethanol and RY023. RY023 has a better solubility profile compared to the other RY drugs (personal communication with Dr. H. June, University of Maryland, Baltimore, MD) and well-characterized responses in rats and hence this drug was chosen for use in our experiments.

3.2 Materials and Methods:

3.2.1 Drugs and Solutions:

Solid RY023 (tert-butyl 8-[trimethylsilyl] acetylene-5,6-dihydro-5-methyl-6- oxo-4H-imidazo [1,5a] [1,4] benzodiazepine-3-carboxylate) was provided by Dr. J. Cook (Dept. of Psychology, Indiana University-Purdue University, Indianapolis, Indiana) and Dr. H. June (University of Maryland, Baltimore, MD). On the day of the experiment, the solid drug was weighed directly in the final container and suspended in the vehicle solution. Vehicle solution was prepared by mixing 0.1ml of Tween 80 (Sigma Aldrich, St.Louis, MO) in 100ml of saline. The drug suspension was briefly vortexed and sonicated by a probe sonicator (Branson Sonifier 250, Duty cycle-30%, Output control-3) for three 15 sec periods while on ice. Ethanol solutions were prepared by mixing absolute ethanol (Pharmco, Brookefield, CT) with saline to arrive at doses of 2g/kg and 3.5g/kg. Ethanol and RY023 solutions were injected at 0.01ml/gm body weight for all experiments except LORR, for which ethanol solution was injected at 0.02ml/gm body weight.

In all assays, males and females of all three genotypes (WT, HET and KO), aged 8-16 weeks were used. However, due to limited number of subjects and multiple treatment-genotype combinations, sex differences were not analyzed.

3.2.2 Intrinsic effects and RY023 antagonism of ethanol-induced motor ataxia:

Both intrinsic effects and ethanol antagonistic effects of RY023 were determined. Because intrinsic effects could confound the interepretation on ethanol antagonism assays, I sought to determine the highest dose of RY023 that produced minimal intrinsic effects. Hence, different dose combinations of RY023 and saline were tested on HET mice to determine the dose of drug to be used in WT and KO mice.

Mice were weighed and subjected to training trials on the previous day on the fixed speed rotarod (Ugo Basile 7650, Varese, Italy) at 8RPM. Mice were given at least 4 training trials to achieve a performance of 100s on the rotarod. Training trials were administered 15 minutes apart. Following training, mice were housed overnight in the testing room. On the test day, mice were tested twice on the rotarod to ensure compliance with criteria. Mice that did not achieve the criterion of 100s on two consecutive trials were excluded from the experiment. Two injections were administered - RY023 and saline (RY dose: 8, 10, 15, 20, 30mg/kg), All injections were administered i.p., in a volume of 0.01mls/g body weight of subject. The order of the injections was RY023 followed by saline injection, 5 min later. Ten minutes after the second injection, performance on rotarod was tested. Trials were then conducted every 15 min for 60 min.

Dose-determination experiments were conducted on HET mice first, followed by testing on WT and KO mice. Only 10mg/kg of RY023 with and without 2g/kg of ethanol was tested in WT, HET and KO. Area under curves (AUC- area under curve refers to area between curve and line at 100sec) were compared at each dose-combination by one-way ANOVA and Fisher's post hoc tests.

3.2.3 RY023 antagonism of ethanol-induced LORR:

Antagonism of ethanol-induced LORR was tested using 15mg/kg RY023 and 3.5g/kg ethanol. Mice were injected with RY023 (i.p, 0.01ml/g body weight) or saline, followed by ethanol 5 minutes later (i.p, 0.02ml/g body weight). Thus, two treatment groups were tested – saline-ethanol (SE) and RY023-ethanol (RY-E). Upon losing their righting reflex, mice were placed in a supine position on V-shaped troughs. Mice were considered to have regained their righting reflex if they were successful in righting themselves three consecutive times within 30sec. A heat lamp was used during the course of the experiment

to ensure normothermia. Data were analyzed by two-way ANOVA and Fisher's post hoc tests.

3.2.4 RY023-induced impairment of locomotor behavior:

Intrinsic effects of RY023 on locomotor activity were tested at a dose of 15mg/kg. Pilot studies were conducted to confirm that this dose of RY023 produced a sedating effect and reduced locomotion compared to vehicle-treated mice. Mice were placed in the testing room one day prior to the experiment. On the morning of the experiment, mice were weighed, injected with RY023 or vehicle and placed separately in new cages. Fifteen minutes later, mice were placed in the center of a plexiglass walled arena each, (43.2 cm X 43.2 cm X 30.5 cm) for automated recording of locomotor behavior. The chambers were placed within sound-attenuating cubicles (Med Associates, St.Albans, VT) that were equipped with a red light and a fan (top right corner). Distance traveled (cm) over a 10 minute period was recorded by an automated program (Med Associates, St.Albans, VT). Between mice, the chambers were cleaned with 70% ethanol followed by water to remove any evidence of smells and excrements left behind. Data were analyzed by two- way ANOVA and Fisher's post hoc tests.

3.2.5 Immunoblotting:

Untreated WT, HET and KO mice were sacrificed, and hippocampi and cortex were isolated from whole brains. Brain regions were flash frozen and stored at -80°C until processing. Samples were homogenized in a sucrose homogenization buffer (10.95g sucrose in 100ml 1X PBS, pH 7.2). The homogenates were centrifuged at 1000g for 10 min followed by spinning the supernatant at 10,000g for 25 min. The resultant pellet was resuspended in 1X PBS (Gibco-phosphate buffered saline, pH 7.2, Invitrogen, Carlsbad, CA) and consisted of the P2 membrane fraction. Protein concentrations for each sample

were determined spectrophotometrically via a bicinchonic acid method. Known protein concentrations were used to generate a standard curve and resulting sample protein concentrations were determined. Stock protein solutions were diluted to a concentration of 2.5μg/μl and stored at -80°C for further use. Twenty-five μg of protein per sample was electrophoresed on precast SDS-10% polyacrylamide gels (Bio-Rad, Hercules, CA) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) for detection by subunit-specific antibodies. The PVDF membranes were blocked overnight with 3% non-fat blotting grade dry milk buffer (Biorad, Hercules, CA) in PBS-comtaining 0.1% Tween 20 (Biorad, Hercules, CA). The following day, membranes were incubated overnight with GABA A receptor anti-α4 (1:1000, NB 300-193, Novus Biologicals, Littleton, CO) antibody. On day 3, membranes were washed three times with PBS-containing 0.1% Tween-20 (PBS-T) for 15 min each. Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1:5000, NB-730H, Novus Biologicals, Littleton, CO). Secondary antibodies were detected by enhanced chemiluminescence (Western Lightning; PerkinElmer Life and Analytical Sciences, Boston, MA). Blots were stripped using Re-blot (Chemicon International, Millipore, Billerca, MA) and reprobed with an anti-β-actin polyclonal antibody (1:10,000, ab8227-50; Abcam, Cambridge, MA) 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 (PDSI, Personal densitometer, Amersham Biosciences, Piscataway, NJ) and analyzed by ImageQuant software (Amersham Biosciences, Piscataway, NJ). Each sample was analyzed on two different blots. Data were analyzed by unpaired Student's t-test.

3.3 Results:

3.3.1 Intrinsic effects of RY023 on motor ataxia:

To determine the highest dose of RY023 that had minimal intrinsic effect on motor ataxia, HET mice were first tested with various doses of RY023 in a pilot study. From previous experience it was known that untreated mice were capable of achieving the criterion of 100 sec, every 15 min for 1 hour on the rotarod. Hence, I compared the impairment produced by different doses of RY023 in HETS to baseline performance (Fig. 3.1 A). Next, area under the curve (AUC) for the performance on rotarod at different doses of RY023 was calculated. AUC's were calculated as the sum of the area between the curve and baseline performance over a 60 minute period. Doses of 8 and 10 mg/kg had the smallest AUC among the drug-treated mice. Since the goal of this assay was to determine the highest dose of RY023 that had the least intrinsic effect, the 10mg/kg dose of RY023 was chosen for further study in WT and KO.

To test the hypothesis that α4-containing receptors are involved in the actions of RY023, WT and KO mice were injected with 10mg/kg dose of RY023 and tested for performance on rotarod. AUC's for WT and KO mice were calculated and compared with data from HET mice. Analysis of AUC by a one-way ANOVA revealed a main effect of genotype [$F(2, 22) = 6$, $p < 0.01$]. Fisher's post hoc tests revealed that HET mice showed significantly lowered impairment compared to WT ($p < 0.005$) and KO ($p < 0.05$). WT and KO mice did not differ from each other. Thus, HET mice were less sensitive to the motor ataxic effects of RY023 compared to WT and KO.

Note: HET mice data used for this comparison was the same as that obtained from preliminary tests, shown in Fig. 3.1 A, B.

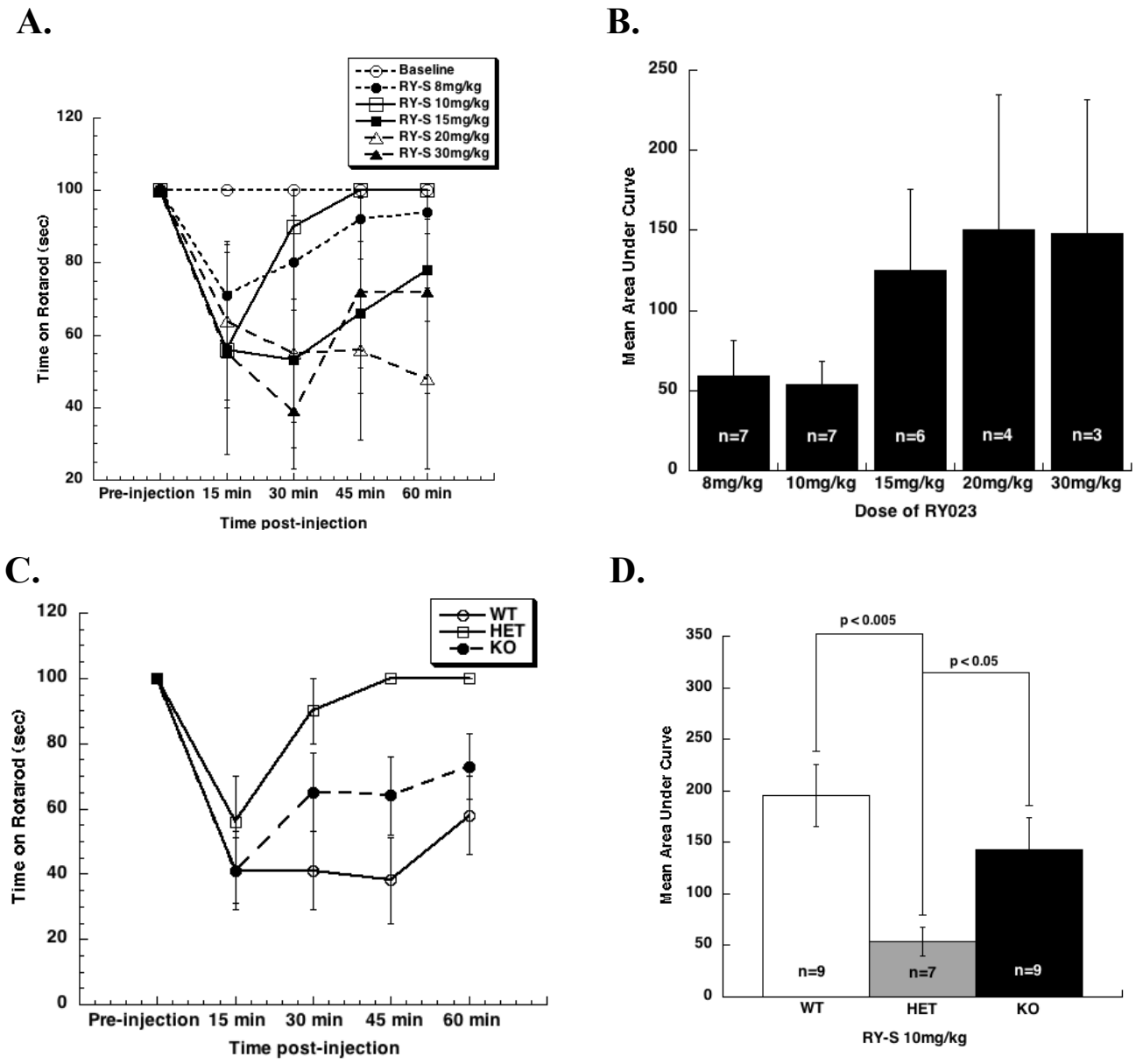

Figure 3.1 Intrinsic effects of RY023 on motor ataxia (Data are expressed as Mean ± SEM)

(A) Intrinsic effects of RY023 in HET mice at different doses of RY023 (8-30mg/kg). All doses produced motor ataxia.
(B) AUC of performance on rotarod at all doses of RY023 in HET mice. Doses 8 and 10mg/kg produced the least impairment of motor performance.
(C) Line graph of performance on rotarod at a dose of 10mg/kg of RY023 in WT, HET and KO mice
(D) AUC of performance on rotarod of WT, HET and KO mice at 10mg/kg of RY023 showed a significant difference between HET and WT ($p < 0.005$) and between HET and KO ($p < 0.05$). KO and WT did not differ from each other in the intrinsic effects of RY023 on motor ataxia.

3.3.2 RY023-induced antagonism of ethanol impairment on motor ataxia:

To test the hypothesis that α4-containing receptors are required for the ethanol antagonistic actions of RY023, WT, HET and KO mice were assayed for response to RY023 antagonism of ethanol-induced motor ataxia. Prior assays had been conducted to confirm that WT, HET and KO did not differ in their response to ethanol alone under the conditions of this assay (not shown). The same result was obtained under slightly different conditions by Chandra *et al* [186]. Line graphs of performance on rotarod over 60 minutes for all three genotypes are shown in Fig. 3.2 A. A one way ANOVA on the AUC's (Fig. 3.2 B) did not show a difference between genotypes.

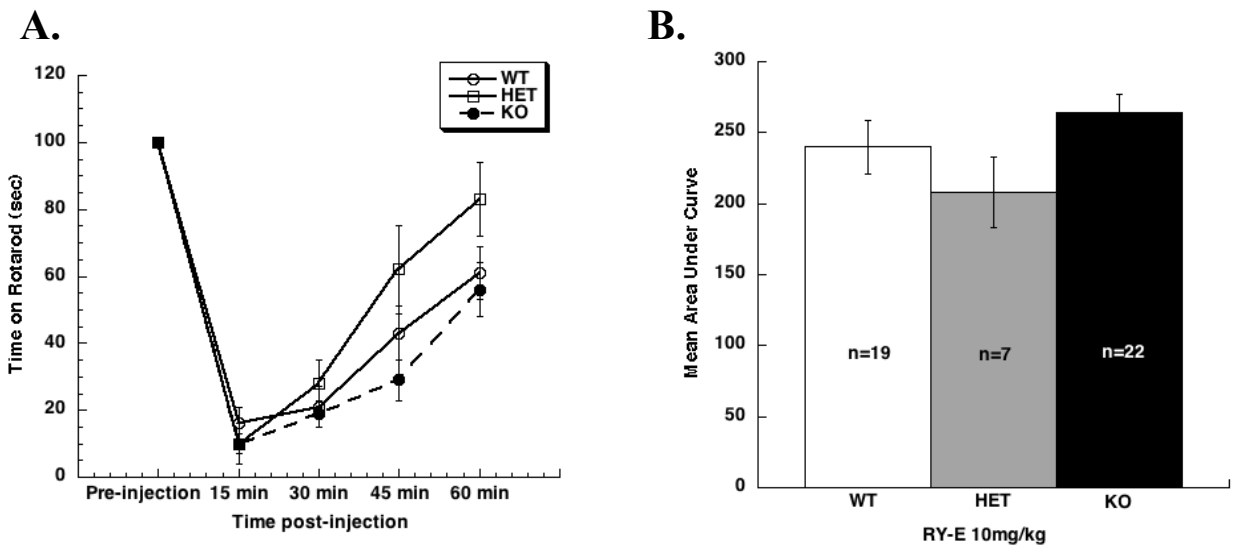

Figure 3.2 Effects of RY023(10mg/kg) on ethanol-induced (2g/kg) motor ataxia

(A) Line graph of performance on rotarod by all three genotypes.
(B) AUC's for performance on rotarod by all three genotypes. No differences were observed between genotypes on the effects of RY023.
Intrinsic effects of RY023 preclude an interpretation of antagonistic effects of RY023 on ethanol-induced motor ataxia.
Data are expressed as mean ± SEM.

Antagonism of ethanol-induced ataxia could not be measured in WT, HET and α4 KO. A comparison of the AUC's from ethanol-induced motor ataxia and from RY-E induced motor ataxia in HETS (recorded as pilots) indicated that 10mg/kg RY023 did not antagonize the effects of 2g/kg ethanol. Based on pilot experiments, higher doses of RY023 would also not be suitable for ethanol-antagonism owing to strong intrinsic effects. Since intrinsic effects of 10mg/kg RY023 differed among genotypes, I sought to determine if the effect of 10mg/kg RY023 on 2g/kg ethanol was different between genotypes. However, WT, HET and KO did not differ in their response to 10mg/kg RY023 and 2g/kg ethanol on motor ataxia.

3.3.3 RY023 antagonism of ethanol-induced LORR:

The ethanol antagonistic potential of RY023 was tested at a dose of 15mg/kg with 3g/kg ethanol. An overall two-way ANOVA showed a significant effect of treatment [$F(1,67) = 40$; $p<0.0001$] but not of genotype (Fig. 3.3 A). However, there was a significant interaction of treatment and genotype [$F(2,67) = 4$; $p<0.05$]. Fisher's post hoc tests revealed a significant effect of treatment within each genotype (Fig. 3.3 A). Thus, all genotypes showed a reduction in ethanol-induced LORR with RY023. A significant difference was found between KO and HETS on the SE group ($p<0.05$) but no difference between genotypes on the RY-E group.

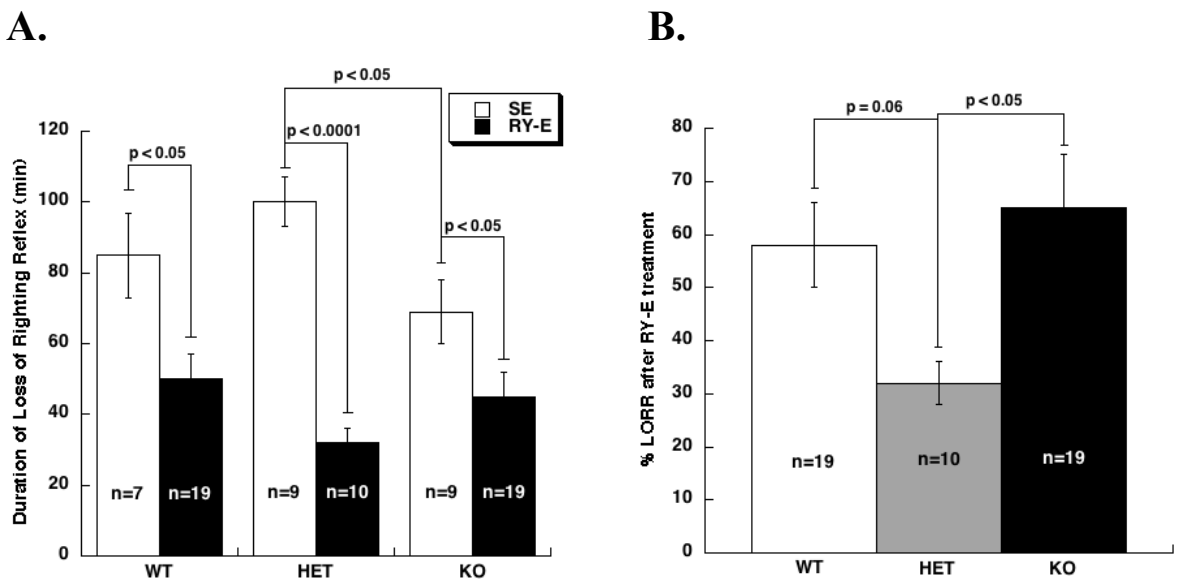

Figure 3.3 Effect of RY023 (15mg/kg) on ethanol (3.5g/kg) -induced loss of righting reflex (LORR)

(A) Durations of LORR in WT, HET and KO mice with saline and ethanol (SE) and with RY023 and ethanol (RY-E). A significant effect of treatment was observed ($p < 0.0001$) in all three genotypes. In addition, a baseline difference between SE-HET and SE-KO groups was observed. Data are expressed as Mean ± SEM.

(B) Durations of LORR with RY023 expressed as a percentage of durations of LORR with ethanol alone for each genotype. A significant difference was observed in the magnitude of reversal of ethanol-induced LORR by RY023. HETs had the greatest reductions in durations of LORR compared to WT ($p = 0.05$) and KO ($p < 0.05$). No differences were observed in % LORR between WT and KO mice.

Due to the baseline difference on the durations of LORR between KO and HETS, RY-E - durations of LORR were also analyzed as percentages of the mean baseline LORR (SE) within each genotype (Fig. 4.3.B). Analysis of the percent sleep times (% LORR) showed a difference between genotypes that was borderline significant [$F(2,45)=3$, $p = 0.06$]. Post hoc tests revealed that HETS were significantly different compared to the KO ($p<0.05$). The difference between WT and HET % LORR approached significance at $p = 0.06$. No difference was seen between WT and KO % LORR.

3.3.4 Intrinsic effects of RY023 on locomotor behavior:

Because study of motor ataxic effects of RY023 on rotarod presented some difficulties due to overlap of intrinsic effects, the study was extended to include intrinsic effects of RY023 on locomotor behavior at a dose of 15mg/kg. This dose of RY023 had a stong sedating effect on mice compared to vehicle treatment as evidenced by the reduction in distance covered over the 10 min period between the two treatment groups. A two-way ANOVA with genotype and treatment as between factors measures indicated a strong effect of treatment [$F(1,81) = 23$; $p<0.0001$], but not an effect of genotype (Fig. 4.4) or interaction between genotype and treatment.

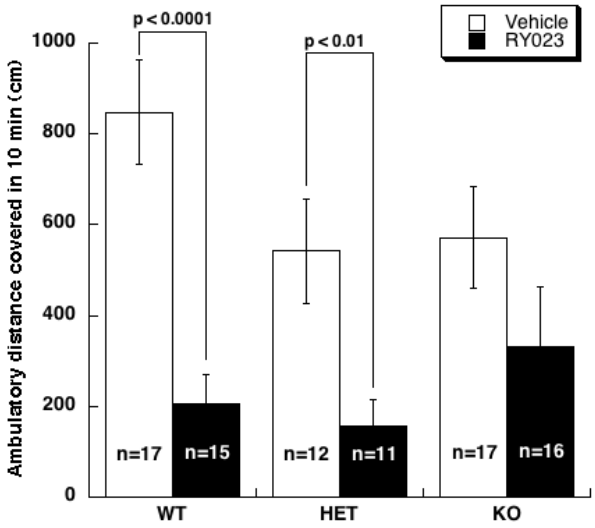

Figure 3.4 Intrinsic effects of RY023 (15mg/kg) on locomotor behavior over a 10 minute period.

A 15mg/kg dose of RY023 produced significant sedation in WT ($p<0.0001$) and in HET ($p< 0.01$) but not in KO compared to vehicle-treated mice. Thus, KO did not respond to the intrinsic sedating effects of RY023. Data are expressed as mean ± SEM.

Fisher's post hoc tests were conducted to determine where the differences in treatment lay. Post hoc tests revealed that a significant effect of treatment was noted in HETS, $p<0.01$ and WT, $p<0.0001$ but not in KO. Thus, while WT and HET mice responded to the sedating

effects of RY023, KO mice did not indicating that they were resistant to this effect of RY023. Post hoc tests also revealed near-significant differences on the baseline response to vehicle between the genotypes. While KO and HET mice were comparable on their baseline response, they differed slightly from the WT (WT and HET: $p = 0.07$; WT and KO: $p = 0.07$). However, previous experiments with different vehicle controls have not yielded significant differences in baseline behaviors (saline controls -[186]; 22.5% HBC, Fig. 2.7A). Hence, it is unlikely that this difference represents a role for α4-containing receptors in baseline locomotor behavior.

3.3.5 Immunoblotting:

While WT have normal levels of α4 protein expression and KO have none [339], the level of expression of α4 protein in HET mice was unknown. Since HET mice showed reduced motor ataxic effects with RY023 compared to WT and KO mice, we hypothesized that differences in levels of protein may be responsible for the same. Semi-quantitative analysis of α4 subunit protein from hippocampi and cortex of WT and HET mice revealed that HETs had significantly lower levels of protein relative to WTs in the hippocampus but not in the cortex ($p<0.05$). This suggests that protein levels maybe differentially compensated in different brain regions. However, it is important to note that this data was collected from a small sample set (n=2-3/geno) run in duplicate. It is likely that the reduction of α4 protein in HET mice is overestimated here due to the small sample set and blot irregularities.

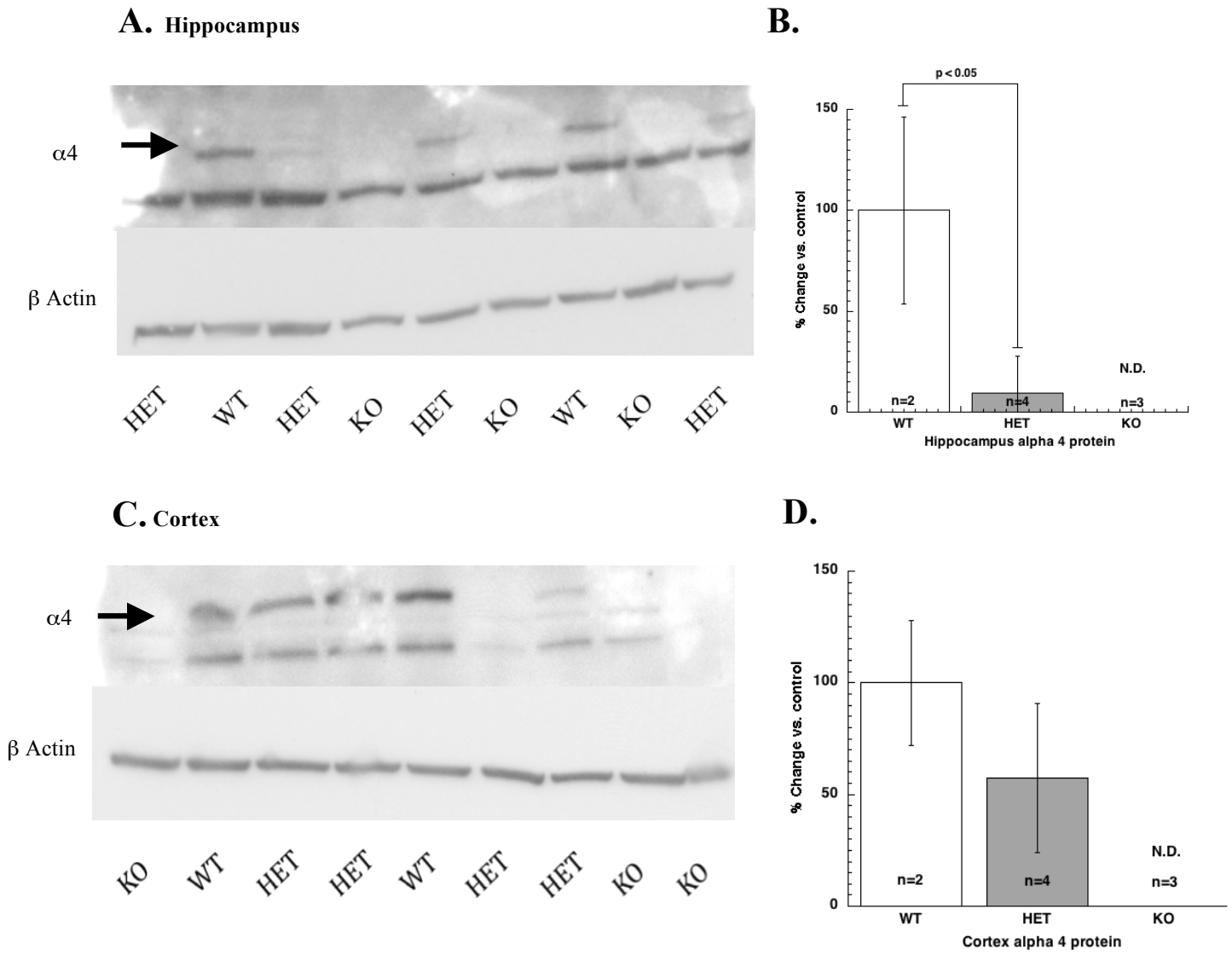

Figure 3.5 Western blot analysis of α4 protein from hippocampus and cortex of WT, HET and KO mice.

(A) Representative blot of hippocampal α4 protein from WT, HET and KO mice. Band intensities appear lower in HET lanes compared to WT. KO lanes do not show discernible levels of α4 protein.

(B) Summary data for levels of hippocampal α4 protein in WT, HET and KO. α4 levels in HETs were significantly reduced compared to WT ($p < 0.05$).

(C) Representative blot of cortex α4 protein from WT, HET and KO mice.

(D) Summary data for levels of cortical α4 protein in WT, HET and KO mice. α4 levels in HETs were not significantly reduced compared to WT.

Data are expressed as mean ± SEM of % change in band intensity relative to WT controls following normalization to β actin.

3.4 Discussion:

The goal of this series of experiments was to determine if α4-containing receptors were required for the intrinsic and the ethanol-antagonistic effect of RY023. Different parameters of RY023 effect were studied – motor ataxia by RY023 alone, reversal of ethanol-induced motor ataxia by RY023, reversal of ethanol-induced LORR and sedating effect of RY023 alone. Results reveal that α4-containing receptors may be involved in mediating the intrinsic effects of RY023 in motor ataxia and sedation but not in its antagonistic effect on ethanol-induced LORR.

3.4.1 Role of α4-containing receptors in intrinsic and ethanol antagonistic effects of RY023 on motor ataxia:

All tested doses of RY023 produced intrinsic impairment of motor performance on the rotarod. This is in contrast to the results reported by Cook *et al* [253]. Cook and collegues used an oscillating bar test to assess the ataxic effects of a 15mg/kg dose of RY023. This dose of RY023 did not alter the baseline performance of rats on the oscillating bar test. In contrast, all tested doses of RY023 (8mg/kg-30m/kg) produced intrinsic effects in mice on the rotarod assay in this study. The difference in response to RY023 between studies may have arisen due to a species-specific or behavior-specific effect. The level of complexity for motor ability on the rotarod assay is higher than that on the oscillating bar assay. Therefore, behavior-specific effects may be confound the response to RY023.

Surprisingly, the intrinsic effect of RY023 was the least in α4 HET mice compared to WT and KO mice. WT and KO mice, themselves, did not differ in the degree of impairment produced by RY023. This implied that a difference in amount of α4 protein

between HETs and WT may be responsible for a lowered response to the motor-impairing effects of RY023 in HETs. To determine this, membrane protein fractions from hippocampus and cortex of WT, HET and KO mice were compared. Lowered levels of α4 protein were observed in hippocampus of HET mice compared to WT. Reduction in GABA A receptor subunit mRNA in HETS of other GABA A receptor mutants is a common observation [17, 120, 388]. As expected, no α4 protein was observed in KO brain samples. While it is not known definitively how much of a role the hippocampus plays in regulation of motor activities, it is plausible that the reduction in α4 protein in this region could be mirrored in other brain circuitry involved in motor co-ordination. This may be responsible for the reduced response to RY023 on intrinsic motor ataxic effects.

Such differences in region-specific expression of proteins have been noted in other mutant mouse models as well. In an α1 KI mouse model, α1 protein expression in the cerebellum of homozygous KI was comparable to that in WT [153]. However, α1 protein expression in cortex of homozygous KI was reduced compared to WT [153].

Given the reduction of α4 in the HET mice and the reduced motor ataxic response to RY023, KO mice would be expected to have an even greater reduction in RY023-induced motor ataxia compared to HET mice. However, this was not the case. KO mice were similar to WT in their response to the motor ataxic effects of RY023. It is conceivable that reduction of α4 in HET fails to induce marked compensation and this allows for the role of α4 in RY023 response to be visible. In contrast, in KO mice, compensation is possibly much more extensive and this compensation masks the role of α4 in the actions of RY023.

Due to limited number of mice and nature of the study design, I was unable to compare directly the effects of ethanol-induced ataxia and the effects of RY023 antagonism

on ethanol induced ataxia between genotypes. However, ethanol-induced motor ataxia was assessed separately in HETs, WT and KO and found not to differ between the genotypes (data not shown). Antagonism of ethanol-induced motor ataxia by RY023 was tested at multiple doses of RY023 in HET mice (not shown). At no dose of RY023 was an antagonism of ethanol-induced motor ataxia observed on rotarod. In addition, combined RY023 and ethanol treatment produced hyperactive effects in mice as a result of which motor ataxic effects on rotarod could be assessed effectively.

Nevertheless, because intrinsic effects of 10mg/kg RY023 differed among the genotypes, the effect of 10mg/kg of RY023 on ethanol-induced motor ataxia in all three genotypes was assessed. In contrast to intrinsic effects, no differences between genotypes were observed on the effects of RY023 on ethanol-induced motor ataxia. These observations imply that RY023 was not capable of antagonizing the effects of ethanol effectively on motor coordination at the dose tested.

These data are in contrast to data reported by Cook *et al* [253] in rats. The said study involved the use of an oscillating beam test to assess ethanol-induced motor incoordination and its antagonism by RY023. Under those conditions, RY023 was successful in antagonizing the effect of ethanol. While the doses of RY023 used were 7.5mg/kg and 15mg/kg, a lower dose of ethanol (0.75-1.25 g/kg) was used in that assay. Although the dose of RY023 used in our experiments (10mg/kg) was in the same range as that used by Cook *et al* [253], an antagonizing effect of RY023 was not observed on ethanol-induced (2g/kg) motor ataxia tested by the rotarod assay. The higher dose of ethanol in my assay could affect multiple receptor combinations which may have varying affinities to RY023 and hence reversal of ethanol effects was not apparent. Interestingly, in the oscillating bar assay, a 15mg/kg dose of RY023 alone did not alter the time spent on the

oscillating bar, indicating that this dose was without intrinsic effect in rats. This suggests either a species difference in response or a behavior-specific modulation of the RY023 effect. Thus, the effects of RY023 may be both ethanol dose specific as well as behavior specific.

Results obtained from experiments on HET mice suggest that α4-containing receptors may be involved in the intrinsic motor-ataxic effects of RY023. Because ethanol-antagonistic effects of RY023 on motor ataxia could not be tested here, it is difficult to comment about the role of α4-containing receptors in this effect of RY023. The use of multiple behavioral assays for motor ataxia (oscillating bar test, rotarod, beam walking, pole walking etc.) coupled with different dose combinations of ethanol and RY023 are required to be able to dissect the role of α4-containing receptors in RY023 antagonism of ethanol-induced motor ataxia.

3.4.2 Role of α4-containing receptors in effects of RY023 on ethanol-induced LORR:

RY023 was effective in reversing the effect of ethanol-induced LORR in all three genotypes. This observation implies that α4-containing receptors are not required for this effect of RY023 and that the drug acts at non-α4 sites to reverse the effects of ethanol. Surprisingly, the greatest reduction of ethanol-induced LORR by RY023 was observed in HETS. At present, the reasons for this dramatic response in HETS are not understood. Regardless, it is safe to conclude that α4 subunit-containing receptors are not essential for the ethanol-antagonizing effect of RY023 on LORR.

This result is in contrast with that obtained with Ro15-4513. The antagonizing effect of Ro15-4513 on ethanol-induced LORR was absent in α4 KO indicating that α4-

containing receptors are required for the effects of Ro15-4513. Similar results were seen on the antagonizing effects of Ro15-4513 on motor ataxia. In 2006, Hanchar *et al* proposed that the RY series of drugs and Ro15-4513 act via a common site, namely α4δ- and α6δ-containing receptors to mediate their ethanol-antagonistic effects [262]. However, the results obtained here indicate that in contrast to Ro15-4513, RY023 does not require α4-containing receptors for its antagonistic effects on ethanol-induced LORR.

In the LORR assay, WT and KO mice did not differ in their responses to ethanol-induced LORR. This is consistent with the effects noted by Chandra *et al* [186]. Of course, this automatically begs the question of whether α4-containing receptors are even involved in LORR induced by ethanol. Since previous studies indicate that the KO did not differ from the WT in their response to high doses of ethanol [186], one might conclude that α4-containing receptors are not involved in ethanol effects. However, the changed cellular responses to ethanol indicate that the lack of α4-containing receptors results in functional reorganization such that synaptic currents are more sensitive to ethanol in the α4 KO. This is likely responsible for maintained ethanol responses in the α4 KO [340]. The absence of discernible behavioral differences between WT and KO may be attributed to extensive synaptic compensation by α1, α2 and γ subunit-containing receptors in the KO [349, 350]. It is also possible that only low dose behavioral effects of ethanol such as impariment of cognition may be affected by α4-containing receptors. Behavioral effects of sedation, motor ataxia and hypnosis refer to moderate to high levels of ethanol intoxication. Unfortunately, very few behavioral assays are available for the assessment of low dose effects of ethanol. The development of a novel behavioral assays, such as the test for ethanol-induced cognition impairment developed by Moore *et al* [383], will enable a more detailed study of low dose ethanol responses is required.

A significant baseline difference was noted in the SE group with the HETS having durations of LORR greater than that experienced by KO. This observation suggests that moderate reduction of α4 in HETS likely unmasks a more sensitive target of ethanol. Thus, it may be possible that α4-containing receptors regulate the effects of ethanol due to their increased sensitivity. Given the limited evidence of this in my studies, I acknowledge the need to better study HET mice with ethanol to confirm my hypothesis.

3.4.3 Role of α4-containing receptors in RY023-induced sedation:

Last, the sedating effects of RY023 alone were evaluated. A dose of 15mg/kg produced marked reduction in distance traveled over a period of 10 minutes in a novel environment. The sedative response to this dose of RY023 is consistent with that noted by Cook *et al*, [253]. In fact, other RY drugs tested on the open field assay were found to produce similar sedative effects [254, 256].

The sedative effects of RY023 were mainitained in WT and HET mice, but not in α4 KO mice. These results imply that RY023 requires α4-containing receptors for modulation of this behavioral effect in mice.

Due to limited numbers of subjects, it was not possible to include ethanol-treated mice and study the antagonizing effects of RY023 on ethanol-induced locomotor behavior. In rats, RY023 anatagonized the effects of ethanol on locomotor behavior [253].

Slight differences were observed in baseline behavior between genotypes during the study of the sedating effects of RY023. While KO and HET mice had comparable baseline behavior, they both had reduced locomotor activity compared to the WT. However, these differences were found to be only borderline significant. In previous experiments with saline and with 22.5% HBC, WT and KO were not found to differ ([186] and this study,

Fig. 2.7A). Because the same has not been noted in previous experiments with different vehicle controls, it is unlikely that this represents a major physiological difference between WT and KO mice.

3.4.4 Role of α4-containing receptors in RY023 effects:

Evidence gathered so far in relation to intrinsic effects and ethanol-antagonizing effects of RY023 indicate that these two effects are likely mediated by distinct receptor subtypes. My results demonstrate that:

1) α4-containing receptors may be involved in RY023's intrinsic motor ataxic effects, based on the reduced response seen in HET mice, although this warrants further study.
2) α4-containing receptors are not required for RY023's reversal of ethanol-induced LORR.
3) α4-containing receptors are a site of action for the intrinsic sedating effect of RY023.
4) Ethanol antagonistic actions of RY023 on LORR are different from those of Ro15-4513 on the same behavior – while RY023 does not require α4-containing receptors for its effects, Ro15-4513 does require α4-containing receptors for its effects.

It is interesting to note that while HETS differed from WT on certain behaviors (motor ataxia), they were not significantly different on other behaviors where the KO differed from WT (sedation). It is possible that compensation was succesful in recovery of certain behaviors but not of others in the KO (e.g., motor ataxia versus sedation). The behavioral differences between HET and KO suggest that α4-containing receptors may be important for motor behaviors, hence their complete absence triggers widespread compensation that rescues motor behavior in the KO whereas a moderate reduction in

HETs does not trigger the same compensatory response. In contrast, α4-containing receptors may play a lesser role in sedating behavior, hence a moderate reduction does not affect this behavior much but a complete lack of α4-containing receptors has some repercussions that cannot be compensated for.

These contrasting behaviors between HETS and KO resulting from ablation of a target likely hold some clues about the evolutionary roles of particular targets – why compensatory mechanisms exist for some behaviors but not for others. These questions bear further study and from an evolutionary standpoint, it would be interesting to investigate the differences between HET and KO at the gene transcription level.

3.4.5 Summary of α4-containing receptors as targets of ethanol antagonists:

In conclusion, results from this study indicate that α4-containing receptors may be involved in some intrinsic effects of RY023 (as studied on motor ataxia and sedation), but not in its ethanol antagonistic action (as studied on ethanol-induced LORR). This is in keeping with the global hypothesis that different subunit-containing receptors have critical roles in certain behaviors but not all. Hence, α4-containing receptors are involved in the locomotor behavior of RY023 and as noted earlier, of alphaxalone. Results from the above experiments also imply that RY023 is less specific for α4-containing receptors compared to Ro15-4513 for its ethanol antagonistic effects. Ro15-4513 produced a dramatic reversal of ethanol-induced ataxia and ~50% reduction in ethanol-induced LORR in WT but not in KO [341]. The ability of Ro15-4513 to bring about reduction in ethanol-induced effects in WT but not in KO also indicate that α4-containing receptors must be involved in ethanol-induced LORR effect and are specifically targeted by Ro15-4513 to bring about reversal. RY023, presumably, is not as specific for α4-containing receptors, but by virtue of its

potent actions on other receptors involved in this behavior, is able to reduce the effects of ethanol-induced LORR.

It is possible that as originally proposed by Liu *et al* [260], RY023 may be acting primarily through α5-containing receptors. However, given the diverse effects of RY023, it is plausible that other receptor subtypes also contribute to the effects of RY023. RY023 is an inverse agonist with low affinity at α1/2β2γ2 containing receptors [253]. It is possible that the inverse agonist activity at these receptor subtypes modulates some of its behaviors. The effects of RY023 at δ-containing receptors have not been characterized. The recently discovered α1δ-containing receptors in the hippocampus [320] that potentiate tonic curents in response to low concentrations of ethanol are another attractive target for RY023. Further pharmacologic and functional characterization of RY023 at different receptor subtypes is required to better understand its effects.

Finally, Ro15-4513 failed to reverse the ethanol-induced LORR effect in KO but RY023 reversed this effect across all three genotypes. These observations suggest that reversal of ethanol effects can be harnessed through diverse receptor combinations. Hence, further research into these novel ethanol-antagonistic compounds is required to understand the specificity of these compounds and behaviors affected by them.

In summary, while Ro15-4513 requires α4-containing receptors for its ethanol antagonistic effects on LORR and motor ataxia, RY023 does not require α4-containing receptors for its ethanol antagonistic effects on LORR, at least. However, RY023 does require α4-containing receptors for its intrinsic sedating effects and possibly motor ataxia.

Chapter 4: The role of α4-containing GABA A receptors in chronic ethanol withdrawal behaviors and in taurine-induced alleviation of the same.

4.1 Introduction:

Alcohol withdrawal syndrome (AWS) results from an imbalance between excitatory and inhibitory neurotransmission. After cessation from ethanol exposure, a predominantly excitatory system persists with lowered inhibitory neurotransmission and increased glutamatergic neuronal firing leading to AWS which is characterized by irritability, anxiety, insomnia, seizure susceptibility, etc. The effects of AWS are often severe and are thought to be a factor in determining relapse [135].

It is, therefore, proposed that drugs that reduce the negative aspects of withdrawal would be effective in preventing relapse. Among the current pharmacotherapies for alcohol dependence is acamprosate. Acamprosate, approved by the FDA in 2004, is effective in reducing relapse to alcohol. This, it does, in part, by acting on a hyperactive glutamatergic system. While the complete mechanism of action of acamprosate is not known, it is thought that acamprosate alleviates withdrawal by indirectly reducing excitatory glutamatergic neurotransmission (For review, see [389]). These indirect effects could be mediated through GABA transmission, antioxidant mechanisms or by modulating amino acids during withdrawal. Regardless, the prevention of a hyper-glutamatergic state appears to alleviate withdrawal symptoms and protects from relapse. In addition to these effects, acamprosate is thought to prevent changes in β endorphins [390, 391] and leptin [392], both of which are associated with dependence and craving mechanisms. Although the mechanism of

action of acamprosate is not clearly understood, it is speculated that at least some of its actions are mediated mainly by glutamatergic systems at low doses and by GABAergic systems at high doses [393].

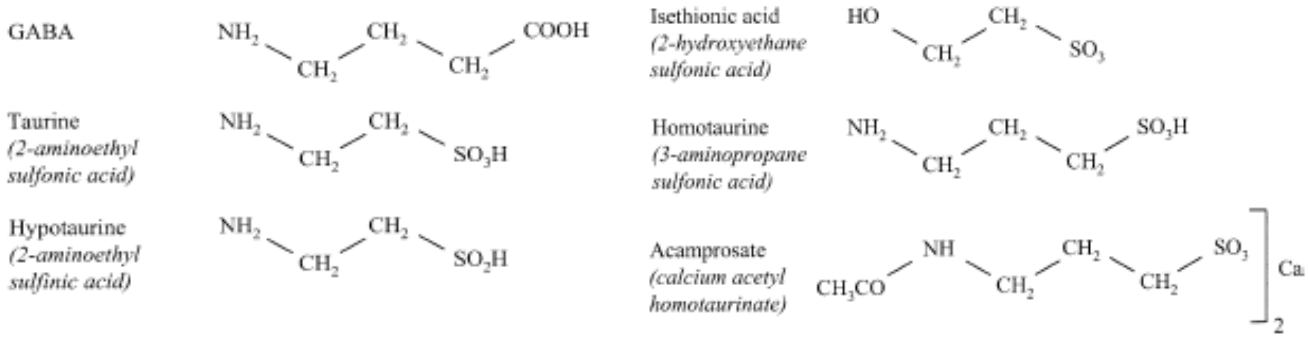

Figure 4.1 Chemical Structures of GABA, Acamprosate, Taurine and associated entities

Chemical structures of GABA, taurine, hypotaurine (precursor), isethionic acid (metabolite), homotaurine and the anti-addiction drug acamprosate. Acamprosate occurs as an acetylated form of homotaurine and is dimerized as a calcium salt, adapted from Olive, M.F. 2002. Interactions between taurine and ethanol in the central nervous system. Amino Acids, 23(4): p. 345-57

Acamprosate exists as a calcium salt of the taurine derivative- homotaurine (Fig. 4.1, adapted from [360]). Interestingly, taurine itself has been implicated in antagonism of acute and chronic alcohol effects. Taurine is a sulphated amino acid that is synthesized from cysteine. It is thought to be involved in mutiple processes such as neuroprotection, antioxidant, calcium homeostasis, immune-modulation, chloride current potentiation and inhibition of excitatory glutamatergic neurotransmission [208]. Taurine bears some structural similarity to both GABA and glycine and acts as a chloride-mimetic agent. Thus, it potentiates inhibitory neurotransmission.

In a study on chronic ethanol effects, Zalud *et al*, showed that taurine treatment reduced the incidence of handling induced convulsions (HIC) during chronic ethanol withdrawal in C3H/HeJ mice [233]. In the untreated group, significant seizure incidences

were noted in addition to a reduction of taurine in several areas of the brain. Thus, a role for taurine in alleviation of ethanol withdrawal effects was proposed. A taurine-depletion study in adolescent mice also showed enhanced corticosterone levels post-chronic ethanol exposure in the taurine-depleted group but not the control group [394]. This indicated a link between increased stress and low levels of taurine following chronic ethanol treatment. In another study, a 7 day pre-treatment of taurine increased the open arm scores for ethanol-withdrawn mice [395]. Thus, taurine alleviated ethanol withdrawal-induced anxiety. In conclusion, multiple studies have pointed out a link for taurine in alleviating biochemical and behavioral effects of chronic ethanol.

However, the mechanism of action of taurine behind these effects is not known. It was previously thought that taurine acted more potently at glycine receptors (200uM-1mM) than at GABA receptors (1-10mM) [204, 213, 215, 218, 396-398]. However, recent work by Jia and collegues elicited that taurine showed greater sensitivity (10-100uM) for extrasynaptic GABA receptors than for glycine or synaptic GABA receptors [234]. This study also showed that taurine-induced current potentiation was due to a direct effect on GABA A receptors and not by indirect increases of ambient GABA. High levels of taurine are present in dentate gyrus, cerebellum and thalamus [203] and these regions also express high levels of α4/α6δ -containing receptors [337, 399]. Interestingly, taurine-induced GABAergic tonic current potentiation was ablated in the ventrobasal thalamic neurons from α4 KO mice confirming the idea that taurine acted on α4-containing receptors to produce chloride current modulation [234]. These findings implied that cellular responses of taurine could be preferentially mediated through α4-containing receptors.

α4-containing receptors undergo remarkable plasticity during ethanol withdrawal. During chronic ethanol withdrawal, increases in the levels of α4 and γ2 subunits are

consistently observed [178, 330]. Insertion of 'new' α4 containing receptors into synaptic locations along with γ2 takes place [331]. In addition, there is decrease of synaptic α1 and decrease of extrasynaptic δ subunits [178]. These changes lead to altered receptor kinetics and sensitivity to ethanol, neurosteroids and classical benzodiazepines [181, 325]. These changes are thought to underlie the changed behavioral responses to ethanol and to classical benzodiaepines [400]. The sensitivity of α4-containing receptors to gaboxadol and Ro15-4513 during chronic ethanol withdrawal [178, 181, 325] suggest that drugs acting on α4-containing receptors retain their activity during this period. Because α4-containing receptors are increased during ethanol withdrawal, it is possible that a drug such as taurine, that is potent at α4-containing receptors, may continue to exert its effects through these receptors.

Because conventional sleep-aids and anticonvulsants have limited success in treatment of withdrawal symptoms, understanding the biochemical changes underlying chronic ethanol withdrawal and identifying drugs that are more effective at the changed receptor compositions may be the route to better treatment and management of chronic ethanol withdrawal and dependence. The availability of the α4 KO mouse and the fact that these mice develop chronic ethanol withdrawal parameters comparable to their WT counterparts (unpublished data) makes this an attractive project, which if succesful, could warrant detailed study and development of similar synthetic compounds.

Because of the relevance of α4-containing receptors during chronic ethanol withdrawal, I hypothesized that α4-containing receptors are required for the effects of chronic ethanol withdrawal. I proposed to expand on previous studies and also examine the effects of taurine on three different withdrawal-related behaviors such as seizure susceptibility (HIC scoring), locomotor behavior, and protracted tolerance to ethanol.

Given the sensitivity of α4-containing receptors to taurine and the fact that taurine improves chronic ethanol withdrawal HICs, I hypothesized that the increased α4-containing receptors during chronic ethanol withdrawal mediate this protective effect of taurine. I predicted that taurine would protect against three withdrawal-related behaviors in WT mice but not in α4 KO mice.

4.2 Materials and Methods

4.2.1 Chronic ethanol withdrawal induction:

WT, HET and KO mice of both sexes were chronically exposed to ethanol by a vapor inhalation method adapted from that described by Dr.Goldstein [401, 402]. Ethanol vapors were conveyed to two inhalation chambers (60 X 82 X 39 cm, Plexiglas, Polycast Technology Corp., Stamford, CT). Each chamber was capable of housing 5 home cages of mice. Each chamber was fitted with an inlet (for ethanol vapor) and an outlet (for venting) port as well as a fan at the top right corner for circulation of vapors. Ethanol (Pharmco, Brookefield, CT) was conveyed at a measured rate (120-140ul/min) by means of a peristaltic pump (Cole Parmer, Model 75 23-20) into heated flasks, maintained at 55°C. Air was passed into each flask at a flow rate of 8L/min. Ethanol vapors were then carried into each chamber by the flowing air. Vapors were vented by means of outlet tubes into a fume hood. Chamber ethanol concentrations were not determined.

Ethanol treatment followed the multiple withdrawal model of Becker and Hale [403]. On day 1, at 3 PM, mice were injected with pyrazole (alcohol dehydrogenase inhibitor) at a dose of 68mg/kg (0.01 ml/g body weight) followed by a priming dose of ethanol (1.5g/kg, 0.01 ml/g body weight). After injections, mice were placed in the inhalation chambers in

their home cages for 16 hours. On day 2 at 7AM, home cages were removed from the chambers and placed on racks. Body temperatures were measured at the start of each abstinence period. A heat lamp was placed in the room to maintain temperature and each cage was provided with transgel for easy access to fluids. Four hours into each withdrawal period, at 11AM, mice were weighed and injected with taurine (500mg/kg, Sigma Aldrich, St.Louis, MO) or saline (0.01 ml/g body weight). This cycle was followed for 3 days. On day 4, mice were removed from inhalation chambers at 7AM and 50µl of blood was collected via the retro-orbital route for measurement of blood ethanol concentrations (BEC). Only HET mice from taurine (T) and saline (S) treatment groups from both chambers were bled. Ethanol-treated mice were excluded from behavioral analyses if BEC was <50 or >300 mg/dl. Mice that had temperatures lower than 32°C, or lost more than 10% of their weight in one cycle or were visibly moribund/comatose were excluded from the experiment.

Previously, α4KO and WT were compared for differences in HIC following chronic ethanol exposure (unpublished results). In that experiment, WT and KO mice that received air exposure (controls) did not differ in their behavior, i.e., baseline HIC was comparable. Chronic ethanol exposure produced comparable HIC in both WT and KO. In the chronic ethanol-taurine experiments, due to availability of limited numbers of subjects, and prior experience in inducing chronic ethanol withdrawal in this line of mice, Air-taurine and Air-saline controls were omitted. For each of the behavioral experiments, data were analyzed by two-way ANOVA with genotype and treatment as main effects. Although, mice of both sexes were used towards this assay, due to limited numbers, data were not analyzed by sex.

4.2.2 Blood ethanol concentration (BEC) determination:

BEC were determined by means of a spectrophotometric process described elsewhere

[404]. HET mice (n≥3 per treatment group per replicate of assay) from each cohort were bled at the start of the final withdrawal period. Approximately 50μl of blood was collected by heparinized capillary tubes via the retro-orbital route at the end of the third cycle of chronic ethanol exposure. Fifteen μl of blood was added to 1ml of ice-cold 0.55M perchloric acid. Samples were thoroughly mixed and centrifuged at 1500 X g for 10 min 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. BECs were calculated using a set of ethanol standards. BECs were averaged for each treatment group and means compared by unpaired Student's t-test.

4.2.3 Ethanol withdrawal-induced hyperexcitability (HIC scoring):

Beginning the second hour following termination of the third and final ethanol treatment cycle, mice were scored for handling induced convulsions (HIC) at hourly intervals for nine hours, (9AM-5PM) of the withdrawal period using the scale of Becker and Hale [403]. Scoring was conducted by two observers who were unaware of the genotype or treatment of the mice being tested. Briefly, mice were scored 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, that 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, that may result in death. Responses from both observers were averaged to arrive at the final scores. Mean hourly HIC scores were used to generate HIC curves for each genotype-treatment combination, e.g., WT-S; WT-T; KO-S; KO-T; HET-S; HET-T. Area under curve (AUC) was calculated as the sum of area between baseline and scores over time

(Hours 2-24). AUC's for each treatment group were compared by two-way ANOVA.

4.2.4 Ethanol withdrawal-induced locomotor behavior:

Twenty four hours into the final withdrawal period, mice were scored for HICs at the 24th hour time point. Following the scoring, mice were sequentially introduced into a novel environment to record exploratory locomotor behavior during withdrawal. Mice were placed in the center of a plexiglass walled arena, (43.2 cm X 43.2 cm X 30.5 cm) for automated recording of locomotor behavior. Two such chambers were used so that two subjects could be tested at a given time by placing within each chamber. The chambers were placed within sound-attenuating cubicles (Med Associates, St.Albans, VT) that were provided with a red light and a fan (top right corner). Distance traveled (cm) over a 5 minute period was recorded by an automated program (Med Associates, St.Albans, VT). Between mice, the chambers were cleaned with 70% ethanol followed by water to remove any evidence of smells and excrements left behind. Total distance traveled in the 5 min period as well as percent distance traveled in center zone was calculated. The center zone was demarcated as the central portion of the open field (11.25 X 11.25cm). Center zone activity was expressed as percent distance covered in center zone. Data were analyzed by two-way ANOVA. Mice that had 'total distance' scores of 0 were excluded from the analysis.

4.2.5 Protracted tolerance to ethanol:

Following measurement of locomotor activity, each mouse was injected with 3.5g/kg of ethanol (0.02mls/g body weight) to assess tolerance to ethanol-induced loss of righting reflex (LORR). This testing took place between hours 24-26 of the final withdrawal period. For the duration of LORR, mice were placed on V-shaped troughs and under a heat lamp in order to maintain normothermia. Righting reflex was regained when mice were able to

right themselves consecutively three times within 30 sec. Duration of LORR for each genotype and treatment combination were analyzed by two-way ANOVA and Fisher's post hoc tests.

4.3 Results

4.3.1 Effect of taurine on blood-ethanol concentrations:

At the start of the third withdrawal period, blood was collected from a subset of chronic ethanol-treated mice with and without taurine treatment. From pilot experiments, it was known that ethanol blood levels do not vary between genotypes. Due to the stress associated with the experiment, only HET mice were bled from each chamber per treatment group (S and T) as representative of the cohort of mice on the day of the experiment. Mean BECs for saline and taurine treated groups were **228.36±18.66 mg/dl** and **244.8±13.4mg/dl**, respectively. No differences were seen in the blood ethanol levels in saline and taurine treatment groups.

4.3.2 Effect of chronic ethanol treatment on body weight:

Analysis of body weight in mice (from day 1 to day 4) undergoing chronic ethanol treatment by two-way ANOVA showed no effect of treatment but a strong effect of genotype [$F(2,110) = 9$; $p < 0.001$]. No interaction between genotype and treatment was observed. Pair-wise comparisons revealed that percent weight loss in saline-treated KO mice was significantly lower than HET mice ($p<0.05$). Significant differences between genotypes on taurine-treated mice was also observed. Percent weight loss in KO was significantly reduced compared to WT ($p < 0.05$) and HET ($p < 0.001$) mice. A treatment

effect within each genotype was not observed. The percent weight loss for all three genotypes was below 10%. As mentioned earlier, mice that suffered >10% weight loss through the ethanol exposure period were not included in the withdrawal tests.

Table 4.1 Percent change in body weight in WT, HET and KO mice undergoing chronic ethanol exposure with saline and taurine treatment (* $p < 0.05$, comparing with saline-treated HET; ** $p < 0.05$, comparing with taurine-treated WT; # $p < 0.001$, comparing with taurine-treated HET).

Genotype	Saline (%)	Taurine (%)
WT	-8.3 ± 0.7	-8.1 ± 0.8
(n)	(16)	(19)
HET	-9.6 ± 0.6	-9.3 ± 0.5
(n)	(21)	(20)
KO	-6.8 ± 0.9*	-6.2 ± 0.7 ** #
(n)	(17)	(23)

4.3.3 Effect of taurine on withdrawal-induced hyperexcitability (HIC scoring) :

Handling-induced convulsions following chronic ethanol withdrawal were used to assess two parameters – the role of the α4-containing receptors in ethanol withdrawal-induced HICs and the role of α4-containing receptors in effects of taurine on HIC. The time-course of ethanol withdrawal hyperexcitability in saline- and taurine-treated mice is depicted in Fig. 4.2A and 4.2B, respectively. The time-course of HIC scoring shows that withdrawal-induced HICs increased reliably over a 10 hour period with a decrease at the 24 hour time point. This pattern of severity is consistent with withdrawal-induced HIC severity in other strains of mice [187, 191]. An overall two-way ANOVA was performed on AUCs (Table 4.2) of saline-treated and taurine-treated genotypes. Main effects of treatment, genotype or an interaction between the two were absent. Thus, HIC scores in α4 KO mice did not differ from WT and HETs. Therefore, the lack of α4 containing receptors did not affect

withdrawal-induced HIC. The lack of difference between treatment groups indicates that taurine treatment did not improve withdrawal-related HICs in any genotype.

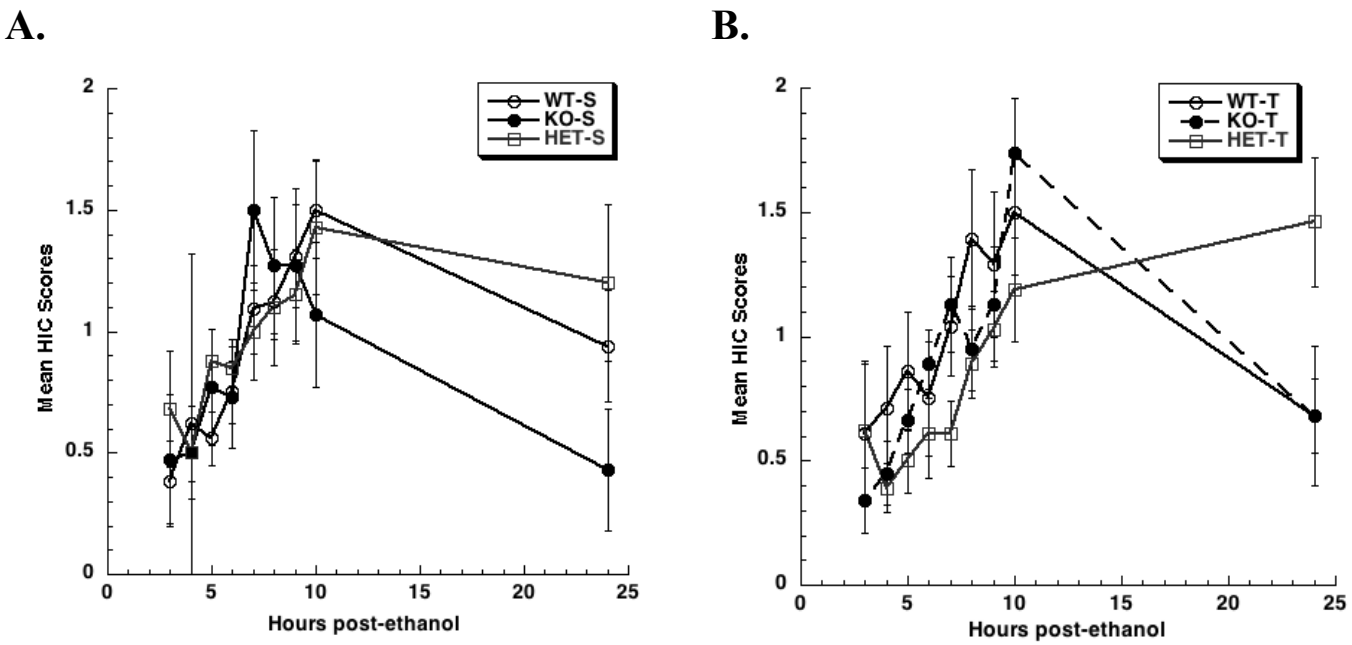

Figure 4.2 Time course of Handling induced convulsions:

(A) Saline-treated and (B) Taurine-treated WT, HET and KO mice. No differences were observed between the genotypes within either treatment group. A between-treatment analysis also did not reveal any differences between genotypes. (n=12-18 per genotype-treatment combination). Each time point represents mean score ± SEM.

Table 4.2 Mean AUC's for saline and taurine-treated WT, HET and KO mice.

Mean AUC's	Saline	Taurine
WT	25 ± 2	23 ± 4
(n)	(16)	(14)
HET	25 ± 4	27 ± 3
(n)	(15)	(12)
KO	18 ± 4	24 ± 3
(n)	(15)	(18)

3.3.4 Effect of taurine on withdrawal-induced locomotor and anxiety-like behavior:

Locomotion and anxiety-like behavior in WT, HET and KO mice during ethanol-withdrawal and the effect of taurine on this parameter was determined using the open field activity assay. It was expected that mice would have reduced locomotor activity during withdrawal and further that center zone locomotion compared to the total would be reduced as an indicator of anxiety-like behavior. Several groups have noticed similar reductions during ethanol withdrawal (See review [405]). Taurine treatment was expected to alleviate ethanol withdrawal – induced anxiety-like behavior.

Following measurement of 24th hour HIC scores, mice were subsequently subjected to measurement of locomotor behavior by an automated activity monitor. An overall two-way ANOVA on total distance covered over a 5 minute period revealed no effect of treatment, genotype or interaction. Thus, WT, HET and KO mice did not differ in the total distance covered with saline or taurine treatments (Fig. 4.3.A). This indicates that the lack of α4-containing receptors does not affect locomotor behavior during withdrawal. In addition, taurine-treatment did not produce any changes in locomotor behavior. Although air-exposed control mice were not tested in this assay, previous locomotor behaviors recorded on treatment-naive mice over a 5 minute period [186], were used to determine if chronic ethanol treatment affected locomotor behavior. As expected, a decrease in distance covered was noted with chronic ethanol treatment.

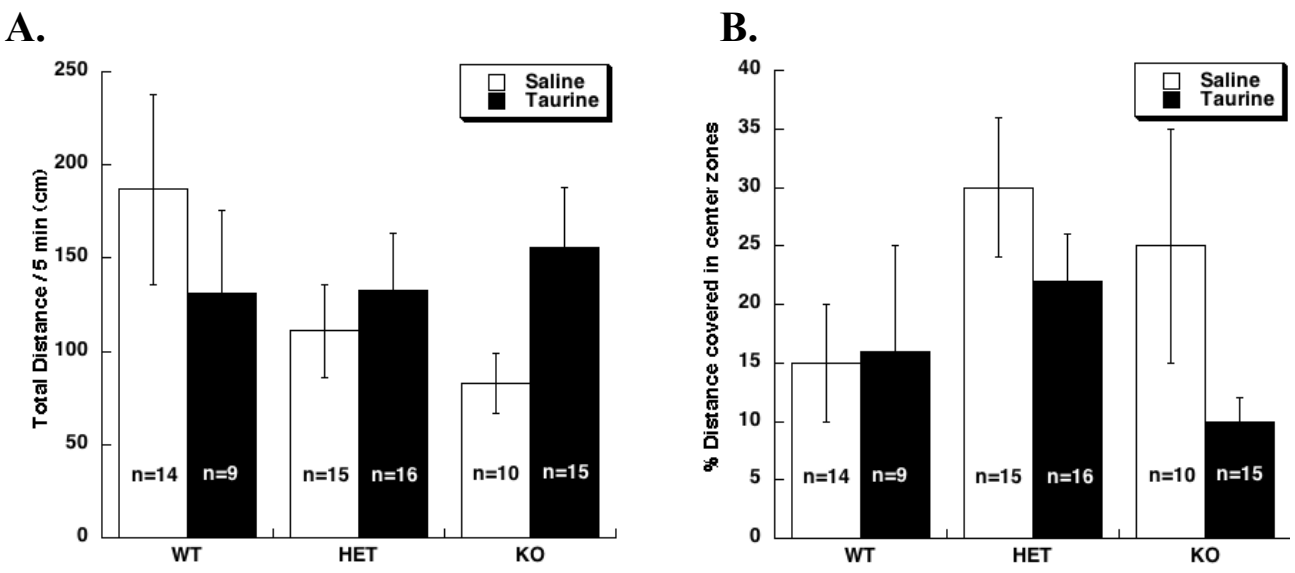

Figure 4.3 Withdrawal-induced locomotor and anxiety-like behavior

(A) Total distance covered did not differ due to treatment or genotype.
(B) Percent distance covered in center zone also did not differ by treatment or genotype.
Data are expressed as mean ± SEM.

Next, percent distance covered in the center zone was compared between the three genotypes with saline or taurine treatment (Fig. 4.3.B). An overall two-way ANOVA of the percent distance covered in center zones did not reveal a significant effect of treatment, genotype or interaction. The percent distance covered in center zones in saline- treated ethanol withdrawn mice was not reduced compared to that experienced by treatement-naive mice (recorded previously). This suggested that saline-treated ethanol withdrawn mice likely do not experience an anxiety-like effect during withdrawal. The lack of a difference by treatment also suggested that taurine treatment did not produce any changes to anxiety-like behavior during withdrawal. Hence, the contribution of α4-containing receptors to anxiety-like behavior during withdrawal could not be measured.

4.3.5 Effect of taurine on protracted tolerance to ethanol:

Following testing of withdrawal-induced locomotion, between 24 to 26 hours post ethanol exposure, mice were assessed for tolerance to ethanol-induced LORR. An overall two-way ANOVA revealed no effect of treatment, however a near-significant main effect of genotype was observed [$F(2,95) = 3$, $p = 0.078$]. An interaction between genotype and treatment was not observed. Fisher's post hoc tests revealed no difference between the genotypes in the saline treated (S) groups indicating that genotypes did not differ in their response to ethanol-induced LORR. However, a significant difference between KO and WT ($p< 0.05$) and HET and KO ($p< 0.005$) was observed in the taurine-treated (T) groups (Fig. 4.4). Taurine-treated KO mice had reduced sleep times compared to WT and HET mice, suggesting that KO mice responded to taurine.

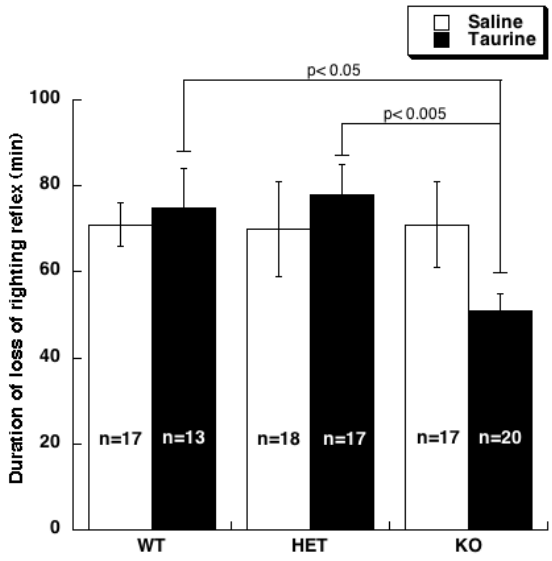

Figure 4.4 Protracted tolerance to ethanol with saline- and taurine-treated WT, HET and KO mice.

Protracted tolerance to ethanol-induced LORR was not different between the genotypes on saline-treated mice. However, tolerance to ethanol-induced LORR was different between the taurine-treated genotypes. Taurine-treated KO mice had significantly reduced sleep times compared to WT and HET mice. Taurine-treated WT and HET mice were not different. Data are expressed as mean ± SEM.

3.4 Discussion:

The purpose of these experiments was to determine if α4-containing GABA A receptors were essential for the withdrawal-alleviating effect of taurine and for ethanol withdrawal in general. According to the withdrawal model proposed by Liang *et al* [358], during chronic ethanol withdrawal, α4 levels increase and α4 specific agonists have enhanced effect during this time. Because α4 undergoes changes in expression and localization, I hypothesized that α4-containing receptors are critical for ethanol withdrawal behavior. In addition, since taurine is hypothesized to act through the α4-containing GABA A receptors [234] and has a protective effect during withdrawal [233], I proposed that lack of α4-containing receptors would nullify the effect of taurine during withdrawal. Several parameters of withdrawal effect such as HIC, locomotor behavior and tolerance to ethanol were evaluated in α4 WT, HET and KO mice. I also explored the effect of taurine on these withdrawal behaviors. In contrast to my expectations, α4 WT, HET and KO mice did not differ in the ethanol withdrawal behaviors assessed here. Further, a protective effect of taurine on chronic ethanol withdrawal was not observed on most ethanol withdrawal effects.

3.4.1 Role of α4 in chronic ethanol withdrawal-induced HIC and effect of taurine:

HIC scores between saline (S) and taurine (T) groups were compared from hours 2-10 and at 24 hours of the final withdrawal period. The intensity of withdrawal-induced HICs followed a time course similar to that observed in other strains of mice [187, 191]. Following saline treatment, withdrawal-induced HICs did not differ between WT, HET and KO mice. The lack of difference on HICs between genotypes is consistent with previous

observations on this line of mice and suggests that lack of α4-containing receptors does not affect this withdrawal phenotype.

Reduction in levels of α4δ-containing receptors has in been linked to catamenial epilepsy. Several studies have shown a role for α4-containing receptors in seizure activity associated with fluctuating neurosteroid levels [288, 406, 407]. The reduction in δ subunits and the subsequent pairing of α4 subunits with γ2 subunits during ethanol withdrawal also results in overall reduced charge transfer during potentiation of tonic current [178, 179, 181]. This is thought to result in the overall reduction of inhibitory neurotransmission and predominance of excitatory neurotransmission, leading to seizures. From these results, it appears likely that α4-containing receptors must be involved in some manner in mediating withdrawal-induced seizures. Therefore, it is quite surprising that deletion of these receptors did not affect HIC. It is possible that residual δ containing receptors dominate this behavior during withdrawal. It is also possible that compensatory responses in the global KO model result in unchanged HIC in the KO.

Surprisingly, a protective effect of taurine on HICs was not observed in any of the genotypes. Given the results presented by the Diaz-Granados' group [233], this was surprising. However, it is possible that differences in our results arise from the different background of mice used in our experiments. In our study, a mixed background of C57BL/6J and Strain129S1/X1 mice was used, whereas the Diaz-Granados group used C3H/HeJ mice. Previous studies by independent groups have revealed that the C3H/HeJ strain is particularly susceptible to handling-induced seizures. It has been shown previously that C57BL/6J are recalcitrant to seizures whereas Strain 129S1/X1 are susceptible to seizures [187]. Thus, our mixed background is likely only moderately susceptible to

seizures. It is possible that a greater effect of taurine may be apparent only on severe seizures.

An alternate explanation could be inherent differences in our methods of withdrawal induction. Difference in behavioral parameters owing to the method of induction of chronic ethanol withdrawal is a problem that has been chronicled in the literature (for review see [405]). Dr.Diaz-Granados' group utilized the chronic continuous ethanol vapor inhalation paradigm whereas our treatment consisted of a chronic intermittent ethanol vapor inhalation paradigm. Differences in the intensity of withdrawal could have resulted due to this. The mean BECs in this study (~230mg/dl) were greater than that observed by Dr.Diaz-Granados' group (~150mg/dl). The higher levels of blood ethanol concentrations in our study may translate to more severe withdrawal. This could also have precluded an effect of taurine at the dose used.

Differences in sensitivity to taurine may also contribute to the lack of effects seen in this study. One group reported that acute taurine treatment produced different effects in two different lines of mice [408], namely C57BL/6J and DBA/2J mice implying a possible difference in strain responses to taurine. Some groups have also noted differences in endogenous levels of taurine in certain strains of mice [409]. Thus, apart from differences in susceptibility to convulsions, the lack of an effect of taurine may also be linked to the endogenous levels of taurine and lowered basal responses in different lines of mice. As a result of such differences, an effect of taurine may be more closely linked to the dose of taurine used. The dose of taurine in the assays, here, was decided following personal communication with Dr.Diaz-Granados. Because the methods of induction of ethanol withdrawal differed between the two groups, it is possible that a 500mg/kg dose of taurine may have been insufficient to counter the withdrawal effects seen in our experiments. If

this is indeed the case, further studies with different levels of intoxication and different doses of taurine should be conducted. This will help determine if taurine alleviates withdrawal in a dose-dependent manner.

3.4.2 Role of α4-containing receptors in locomotion and anxiety induced by chronic ethanol withdrawal and effects of taurine on the same:

Locomotor behavior and anxiety-like behavior in mice that were exposed to chronic ethanol vapors with and without taurine administration were assessed at ~24 hours into withdrawal. Initially, pilot assays using the elevated plus maze experiment to study withdrawal-induced anxiety-like behavior were performed. However, anxiety-like behavior could not be measured on this assay due to highly reduced locomotor behavior on the plus maze. Hence, percent distance covered in center zone of an open field was used as a surrogate of anxiety-like behavior instead. Comparison with naïve mice previously subjected to the open field assay indicated that naïve mice have greater ambulation compared to ethanol-withdrawn mice in this assay (not shown). All genotypes of mice undergoing chronic ethanol exposure, thus, had reduced locomotion.

Once again, saline-treated α4 KO and HET mice did not show differences in ambulatory behavior compared to WT mice. This indicates that lack of α4-containing receptor does not affect locomotor behavior during withdrawal. Next, percent distance covered in center zones was assessed. This measure is often used as a surrogate of anxiety-like behavior in rodents. However, comparison with previously tested naïve mice indicated that ethanol-withdrawn mice did not differ in the percent distance covered in center zones. Hence, it is not certain if ethanol-withdrawal produced anxiety in these mice. Regardless, no differences were seen among saline-treated WT, HET and KO mice.

Hence, I conclude that based on the evidence here, α4-containing receptors are not involved in this withdrawal effect.

A reduction in inhibitory neurotransmission has been linked to anxiety during withdrawal. Similar reductions in inhibitory neurotransmission are observed in disorders associated with hormonal imbalance. α4δ-containing receptors are hypothesized to play a major role in the behavioral effects resulting from fluctuations in endogenous neurosteroid levels [291, 306, 315, 329, 410, 411]. In fact, the incidence of anxiety, irritability (associated with premenstrual syndrome) and postpartum depression has been linked reduced levels of δ-containing receptors [286, 288, 311]. Chronic ethanol withdrawal leads to changes in levels of neurosteroids [412]. The reduced δ subunits, changes in levels of neurosteroids coupled with the increase in α4-containing receptors during ethanol withdrawal may contribute to increased anxiety and associated locomotor behavior.

Based on the results obtained in the locomotor behavior assay, it appears that α4-containing receptors are not required for reduced locomotor behavior observed during ethanol withdrawal. Whether α4-containing receptors are important for anxiogenic effects could not be tested here due to the lack of anxiety like behavior in ethanol-withdrawn mice. Hence, an assay that is sensitive to anxiogenic effects of withdrawal is required to assess the involvement of α4-containing receptors in this effect.

No differences were observed on either measure (total distance or percent distance in center zone) between saline- or taurine-treated WT, HET and KO mice. This result suggests that taurine treatment did not improve ethanol withdrawal-induced locomotor suppression or anxiety-like behavior. This is in contrast to the results reported by Helfand *et al*, although their model differed from that used in the current study. Helfand *et al*

showed that pre-treatment with taurine (50mg/kg) 7 days prior to continuous chronic ethanol withdrawal induction, alleviated withdrawal-induced anxiety-like behavior on the elevated plus maze assay [395].

Interestingly, another study by the same group where taurine was depleted by pre-treatment with guanidinoethane sulfonate (a taurine uptake antagonist) for 7 days prior to chronic ethanol withdrawal induction did not elicit dramatic behavioral changes. Depletion of taurine did not significantly alter the withdrawal-induced anxiety on elevated plus maze although plasma corticosterone levels were increased [394]. Thus, although biochemical changes indicative of increased stress were observed, no differences in behavior were observed in mice due to taurine depletion in the latter study. This suggests that the nature of interaction of taurine with ethanol effects may be weak. While biochemical changes due to taurine administration may be observed, behavioral effects of taurine are likely subtle. The lack of differences in behavior in the mice used in this study could result from a similar situation, although this needs to be confirmed. Biochemical characterization of levels of taurine and stress indicators such as plasma corticosterone in ethanol vapor-treated mice would help shed some light on this possibility.

3.4.3 Role of α4-containing receptors on protracted tolerance to ethanol in chronic ethanol withdrawn mice and effect of taurine on the same:

Lastly, I wanted to determine if lack of α4-containing receptors affected protracted tolerance to ethanol. In addition, I wanted to determine if taurine treatment is able to alleviate the effects of tolerance to ethanol and if these effects depend on α4-containing receptors. Mice were exposed to a single intoxicating injection of 3.5g/kg of ethanol between 24 - 26 hours of chronic intermittent ethanol withdrawal. The resulting duration of

LORR was recorded. Previous experiments with α6 WT and KO had revealed that tolerance to ethanol was reliably observed at 26 hours post-ethanol vapor cessation [187]. Hence, the same timeline was followed in the current study for assessment of tolerance to ethanol.

Ethanol-withdrawn WT, HET and KO mice did not differ in their responses to the acute ethanol suggesting that α4-containing receptors are not required for this effect. Given the dynamic changes that the α4 subunit undergoes during ethanol and the vast amount of evidence linking it to tolerant behavior, it is surprising that no difference in sensitivity to ethanol was observed in ethanol-withdrawn α4KO mice. This result is similar to the lack of difference in response to acute ethanol-induced behaviors [186]. This result is surprising given the changes in α4 expression during ethanol withdrawal [178, 181, 330, 413]. An increase in α4γ2-containing receptors and a decrease in α4δ-containing receptors is observed during ethanol withdrawal [178, 179]. The reduction in ethanol-sensitive α4δ-containing receptors and the subsequent pairing of α4 subunits with synaptic γ2 subunit [178, 179, 181] could contribute to tolerance to ethanol observed during withdrawal. Because α4-containing receptors are deleted in the KO mice, I hypothesized that tolerant behavior during ethanol withdrawal would be changed. The lack of difference between saline-treated WT, HET and KO mice suggests that α4-containing receptors are not absolutely required for the changed sensitivity to ethanol during withdrawal.

However, it is possible that compensation in the α4 KO mice may have masked a role for α4-containing receptors in ethanol responses. The unchanged responses to acute ethanol in the α4 KO mice are hypothesized to result from compensation in the global KO [186]. These compensatory changes may minimize a role for α4-containing receptors during ethanol withdrawal.

Administration of taurine did not significantly affect durations of LORR compared to the saline-treated group (although a genotypic effect in the taurine treated group was apparent, discussed below). The lack of an effect of taurine in the WT and HETs suggests that taurine may not be effective in reducing tolerance to ethanol in chronic ethanol-withdrawn mice.

A significant difference between genotypes on the taurine-treated groups was observed. Taurine-treated KO had reduced LORR compared to taurine-treated WT and HET mice. The physiological significance of this is not clear immediately. It is not known how taurine may act to modulate tolerant responses to ethanol in chronic ethanol-withdrawn mice. In acute ethanol experiments, taurine is known to antagonize the effects of ethanol [196, 219, 221, 414]. Hence, the reduced response to ethanol in taurine-treated KO may be the result of such antagonism. However, the lack of a reduced response to ethanol in taurine-treated WT and HETs suggests a changed response response to taurine unique to the KO. Apart from extrasynaptic α4-containing GABA A receptors, glycine receptors are also known to be sensitive to taurine, even more so than synaptic GABA A receptors [215, 396, 398, 415]. It is possible that compensatory responses in the KO may include changes in the glycinergic system, which in turn may be responsible for the response to taurine. However, at present, evidence supporting this hypothesis is lacking and a gene array study of the α4 KO may provide some much needed answers toward this possibility.

4.4.4 Summary of role of α4-containing receptors in chronic ethanol withdrawal and actions of taurine:

The above experiments were designed to assess the involvement of α4-containing GABA receptors in ethanol withdrawal behavior and in the withdrawal-alleviating effects

of taurine. Although the α4 subunit undergoes increases in expression, relocalization and displays sensitivity to α4-specific ligands during ethanol withdrawal [178, 180, 181], it is unclear if changes in α4-containing receptors are the cause or the effect of chronic ethanol withdrawal. By understanding if ethanol withdrawal behaviors are differently modulated in the global α4 KO mice, I sought to resolve the conundrum of involvement of α4-containing receptors in ethanol withdrawal.

Withdrawal behaviors in saline-treated WT, HET and KO mice were comparable on all three endpoints of HIC, locomotor and anxiety-like behavior, and tolerance to ethanol. This indicates that α4-containing receptors are not absolutely required for the chronic ethanol withdrawal effects assessed here. In spite of changes in expression and altered kinetics, α4-containing receptors may not be a critical mediator of these ethanol withdrawal effects. It must be noted that the changes observed in α4 subunit expression and relocalization have been studied mainly in the hippocampus [178, 180, 181]. At present it is not known how α4-containing receptors are changed in other parts of the brain. It is possible that the changes in α4-containing receptors in the hippocampus are but a small part of the vast changes occurring in the brain. Hence, the contribution of hipoocampal changes to behavior during withdrawal may be limited. It is also possible that α4-containing receptors are involved in other withdrawal behaviors that were not assessed as part of this study. Thus, results from this study can only add another piece of information to what is already known about the modulation of withdrawal effects – that α4-containing receptors are not essential for ethanol withdrawal behaviors studied here.

A second goal of the above experiments was to ascertain if α4-containing receptors were required for the actions of taurine during chronic ethanol withdrawal. However, I was unable to determine this due to lack of an effect of taurine on most

behaviors. Results obtained in this study suggest that taurine does not alleviate the ethanol withdrawal effects of seizure susceptibility, anxiety and tolerance under the given experimental conditions. Although responses to ethanol-induced LORR in taurine-treated α4 KO were changed, it is unclear if this represents a major role for α4 receptors. Rather, this effect may be an expression of compensatory responses in the α4 KO mice.

A hypothesis for the relatively minor effects of taurine in this study is put forth. The premise of the above experiments was that increases in α4 in the aftermath of ethanol exposure could be harnessed by taurine for alleviating withdrawal endpoints. A positive effect of taurine on chronic ethanol withdrawal-induced seizures was demonstrated in C3H/HeJ mice [233]. But in my hands, no major effect of taurine was visible in our mice on three different endpoints of chronic ethanol withdrawal.

It has been noted that different background strains exhibit behavioral differences that can be linked to differences in particular receptor types [416, 417]. Thus, while increases in α4 maybe dramatic and sufficient for an effect of taurine in C3H/HeJ mice, levels of α4 may not be increased to the same degree in a mixed background of C57BL/6J and Strain 129X1/SvJ mice. It is noteworthy that α4 protein levels were measured at 30 hours into withdrawal in WT mice that had been subjected to chronic ethanol and air-saline treatment (data not shown). Analysis of Western blots of α4 protein from these mice revealed an increase in levels of α4 in the ethanol-treated mice (n=3). However, this increase was not significantly different from air-saline treated mice (n=3), given the small sample size. This suggested that α4 levels were increased in our strain of mice with the chronic ethanol treatment paradigm used here.

A comparison of gene expression profiles and concurrent biochemical changes in the aftermath of chronic ethanol exposure in C3H/HeJ and the mixed background strain of

mice used in this assay may help explain the reasons for differences in behavioral responses. In addition, an analysis of gene expression profiles in the global α4 KO may also explain how response to ethanol withdrawal is maintained in these mice. In addition to changes in expression of GABA A receptor subtypes, it is possible that other receptor families compensate for the lack of α4-containing GABA A receptors.

It is noteworthy to mention that α4 subunit has been identified as being increased and combined with γ2 subunit in synaptic locations whereas δ subunit is reduced following chronic ethanol exposure [181, 358]. In a previous study by Jia *et al*, [234] taurine was shown to act potently at α4-δ containing extrasynaptic receptors. However, its effects of novel α4 combinations have not been studied. Although α4δ-containing receptors are very sensitive to taurine, α4γ2-containing receptors may not be as sensitive to taurine. Thus, during chronic ethanol withdrawal, some other distinct targets may mediate taurine effects. Hence, further electrophysiological characterization of mice exposed to chronic intermittent ethanol paradigm is essential to understand if taurine affects current potentiation through α4-containing receptors.

Although my results do not provide much insight, the interactions of taurine with ethanol certainly warrant more consideration. Given the fact that taurine is an important ingredient of energy drinks and energy drinks are frequently combined with ethanol, a better understanding of this interaction is required. A study of drinking behavior among college students revealed that close to 50% of students frequently combined energy drinks with alcohol [418]. An independent study showed that combination of energy drinks with alcohol only served to reduce the perception of headache, weakness, dry mouth and motor impairment [419]. However, performance on objective motor coordination and visual reaction time were not changed due to combination of energy drinks with alcohol [419].

These observations suggest alarming implications for modification of alcohol drinking behavior due to combination with energy drinks. The perception of reduced impairment may actually foster drinking behavior although reductions in behavioral impairment are not observed. Interestingly, the lack of behavioral effects in humans upon combining alcohol with energy drinks is similar to the lack of overt differences in behavior in this study.

In conclusion, my efforts reveal that chronic ethanol withdrawal behaviors of HIC, locomotion and anxiety, and tolerance do not require α4-containing receptors. In addition, the effects of taurine on withdrawal parameters are ambiguous at best. Strain differences, differences in ethanol treatment paradigms may have precluded observing a robust effect of taurine. Although behavioral differences were not apparent between the genotypes, electrophysiological and biochemical characterization of taurine responses are required to conclude if α4-containing receptors are a behaviorally relevant target of taurine.

Chapter 5: Summary and Conclusions

The goal of this thesis was to elucidate the role of α4-containing receptors in 1) volatile and injectible anesthetic actions; 2) neurosteroid actions; 3) chronic ethanol withdrawal effects; 4) actions of taurine during chronic ethanol withdrawal.

5.1 Potential mechanisms of a4 GABA receptors in anesthetic actions:

α4 global KO mice were studied with different classes of anesthetics to understand the role of α4-containing receptors in anesthetic actions. Behavioral analyses revealed that α4-containing receptors are involved in the low dose amnestic effects of isoflurane and in the locomotor stimulatory effects of alphaxalone. However, in spite of evidence linking α4-containing receptors to the actions of etomidate and propofol, α4 KO mice did not differ in their responses to etomidate or propofol.

Behaviors are mediated by complex circuits. Drugs that modify behavior can act at different levels of circuitry to bring about their effects. In the α4 KO, gaboxadol-induced current potentiation was reduced in the ventrobasal thalamus [16]. Consistent with this observation, behaviorally, a reduction in gaboxadol-induced LORR, motor-incoordination and analgesic effects was observed [16]. However, the same behavioral resistance was not seen with isoflurane-induced LORR and MAC, although isoflurane reduced current potentiation in the KO ventrobasal thalamic neurons [343]. Consistent with the reduced cellular responses, a decrease in the amnestic effects of isoflurane was observed.

The reduction in the amnestic effects of isoflurane in the KO implies that α4-containing receptors are involved in the amnestic response to this inhaled anesthetic. Although hippocampal current potentiation to isoflurane was not measured, it is possible that current potentiation with isoflurane in the hippocampus shows the same reduction as

that observed in the ventrobasal thalamus. Because the hippocampus is associated with memory and is a region of α4 expression, it is likely that α4-containing receptors in this region modulate isoflurane-induced amnesia. This possibility could be tested by creating a hippocampal specific knockout of the α4 subunit or by hippocampal-specific knockdown of α4-containing receptors by siRNA technique. A hippocampal specific knockout mouse can be created by crossing the floxed α4 gene[16] bearing mice to tissue specific Cre deleter mice where Cre recombinase expression is under the control of a hippocampal specific promoter. α4 expression has been knocked down by siRNA techniques successfully in the nucleus accumbens [333]. Similar knockdown of α4-containing receptors can be achieved in the hippocampus. Studies on amnestic responses in such mice will reveal if hippocampal α4-containing receptors are involved in the amnestic effects of isoflurane and other drugs.

The involvement of α4-containing receptors in amnestic effects opens up new vistas for therapeutic applications. α4-containing receptors are another addition in the list of α subunits that regulate memory processes. Global deletion of α1-containing receptors affected the amnestic effects of isoflurane dramatically [115]. The global deletion of α5-containing receptors produced similar results with etomidate [116]. Thus, it appears that GABA A receptors may be critical in gating memory. Should α4 and α5 specific agonists be developed, such drugs may be of particular interest in the treatment of post-traumatic stress disorders. Such possibilities have already been discussed with regard to the α5-containing receptors. In addition, the improved performance of both α4 [346] and α5 KO [117] on memory-based tasks give rise to the interesting possibility of development of specific nootropic agents. Such drugs are already being discussed as having applications in pathological processes where memory is affected such as Alzheimer's disease [420, 421].

Studies on several mutant mouse models have provided mixed results about the involvement of GABA A receptors in volatile anesthetic effects of LORR and MAC. Mice bearing an isoflurane-insensitive mutation in the α1 subunit showed reduced responses to the LORR effects of isoflurane but unchanged MAC responses [83]. Mice bearing the isoflurane-insensitive mutation in the α2 subunits showed unchanged responses to isoflurane and halothane on LORR and MAC [84]. The RORR responses to isoflurane were, however, increased in these mice suggesting some role for α2-subunit containing receptors in the actions of isoflurane [84]. β3 KO [80] and KI [15] studies have both shown reduced MAC responses to volatile anesthetics. However, the change in response to LORR and MAC effects of volatile anesthetics in the above studies has ranged from 10-25% in these mutant mice. This suggests that the role for these GABA A receptor subunit-containing receptors in the actions of volatile anesthetics is modest. An overview of studies that focused on effects of volatile anesthestics on GABA A receptor mutant mice indicates only moderate changes in LORR and TC responses to volatile anesthetics suggesting that GABA A receptors may not be critical mediators for these effects of volatile anesthetics. Rather, it is now proposed that these volatile anesthetic effects are a result of action of volatile anesthetics on multiple discrete targets.

Because the actions of the intravenous anesthetics, etomidate and propofol, were not changed in the α4 KO mice, I conclude that etomidate and propofol do not require α4-containing receptors at least for the effects studied here. β2- and β3-containing receptors have been implicated in the sedating, immobilizing and LORR effects of etomidate and propofol [15, 82]. It is proposed that half of the α4-containing receptors are associated with a β subunit and no γ or δ subunit [269]. *In vitro* expression of α4β3 receptor combination in human embryonic kidney cells showed that such binary receptors showed high

sensitivity to GABA and increases in current potentiation with etomidate and propofol, similar to α4β3δ receptors [367]. Thus, several lines of evidence pointed towards a possible role for α4-containing receptors in the actions of etomidate and propofol. However, behavioral characterization of α4 KO and WT mice revealed no differences in loss of righting reflex and motor ataxia induced by etomidate and propofol. Lower dose sedating effects of etomidate also did not differ in WT and α4 KO. Thus, α4-containing receptors are not required for the effects of etomidate and propofol on the behaviors tested as part of this study. It is possible that α4-containing receptors may be involved in other behaviors such as those observed at very low doses of anesthetics. For example, etomidate-induced amnestic effects may be changed in α4 KO mice in parallel to the involvement of these receptors in the amnestic effects of isoflurane.

What are the possible sites of action for etomidate? The β3 subunit has been strongly implicated in the actions of etomidate. Quinlan *et al*, showed that a β3 KO mouse model had reduced etomidate-induced LORR [80]. A forebrain specific KO of the β3 subunit also displayed reduced sensitivity to etomidate-induced LORR [125]. Finally, a β3 KI mouse had dramatically reduced responses to etomidate-induced LORR and immobilization [15]. Thus, substantial evidence indicate that β3 subunits are an essential target for at least some effects of etomidate. The binding site for etomidate is proposed to lie at the interface of an α and a β subunit [366]. While β subunits that are likely involved in actions of etomidate have been proposed, the identity of the α subunit, that participates in the actions of etomidate is unknown. Studies with α1, α5, γ2 and δ KO mice revealed no changes in the LORR effects of etomidate [17, 116, 119, 122]. While it is possible that compensation may have masked a role for these receptors in the actions of etomidate, it is possible that other α subunits such as α6, α2 and α3 may be involved in the actions of

etomidate. Conditional knockouts and siRNA techniques could be used to generate selectively ablated subunits and thus the contribution of these subunits to etomidate actions could be evaluated.

Another interesting possibility is that while the β3 subunit is critical for modulation by etomidate, the identity of the α subunit may not be as critical. This may explain why several α subunit KO mice do not show changes in responses to etomidate. However, the *in vivo* contributions of α6, α2 and α3 subunits to the actions of etomidate is required to substantiate this hypothesis. Further studies on an array of different receptor subtypes will have to be conducted to validate this possibility.

A clearer understanding of the pharmacology of etomidate and propofol is required to explain the actions of these drugs. Electrophysiological data from different brain regions of WT and KO mice may also be used to resolve this issue. In order to take into account the effects of deletion of α4-containing receptors on general neurotransmitter-receptor system architecture, it would be worthwhile to conduct microarray studies. For example in the α6 null mutant, cerebellar granule neurons were found to have increased expression of voltage-independent K^+ channels [422]. A similar situation can be envisaged in the α4 KO mice. Hence, it is plausible that compensatory changes are not limited to the GABAergic system alone. Thus, a conclusive interpretation of results obtained here will require a better understanding of changed parameters in the α4 KO.

5.2 α4 GABA receptors in neurosteroid actions:

Behavioral effects of the neurosteroid anesthetic alphaxalone, were studied in the α4 global KO mice. The locomotor stimulatory effects tested at a low dose of alphaxalone

were reduced in the α4 KO mice. However, effects produced by higher doses of alphaxalone such as motor ataxia and loss of righting reflex were not changed in the α4 KO mice.

Because endogenous neurosteroids undergo changes in levels through different hormonal stages, GABAergic receptors are subject to fluctuations in expression and resulting inhibitory neurotransmission. Pubertal mood swings, pubertal learning, premenstrual syndrome, postpartum depression and perimenopause are stages during which GABAergic receptors are particularly critical for their responses to steroids [286, 288, 290, 291, 311]. Progesterone and corticosterone metabolites (THP and THDOC) are released as an early-stress response and are thought to act on GABAergic receptors to modulate inhibitory neurotransmission [308, 423-425].

α4βδ-containing GABA receptors have special significance in modulating effects of neurosteroids. Several lines of evidence support this hypothesis. Compelling evidence towards this was obtained from the δ KO mice. Knockout of δ receptors produced a marked attenuation of neurosteroid effects [17]. δ KO mice had reduced sleep times, reduced anxiolysis and reduced pro-seizure activity with PTZ compared to WT mice [17]. δ KO also could not regulate neuronal excitability during pregnancy in the presence of elevated neurosteroid levels. This was proposed as a mechanism for psychiatric and neurological disturbances associated with pregnancy and postpartum [286]. Consistent with this observation, δ KO mice showed evidence of postpartum depresson due to inability to maintain tonic response to changed levels of neurosteroids (see section 10.1.1) [311]. These results pointed towards a prominent role for δ-containing receptors in the actions of both endogenous and exogenous neurosteroids.

Dentate granule neurons of the hippocampus from α4 KO showed reduced tonic current potentiation in response to alphaxalone [340]. Such reduction in tonic current was likely reflected in the lack of a locomotor stimulatory response in the KO. However, normal response to LORR and motor ataxic effects was observed. Unlike the δ KO mice, α4 KO mice reproduced normally and cared for their offspring, although this was not studied formally. HET mice also produced offspring in keeping with Mendelian ratios. These results raise the question of involvement of α4 in neurosteroid effects. Since levels of δ subunit are also reduced in these mice [350], one would imagine a significant reduction in the effects of neurosteroids. However, this was not the case. This suggests that the presence of residual δ-containing receptors alone in α4 KO may be sufficient for the response of neurosteroids at high doses. Because α4βδ receptor combinations have been implicated in fluctuating steroid responses, the role of α4 may be more clearly apparent only in conditions of changed neurosteroid availability such as premenstrual syndrome, pubertal mood swings and perimenopause.

The possibility that the existing δ subunits could give rise to novel combinations with other α subunits and result in normal behavioral response cannot be excluded. This is especially more plausible due to the compensatory increase in α1 mRNA in regions of α4 and δ expression, such as hippocampus and thalamus in the α4 KO mice [350]. The suggestion of receptors of unknown stoichiometry consisting of α1, α4, β2 and δ mRNA has been proposed previously [36]. Given that α1 subunits are upregulated in the α4 KO [349], the possibiltity of α1 subunits taking the place of α4 subunits is a particularly enticing possibility. In fact, α1δ containing receptors have been discovered in the hippocampus of WT and α4 KO mice [320]. In WT and KO, these α1δ-containing receptors mediate tonic currents and are responsive to tonic current potentiation with low

doses of ethanol [320]. How this receptor combination may affect response to neurosteroids is yet to be investigated. At present, results from our α4 KO study point towards a role for α4 receptor combinations in the low dose actions of neurosteroids studied here. Thus, results obtained from the α4 KO model challenge the current hypothesis of a major role for α4δ-containing receptors in neurosteroid action.

Another interesting possibility which is yet to be evaluated is the role of α4 and its modulation by neurosteroids in a sex-specific manner. No changes were seen in reproductive or maternal behavior, probably due to the residual δ-containing receptors. In behavioral assays with the α4 KO, trends towards significant differences in responses of α4 KO males and females were observed in locomotor behavior with alphaxalone. However, due to limitations of number and design of experiments, I was not at liberty to pursue this design. Because subtle sex differences presented themselves in other assays as well, it is worth considering if the lack of α4 is compensated differently in males versus females. These differences could be particularly critical at lower levels of endogenous neurosteroids which influence anxiety, mood disturbances or seizure susceptibility. What might be the corresponding significance, if any, of these receptors in males? These possibilities raise some interesting questions about possible gender-specific regulation of these receptors and merit further scrutiny.

5.3 α4 GABA A receptors and ethanol antagonists:

The role of α4-containing receptors in the actions of an ethanol antagonist, RY023 was assessed by studying α4 WT, HET and KO mice. α4-containing receptors were involved in the intrinsic effects of RY023 but not in the antagonistic effects of the same on ethanol-induced LORR. On motor ataxia, HET mice had reduced intrinsic response to

RY023, whereas WT and KO were comparable. On the other hand, the intrinsic sedating effects of RY023 was reduced in the KO, while WT and HET were comparable. Upon comparison of expression of α4 levels in WT, HET and KO mice, it was found that compared to WT, HET mice display lowered levels of α4 in hippocampus, but not in cortex. Although this data was obtained from a limited sample set, the differences in expression of α4 in the hippocampus point to the possibility of sub-normal levels of α4 being responsible for the reduced response to RY023 on motor ataxia. The lack of differences in KO mice on the same behavior is likely the result of compensation. Interestingly, the sedating effect of RY023 was maintained in WT and HET mice but not in KO. It is possible that the ablation of α4-containing receptors in the KO was sufficient to decrease response to the sedating effects of RY023 but a reduction in α4-containing receptors (as evidenced in the HET) may not have been sufficient to change response to RY023. Lastly, ethanol antagonistic effects of RY023 were maintained in all three genotypes indicating that reversal of ethanol-induced LORR by RY023 does not require α4-containing receptors.

These results are in contrast to those obtained with the ethanol antagonist Ro15-4513 which suggested a role for α4-containing receptors in ethanol antagonism. In a previous study, Ro15-4513 antagonism of ethanol-induced motor ataxia and LORR was dramatically reduced in the α4 KO, indicating that actions of Ro15-4513 are primarily mediated through α4-containing receptors [341]. Since RY023 is a structural analog of Ro15-4513, it was proposed that RY023 antagonism of ethanol effects would be mediated through α4-containing receptors [262]. However, this was not the case. RY023 antagonism of ethanol-induced LORR was maintained in all three genotypes. This suggests a different mechanism of action for the ethanol antagonistic effects of RY023 compared to Ro15-

4513. These results are contradictory to the proposed theory of α4δ-containing receptors as the common target for ethanol antagonistic actions of these drugs.

The electrophysiological effects of RY023 have been tested only on limited combinations of receptors, namely α1β3γ2, α2β3γ2, α4β3γ2, α5β3γ2 and α6β3γ2 [253, 255]. Since αβδ receptor combinations are key in alcohol behaviors, characterization of responses of RY023 on δ-containing receptor combinations may help explain the differences observed here. Differences in electrophysiological profiles of both Ro15-4513 and RY023 in multiple brain regions will also provide more information about functional differences between both drugs in WT, HETS and KO.

Results obtained here with the HET mice have provided some clues about the effects of sub-normal levels of α4-containing receptors. This experience suggests that the inclusion of HET mice in behavioral assays, where possible, may help uncover the role of the target under study.

5.4 α4 GABA A receptors, chronic ethanol withdrawal and taurine:

α4 KO mice were used to study chronic ethanol withdrawal effects and the ability of taurine to alleviate chronic ethanol withdrawal behavior. HIC, anxiety and locomotion, and tolerance to ethanol were assessed in WT, HET and KO mice that received chronic ethanol treatment with or without taurine. Ethanol withdrawal behaviors did not differ on saline-treated WT, HET and KO mice. This suggests that α4-containing receptors are not required for the withdrawal effects. However, an effect of taurine was also not apparent on most withdrawal behaviors studied in this assay except protracted tolerance. As a result of this, the role of α4-containing receptors in the actions of taurine during chronic ethanol withdrawal remains unanswered.

α4-containing receptors undergo increases in expression and changes in localization following ethanol withdrawal [345]. These changes in α4 expression have been linked to a variety of behavioral alterations in the aftermath of ethanol exposure. The hallmarks of withdrawal include seizure susceptibility, anxiety, insomnia and tolerance to typical benzodiazepines among other effects [3]. Chronic ethanol withdrawal increases α4 and γ2 subunit levels on the one hand and decreases α1 and δ subunit levels on the other [178]. Although α4 levels are increased, its combination with γ2 subunits instead of δ results in decreased total charge transfer and thus reduces the level of inhibition [178]. A decrease in current potentiated by benzodiazepines and ethanol, correlating with decreases in α1 and δ subunits were seen during withdrawal [180]. Concurrent increases in binding of α4-specific drugs such as Ro15-4513 and THIP were also observed [181]. Hence, it was proposed that adminstration of drugs that specifically modulate α4-containing receptors and increase the current mediated through them will be of use during ethanol withdrawal.

Taurine is one such agent linked to ethanol antagonism and alleviation of ethanol withdrawal. Although multiple groups have studied the effects of taurine on ethanol, a resolution about its mechanism of action has not been reached. Recent work by Jia and collegues showed that this amino acid was a potent agonist at α4-containing GABA A receptors and increased tonic current potentiation through them [234]. One group showed that taurine alleviated chronic ethanol withdrawal-induced seizures in mice [233]. To test if taurine acted through α4-containing receptors, α4 KO and WT mice were subjected to chronic intermittent ethanol treatment with and without taurine and subsequent withdrawal behaviors were assessed. Contrary to my expectations, a positive effect of taurine on withdrawal was not noted on any of the behaviors studied. It is possible that differences in assay design and strain of mice used may have contributed to this result. However, it is

surprising that a fairly high dose of taurine (500 mg/kg) given over three successive days failed to elicit any effect in contrast to a single dose (500 mg/kg) of taurine used in the previous study [233]. Due to these discrepancies, the role of α4 in actions of taurine could not be tested conclusively in these assays.

The fact that behavioral responses to acute [186] and chronic ethanol exposure were maintained in the α4 KO imply that α4-containing receptors are not be necessary for mediating these effects of ethanol. It is important to note that acute effects of motor ataxia, sedation and hypnosis represent moderate levels of alcohol intoxication. It is possible that lower doses of ethanol do depend upon α4-containing receptors for their effects. However, these have not been assessed as yet by an assay sensitive to the low dose impairment by ethanol. The low dose assay developed by Moore *et al* to assess cognition-impairing effects of ethanol should be useful in this regard [383].

It is also possible that the extent of compensation observed in the α4 KO may be responsible for masking the true role of α4-containing receptors. In fact, Liang *et al* demonstrated changed synaptic receptor dynamics in the α4 KO mice that had increased sensitivity to ethanol [340]. Behavioral responses to acute ethanol were also unchanged in α4 KO mice. Hence, although the results obtained from chronic ethanol studies in α4 KO do not elicit a role for α4 in ethanol withdrawal behaviors, this merits a closer scrutiny.

It is not known currently how the synaptic changes occuring in α4 KO affect current kinetics during ethanol withdrawal. It is possible that these synaptic changes rescue deficits in the α4 KO and prevent an apparent role for α4-containing receptors in ethanol withdrawal. Given the lack of behavioral changes in ethanol withdrawn KO, it would be particularly interesting to understand how changes in subunit composition and GABA A receptor-mediated current during ethanol withdrawal are modulated in the α4 KO.

Studying these parameters will illuminate our understanding of the other receptor combinations that may be sensitive to ethanol.

Conditional knockout of α4-containing receptors could be used to elucidate its role in ethanol behaviors. For example, a conditional mutant in which α4 subunit expression can be turned off in adult mice a few days or weeks prior to studying ethanol withdrawal behavior will help exlude compensatory effects that may have arisen due to developmental deficit of α4 in the global KO. In fact, Rewal *et al* [333] showed that reduction of the α4 subunit-containing receptors in adult rats influenced ethanol drinking behavior markedly. Knockdown of α4 mRNA specifically in the nucleus accumbens was achieved by means of siRNA technique. This study showed that a 75% reduction of α4 expression in the nucleus accumbens did not alter the expression of other subunits such as γ2, α1, β2 and δ [333]. Thus, transient reductions in α4 protein expression did not produce compensatory changes as evidenced in the global KO mice. This study elucidated that reduction in α4-containing receptors was responsible for reduced preference for ethanol [333]. Similar studies could be embarked upon to understand the role for α4-containing receptors in acute and chronic ethanol actions. If conditional mutants of the α4 subunit are created in future, a side by side comparison of global and conditional knockout mice may be instrumental in resolving the results obtained here.

As for taurine, the contradictory lines of evidence and possible strain differences relating to ethanol behavior confound the resolution of its role. Yet, the overwhelming evidence for effectiveness of acamprosate, a close analog of taurine, and its hitherto unknown mechanism of action, underscore the importance of studying these agents.

5.5 Compensation and its significance:

Global α4 KO mice show evidence of compensation that needs to be taken into account while interpretating behavioral changes in these mice. A prominent decrease in δ subunits was observed in thalamic nuclei [349]. In addition, an upregulation of α1 and α2 subunits was noted [350]. These increases were greatest in regions of δ subunit expression. A subtle increase in γ2 subunits was also seen possibly as a result of decreased δ subunit expression [350]. Ultrastructural studies also revealed that the δ subunit was localized within the cytoplasm, specifically in the endoplasmic reticulum and the Golgi complex [349]. This suggested that lack of α4 subunits affected the trafficking of δ subunits to the surface. A reduction in tonic current was observed in the hippocampus and dentate gyrus of these mice, consistent with a loss of α4-containing receptors [16]. Deletion of α4-containing receptors did not affect the charge transfer during synaptic current potentiation [340]. However, marked slowing of the rise and early decay of mIPSC was noted in the α4 KO mice [339]. Further characterization of changes in α4 KO mice is underway.

It is necessary to take these changes into consideration while understanding changes that may be observed in response to drugs in the α4 KO mice. Compensatory changes may also mask the isolated role of the α4-containing receptors. As stated in the relevant chapters, novel combinations of α1 and α2 subunits with δ subunits may be responsible for masking the effects of neurosteroids. In addition, increased populations of α1 and α2 subunits with γ2 subunits may also be responsible for maintaining responses to some anesthetics and intrinsic effects of RY023.

To assess the contribution of novel receptor combinations and increased population of certain receptor subtypes to drug responses, several approaches can be used. The α4 KO

mice were created from mice that possessed a floxed allele of the α4 gene by crossing with a global Cre-deleter strain of mice [16]. It is possible to create conditional mutant mice by crossing the floxed mice to tissue specific Cre-deleter strains where expression of the enzyme Cre-recombinase is under the control of a tissue specific promoter. In this way, α4 expression can be deleted in specific brain tissues. It is also likely that region-specific deletion of α4-containing receptors will limit extensive compenstion. Creating region-specific KO mice where α4 expression is ablated in the cortex, straitum, hippocampus and/or thalamus will give us information about the specific behaviors controlled by these receptors in different brain regions. In addition, temporal α4 expression could be achieved by breeding floxed α4 mice to inducible Cre-recombinase expressing mice. By turning on Cre-recombinase a few days before behvioural testing will likely reduce the possibility of compensatory responses (These possibilities have already been discussed, see [426])

Electrophysiological characterization for specific brain regions could be performed with a variety of agonists and antagonists to delineate the contribution of specific receptor subtypes to current modulation. The limited availability and the reduced specificity of GABA A receptor subtype-specific ligands allows identification of receptor subtypes only to a moderate degree of accuracy. This problem is especially confounded in the presence of hitherto unknown receptor combinations. Hence, multiple agonists and antagonists at varying concentrations are used to determine the identity and characteristics of currents modulated by specific brain regions.

For example, to understand if α1γ2-containing receptors are compensating for α4 loss and contributing to current modulation and subsequently to behavior linked to a specific brain region, one could apply an α1 preferring agonist such as zolipidem. Zolpidem shows high affinity for α1 containing receptors [427]. Native and recombinant

receptors containing α1γ2 combinations have been studied extensively and hence their characteristics are known [428-431]. By comparing mIPSC characteristics of zolpidem-induced current in the same brain region from an α4 KO, α4 WT, conditional α4 KO and conditional α4 WT, one could hope to understand the relative contributions of native and compensatory α1γ2-containing receptors to current modulation in a specific brain region. Several such agonists and antagonists could be used for a detailed characterization. Finally, studying behavior in the an α4 KO, α4 WT, conditional α4 KO and conditional α4 WT mice will allow comparison of whether changes observed at the cellular level (if any) translate to behavioral differences.

Similar studies could be performed with regions that express α1δ receptor combinations. It is important to note that the native α1δ receptor combination has been recently identified in one brain region, namely, the hippocampus [320]. As yet, this receptor combination has been assessed for tonic current contribution and responses to ethanol [320]. However, a thorough characterization of responses of such receptors to neurosteroids, α1-specific (zolpidem) and δ-specific (gaboxadol, neurosteroids) drugs is required. Hence, understanding the contribution of an increased population of such α1δ receptors to compensatory responses in the α4 KO will require the characterization of native α1δ-containing receptors first.

While this method is complicated, it is a sound approach to determine how changes at the cellular level may mask or unmask responses at the behavioral level. Take the case of ethanol responses in the α4 KO and WT mice at the cellular level – hippocampal dentate gyrus neurons were evaluated for current modulation by different concentrations of ethanol [340]. Tonic currents in response to 10-100mM of ethanol were detected in α4 WT neurons [340]. However, tonic current potentiation in response to ethanol was not observed in the

α4 KO mice with the above concentration range [340]. Synaptic current potentiation in the presence of ethanol was also recorded. In WT dentate gyrus granule neurons, mIPSC were slightly potentiated with 10-100mM of ethanol [340]. However, KO showed a large potentiating effect of ethanol on mIPSC recorded from the same region [340]. This suggested that deletion of α4 containing receptors had ablated tonic current potentiation by ethanol but at the same time produced changes in the synaptic machinery that conferred increased sensitivity to ethanol. When studied for behavioral responses at moderate doses of ethanol, no differences were observed between WT and KO suggesting that the changed synaptic sensitivity to ethanol retained behavioral effects of ethanol in the KO [186].

Although it is entirely possible that the increased sensitvity of synaptic receptors to ethanol rescues any behavioral differences to ethanol in the α4 KO, this explanation does not take into account other compensatory mechanisms that may be at play to restore the behavioral responses of ethanol. Compensatory responses can extend beyond the GABA A receptor system as well. For example, α6 KO mice showed an increase in the voltage-independent K+ leak conductance in the cerebellar granule cells which allowed maintenance of normal granule cell excitability [422]. Similarly, knockout of HCN1 channels (responsible for hyperpolarization-activated mixed cationic currents) led to the compensatory upregulation of GABA A α5 subunit-containing receptors in the cortex [432]. This allowed the normal synaptic excitability control in the KO mice [432]. These examples imply that compensatory responses to deletion of particular targets involve system-wide changes encompassing multiple neurotransmitter- receptor families. Hence, analysis of compensation in the α4 KO must also extend to other neurotransmitter-receptor families. A gene array of WT and KO mouse brain tissue is one step in resolving this issue. In addition to this, functional changes at the cellular level, changes in receptor trafficking

and channel desensitization characteristics should also be taken into consideration to interpret the changes observed in the α4 KO mice.

Although compensation definitely confounds our ability to interpret the role of the the ablated target accurately, such data also gives us some information about built in fallback mechanisms. Study of compensatory pathways will elucidate the inter-dependencies of different receptor systems and how these are regulated. From a more global perspective, this kind of information can be extended to potential gene therapy applications as well where compensatory patterns may be predicted with greater accuracy owing to models developed in mice. In addition, given our limited knowledge about receptor dynamics such as densensitization, assembly and trafficking, genetically engineered models may be used to study changes in these processes. Overall, the results obtained with α4 KO with anesthetics, neurosteroids, ethanol and ethanol antagonists challenge our understanding of the mechanism of actions of these drugs. Already, biochemical techniques coupled with microscopic characterization have aided our understanding of compensatory changes in the α4 KO [349, 350]. Similarly, microarray of the global knockout will help anticipate potential candidates for compensation, thus facilitating a more accurate interpretation of behavioral data. In addition, the creation of conditional α4 knockouts, region-specific or temporal-specific mutations will be useful to bypass compensatory responses and allow for a better understanding of the role of α4.

5.6 Success stories of gene-targeted mice:

While genetically engineered mice have long faced criticism due to compensatory effects that confound interpretation, the knowledge gained from gene-targeted mice make the disadvantages of this technique a small price to pay. Knockout models while being expensive and time-consuming to create, have successfully elucidated the physiological

and pharmacological effects of specific targets. Understanding the compensatory mechanisms or lack thereof provides information about the significance of deleted or modified targets. Dramatic results with some of these knockout models provides answers about the functions of certain targets.

While these benefits are not immediately apparent, the long term study of these models yield interesting results. Take the case of δ KO mice- these mice were generated in the late 1990's. Ten years after their creation, these mice continue to provide answers and elucidate the role of these δ containing receptors in physiological and pathophysiological functions of neurosteroids. Thanks to this model, we now know the implications of lack of δ-containing receptors in processes such as learning during puberty, premenstrual syndrome, pregnancy and post partum depression [286, 288, 290, 311]. Similarly, the β3 KO mice that were generated in 1997 had severe deficits such as cleft palate, seizures and hypersensitivity [388]. Studies on these KO mice have revealed that apart from a role in anesthetic sensitivity to etomidate and midazolam [80, 125], the β3-subunit containing receptors are involved in epilepsy and autism spectrum disorders [433-436].

Advances in the field of genetic engineering have led to newer methods of assessing the contributions of specific receptor subtypes to drug actions. Site-specific mutations that change response to certain drugs have been identified leading to the creation of knockin mutant mice. These knockin mutant mice have the advantage of maintaining normal responsiveness to endogenous modulators and altered responses only to specific drugs. Normal responsiveness to endogenous ligands allows normal development of these mice and reduces the incidence of compensation. This technqiue has been particularly useful in delineating the mechanism of actions of benzodiazepines. Knockin mutations in the α1, α2, α3 and α5 subunits of the GABA A receptor have successfully outlined the role of these

receptors in the actions of benzodiazepines. α1 KI mice implicated α1-containing GABA A receptors in the sedative, amnesic and anti-convulsant (partly) effects of diazepam [437, 438]. The myorelaxant effects of diazepam were found to be mediated by α2, α3 and α5-containing receptors whereas the anxiolytic effects of the same required α2-containing receptors [118, 439, 440]. α5 KI mice showed an overall reduction in the abundance of α5-receptors in the hippocampus and not surprisingly, an improvement in baseline memory on hippocampal dependent tasks was observed [118]. In addition, β3 KI mice were also critical in delineating the hypnotic and immobilizing effects of etomidate [15].

The above mentioned successes are limited to key studies of the GABA A receptor system. Similar studies have been pioneered with glycine and NMDA receptors and it is hoped that, as with GABA A receptors, these studies will elucidate the physiological and pharmacological roles of glycine and NMDA receptors. In conclusion, although genetically engineered mice present some disadvantages in terms of confounding compensatory changes, possible neonatal lethality as well as increased time and costs, the wealth of knowledge to be gained from the successful creation of viable genetically engineered models far outweighs the risk associated with this technique. Considering that our knowledge of the complex functioning of the CNS is evolving, genetically engineered mice present a good model by which to understand the *in vivo* consequences of alterations in the nervous system.

5.7 Summary:

It is interesting to note that α4-containing receptors are involved specifically in the low dose effects volatile anesthetics and neurosteroids. α4-containing receptors are also speculated to be important for low dose effects of ethanol. Because α4-containing receptors are mediators of tonic current and because tonic current is a critical regulator of neuronal

excitability, the finding that low doses of alcohol, anesthetics and neurosteroids act on these receptors has great significance. It is possible that α4-containing receptors might serve as the earliest responders for effects of these classes of drugs.

Behavioral assays that are uniquely sensitive to low dose effects of anxiety, disinhibition, euphoria, cogntion-impairment by alcohol and anesthetics are currently unavailable. Developing behavioral assays that are more sensitive to low dose effects of anesthetics and alcohol will help dissect the role of α4-containing receptors. In fact, Moore *et al* have recently developed such an assay to assess low dose effects of alcohol [383].

Because α4-containing receptors are involved in regulating memory [346] as well as learning during puberty [290], these receptors may have special significance in anesthetic effects in young children and in adolescents. The side effects of anesthetics such as post-operative cognitive deficits, anesthetic awareness and the trauma associated with these occurances make understanding the mechanism of effects of anesthetics a very important goal. In addition to these side effects, very little is known about how anesthetics mediate the desirable effects of unconsciousness, amnesia and immobility. Genetically engineered models have enabled the study of specific targets thought to be involved in anesthetic effects. The GABA A receptor α4 KO model has helped understand better the mechanism of actions of inhaled anesthetic-induced amnestic effects. This model could be used to evaluate the effects of other anesthetics as well.

Although some behavioral effects of anesthetics have been studied with the α4 KO, the full spectrum of its effects are yet to be understood. The fact that the novel sedative gaboxadol, requires α4-containing receptors for its actions suggests that α4-containing receptors can be harnessed to produced sedative, hypnotic and analgesic effect. This provides some clues for rational drug discovery of novel sedative-hypnotic drugs that have

fewer side effects. Should the structure of α4-containing receptors be elucidated, drugs specific to it (like gaboxadol) can be developed resulting in safer, more specific effects.

Neurosteroids have been implicated in an array of effects from mood disturbances, premenstrual syndrome, epilepsy, postpartum depression, ethanol drinking behavior and recently, learning during puberty [288, 290, 296, 299, 308, 311, 329, 424, 425]. Endogenous neurosteroid levels undergo fluctuations according to hormonal cycles. GABA A α4δ-containing receptors have been identified as a major target for neurosteroid effects and dysregulation of GABA A receptors are thought to result in the above-mentioned syndromes [282, 283, 295, 307, 315]. Studies with the δ KO [17, 286, 311] and the α4 KO have been successful in elucidating the relative involvement of each of these targets in neurosteroid actions. Hence, these receptors provide a potential therapeutic target for alleviating disorders resulting from hormonal disturbances.

The pursuit of a magic drug that would eliminate the ill-effects of ethanol has kept researchers busy for over three decades now. Yet we only have a handful of putative ethanol antagonists to date. Finding a specific ethanol antagonist has only been as difficult as delineating the mechanism of action of ethanol. A clear understanding of the ethanol-antagonistic properties of Ro15-4513 has eluded scientists for two decades now and finally, the α4 KO mice have proven to be instrumental in elucidating its site of action for behavioral ethanol antagonistic effects. Structural analogs of Ro15-4513, namely the RY series of drugs have also been shown to act as ethanol antagonists. Recent work by Hanchar *et al*, suggested that these drugs may be acting through the same GABA A receptor subtype, i.e., α4δ-containing receptors [262]. My work here with α4 KO mice indicates that while some intrinsic effects of the representative drug RY023 are mediated through α4-containing receptors, the ethanol antagonistic effects of RY023 are not.

The renewed focus on these drugs will hopefully lead to a better understanding of the mechanism of actions of these drugs. It is hoped that such knowledge will enable the development of a second generation of better, safer and with a good bit of luck, clinically viable ethanol antagonist. Should such drugs be developed, they will have widespread applications in the reversal of intoxicating ethanol effects, treatment of acute ethanol toxicity and possibly in prevention of ethanol addiction and dependence.

Because of the addicting potential of ethanol and the tremendous toll that alcohol dependence indirectly takes on immediate family as well as society, drugs that enable treatment of ethanol addiction and management of ethanol withdrawal are sorely needed. Understanding the molecular changes that result in ethanol withdrawal are key to achieving this goal. Although studies with the α4 KO have not shed much light on ethanol withdrawal effects, they cannot be excluded as important players in ethanol withdrawal effects just yet. The overwhelming evidence linking changes in α4 to ethanol withdrawal warrant a second look at the role of these receptors. Use of conditional mutants or knockdown models of α4-containing receptors may enable the study of α4 containing receptors without the complications of compensatory mechanisms.

Even with the caveat that accompanies a global knockout model, α4 KO mice have furthered our understanding of physiological regulation of memory and the actions of gaboxadol, isoflurane, alphaxalone and Ro15-4513. In addition, this model has helped challenge notions about involvement of α4-containing receptors in actions of etomidate, propofol and RY023. Thus, the global knockout strategy, although imperfect, continues to provide a wealth of information and advance our understanding about neurotransmitter-receptor functions.

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List of Abbreviations

AWS – Alcohol withdrawal syndrome

BEC – Blood ethanol concentration

CIE – Chronic intermittent ethanol

CNS – Central nervous system

EC_{50} – Half maximal effective concentration

GABA A – γ aminobutyric acid type A

GABA – γ aminobutyric acid

HET – Heterozygous

i.p. – Intraperitoneal

KI – Knockin

KO – Knockout

LORR – Loss of righting reflex

MAC – Minimum alveolar concentration

mIPSC – Miniature inhibitory postsynaptic current

mRNA – Messenger ribonucleic acid

r.o. – Retro-orbital

RORR – Recovery of righting reflex

Ro15-4513 – ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5-α)BZ-3-carboxylate

RY023 – tert-butyl 8-[trimethylsilyl] acetylene-5,6-dihydro-5-methyl-6- oxo-4H-imidazo [1,5a] [1,4] benzodiazepine-3-carboxylate

THIP – Gaboxadol

WT – Wildtype