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Deletion of $GABA_A$ Receptor Alpha-1 Subunit-containing Receptors Alters Responses to Ethanol and Other Anesthetics

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Running Title Page

$GABA_A$ Receptor α1 Knockout Mouse Characterization

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Abstract

$GABA_A$ receptors have been implicated in mediating several acute effects of ethanol including anxiolysis, ataxia, sedation/hypnosis and anticonvulsant activity. Ethanol sensitivity of neurons has been associated with expression of α1 subunit-containing receptors. The objective of this study was to determine the contribution of α1 subunit containing receptors to ethanol responses in comparison to neurosteroids and other anesthetics using $GABA_A$ receptor α1 subunit knockout mice. Deletion of α1 subunit-containing receptors did not alter the anxiolytic, ataxic, anticonvulsant or hypnotic effects of ethanol or acute functional tolerance to ethanol, but did increase sensitivity to the locomotor-stimulating effects of ethanol. The ability of ethanol to potentiate muscimol-stimulated chloride uptake and ethanol clearance was also not altered following α1 subunit deletion. The anticonvulsant and hypnotic effects of neurosteroids as well as their potentiating effect on GABA-mediated Cl^- uptake were unaltered in $\alpha1^{-/-}$ mice. The hypnotic effect of pentobarbital, etomidate, midazolam were reduced while the effect of ketamine was enhanced in $\alpha1^{-/-}$ mice. Thus, $GABA_A$ receptor α1 subunit-containing receptors appear to influence the motor-stimulating effect of ethanol and the sedative/hypnotic effects of some anesthetics, but not ethanol. These receptors do not appear to be necessary for most ethanol responses, suggesting involvement of other $GABA_A$ receptor subtypes or other targets altogether.

γ-aminobutyric acid type A ($GABA_A$) receptors form the major inhibitory neurotransmitter system expressed in the CNS and are the targets of several classes of drugs including alcohols, inhalation anesthetics, neuroactive steroids, barbiturates and benzodiazepines. $GABA_A$ receptors are heteromeric protein complexes consisting of several homologous membrane-spanning glycoprotein subunits. $GABA_A$ receptor subunits cloned from the mammalian CNS have been divided into classes, with some containing several isoforms: 6α, 4β, 3γ, one δ, one ε, one π, and one θ. (Sieghart and Sperk, 2002). Furthermore, α1 subunits are the most abundant α subunit variant expressed in brain and may serve as important targets for ethanol (McKernan et al., 1991a; McKernan et al., 1991b).

Although the behavioral effects of acute systemic ethanol exposure (i.e., anxiolytic, anticonvulsant, ataxic, sedation/hypnosis) are well established, the neuronal mechanisms underlying these actions remain elusive. Similarities between the acute effects of benzodiazepines (BZDs), neurosteroids and ethanol implicate $GABA_A$ receptors as a site of action for ethanol. Indeed, several findings suggest $GABA_A$ receptor involvement in the sedative/hypnotic and anxiolytic actions of ethanol, similar to those of BZDs (Grobin et al., 1998, for review). At the functional level, physiologically relevant concentrations of ethanol (20-60 mM) potentiate muscimol-stimulated chloride uptake in cerebral cortical synaptoneuorosomes of rats (Suzdak et al., 1986a; Morrow et al., 1988a), mouse cerebellar microsacs (Allan and Harris, 1987) and cultured spinal cord neurons (Mehta and Ticku, 1988). Ethanol potentiation of $GABA_A$ receptor-mediated chloride uptake as well as the behavioral effects of ethanol are blocked by $GABA_A$ receptor antagonists and inverse agonists (Suzdak et al., 1986b; Ticku and Kulkarni, 1988). However, direct interaction of ethanol with neuronal $GABA_A$ receptors using patch clamp recording techniques has rarely been observed at these

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ethanol concentrations (Frye et al., 1994; Crews et al., 1996; Marszalec et al., 1998). One recent study demonstrates ethanol (1 mM) enhancement of GABA responses in progesterone-withdrawn rats that exhibit increased α4δ subunit expression (Sundstrom-Poromaa et al., 2002). Although it remains inconclusive whether ethanol's actions through $GABA_A$ receptors are direct or indirect, the subunit composition of the receptor may be an important determinant of ethanol's effects on the CNS.

It has been proposed that the acute effects of ethanol are mediated through α1-containing receptors. Studies using the α1 subunit-selective ligand zolpidem determined a high correlation between [^{3}H]zolpidem binding to neurons and sensitivity to ethanol, suggesting that αl-containing receptors may be involved in mediating ethanol-induced neuronal inhibition (Breese et al., 1993). Ethanol was found to enhance neuronal responses to GABA in the neocortex, medial septum, inferior colliculus, substantia nigra pars reticulata, ventral pallidum and cerebellum, but not in the hippocampus or ventral tegmental area (Palmer and Hoffer, 1990; Soldo et al., 1994). This localization of ethanol-sensitive $GABA_A$ receptors is closely correlated to brain regions exhibiting high affinity zolpidem binding (Breese et al., 1993; Criswell et al., 1995). Moreover, these brain areas were found to preferentially express α1, β2 and γ2 subunits suggesting that the α1β2γ2 subtype confers ethanol sensitivity (Criswell et al., 1995; Criswell et al., 1997).

The effects of chronic ethanol consumption on $GABA_A$ receptor function and subunit expression further supports the role of subunit composition in determining ethanol sensitivity. Chronic ethanol exposure produces reduced efficacy of muscimol agonists, BZDs and ethanol as observed in functional assays (Allan and Harris, 1987; Morrow et al., 1988b). These changes in $GABA_A$ receptor function following chronic ethanol consumption are associated with significant

increases in α4, γ2s and γ1 levels and a decrease in α1 subunit expression (Devaud et al., 1997). Furthermore, the observed changes in subunit expression may explain the behavioral tolerance to ethanol, cross-tolerance to BZDs and sensitization to the anticonvulsant action of neurosteroids following chronic ethanol consumption (Grobin et al., 1998).

Increasing evidence for the role of $GABA_A$ receptors in the acute actions of ethanol have led to the production of several subunit-specific knockout mouse lines used to explore the contribution of subunit subtypes to ethanol action. While knockout mice of the α6 and γ2L subunits exhibited normal responses to ethanol, δ subunit knockout mice were less sensitive to some ethanol responses, possibly due to reduced sensitivity to neurosteroids (Homanics et al., 1997; Homanics et al., 1999; Mihalek et al., 1999; Mihalek et al., 2001). Initial characterization of $\alpha1^{-/-}$ mice revealed a dramatic loss of $GABA_A$ receptor number, BZD sites, a reduction in muscimol-stimulated Cl^- uptake, increased susceptibility to bicuculline-induced seizure and a pathologic tremor (Kralic et al., 2002b). Furthermore, loss of α1-containing receptors resulted in a compensatory increase in α2 and/or α3-containing receptors that may contribute to altered functional and behavioral responses to BZD site agonists including reduced sensitivity to zolpidem which might predict reduced responses to ethanol (Vicini et al., 2001; Kralic et al., 2002b). The goals of the present studies were to identify the effects of α-1 subunit deletion on functional and behavioral responses elicited by ethanol, neurosteroids and anesthetics.

Materials and Methods

Mouse Production. Genetically engineered male and female mice were produced as described (Vicini et al., 2001) and raised in colonies at the University of North Carolina-Chapel Hill and the University of Pittsburgh. Briefly, the control α1 allele was an unrecombined floxed allele in which exon 8 of the α1 gene was flanked by loxP sites. The amount and distribution of α1 protein produced from this floxed allele does not differ from a true wild type allele (unpublished observations). The knockout α1 allele was a floxed allele following Cre-mediated recombination. This recombined allele has been demonstrated to be a true null allele (Vicini et al., 2001; Kralic et al., 2002b). Control ($\alpha1^{+/+}$) and knockout ($\alpha1^{-/-}$) mice of F4-F6 generations on a C57BL/6J X Strain 129Sv/SvJ hybrid genetic background were derived from heterozygous breeding pairs. All mice were genotyped by Southern blot analysis of tail DNA (Vicini et al., 2001). After weaning, mice were group housed, given free access to standard rodent chow and water and maintained on a 12-h alternating light/dark schedule with lights on at 7:00 AM. All studies were conducted with mice between 7 to 12 weeks of age, were carried out in accordance with the Guide for the Care and Use of Laboratory Animals by the U.S. National Institutes of Health, and were approved by the Institutional Animal Care and Use Committees at the Universities of North Carolina-Chapel Hill and Pittsburgh.

Chloride Uptake Assay. Following decapitation, brains were immediately removed and placed in ice cold saline from which cerebral cortices were isolated. Seven sets of cortices per genotype were pooled for each experiment. Synaptoneurosomes were prepared and Cl^- uptake was conducted as previously described (Morrow et al., 1988). The synaptoneurosomal pellet was resuspended in 6.6 volumes of ice cold assay buffer (20 mM Hepes, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4) for a final protein concentration of approximately

5 mg/ml. The homogenate (200 μL) was aliquotted per assay tube and pre-incubated at 30°C for 12 min. Muscimol-stimulated Cl^- uptake was initiated by addition of 0.2 □Ci ^{36}Cl (NEN, Boston, MA) in the presence of an EC_{30} concentration of muscimol (2.5 □M and 5 □M for $\alpha1^{+/+}$ and $\alpha1^{-/-}$ samples, respectively) alone or in conjunction with THDOC (1 nM – 10 □M) (Steraloids; Newport, RI) or 30 mM ethanol (AAPER Alcohol and Chemical Co.; Shelbyville, KY). The respective EC_{30} of muscimol was used for each genotype since it was previously determined by conducting concentration-response curves that the EC_{50} and E_{max} of muscimol were altered following α1 subunit deletion (EC_{50}: 3.7 ± 0.6 and 7.9 ± 1.0 □m; E_{max}: 30.4 ± 1.1 and 21.7 ± 0.7 nmol Cl^-/mg protein in $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice, respectively) (Kralic et al., 2002b). The solution was vortexed and uptake terminated after 5 seconds by addition of 4 mls of ice cold assay buffer containing 100 μM picrotoxin with rapid vacuum filtration over S&S #32 filters using a single manifold filter. The synaptoneurosomes were washed twice with 4 mls buffer; the filter allowed to dry and radioactive counts determined by liquid scintillation spectroscopy. Chloride uptake was measured in the absence of muscimol and subtracted from all tubes to determine muscimol-stimulated chloride uptake at the respective EC_{30} concentrations. Net potentiation by drugs was obtained by subtracting muscimol-stimulated chloride uptake from total uptake obtained from drug plus muscimol. Concentration-response curves were evaluated using non-linear regression by Prism (Graphpad, San Diego, CA) to obtain the EC_{50} and E_{max} values and compared between genotype by Student's t test.

Ethanol Metabolism and Clearance. Mice were tested for ethanol clearance and metabolism following injection of ethanol (3.5 g/kg; i.p.). Blood was collected from the retro-orbital sinus at 1 and 3 h post-injection. Plasma ethanol levels (BECs) were determined using a

commercial kit (Procedure No. 333-UV; Sigma, St. Louis, MO). Data were analyzed using Sigmastat and compared by two-way ANOVA.

Open Field and Elevated Plus Maze. Naïve mice were evaluated for basal anxiety levels and locomotor activity as well as for the anxiolytic and locomotor stimulant effects of ethanol using the elevated plus-maze and open field exploratory observation. Mice were transported to the testing room the night prior to testing. Animals were weighed and tested between 9 and 11:00 AM. Ethanol (0.5, 1.0, or 1.5 g/kg) or saline was injected i.p. after which mice were returned to their home cage for ten minutes. Mice were then placed on the center platform of the elevated plus-maze and five anxiety and motor related behaviors were recorded over a five minute period: (1) number of open arm entries, (2) time in open arms, (3) number of closed arm entries, (4) time in the closed arms, (5) total number of arm entries. Following plus-maze testing mice were placed back in their home cages for a five-minute period. Subsequently, mice were tested in the open field exploratory assay. Each mouse was placed facing outward in a corner of an 18 inch x 18 inch clear Plexiglas box with a grid of 4.5 inch squares under the floor. There were sixteen total squares, with four squares being in the center not bounded by the wall of the box. The mice were observed over a ten-minute period, and the number of crossings was recorded manually. Each time the mouse entered a square with a majority of its body, a crossing was recorded, with differentiation between inner and outer squares being noted. Data were analyzed using SigmaStat using two-way ANOVA with Tukey's post hoc test.

Accelerating Rotarod. Mice were tested for motor coordination and for the ataxic effects of low dose ethanol on an accelerating rotarod (Model 7650, Ugo Basile, Italy). To test motor coordination and motor learning, each mouse performed a single daily trial for nine consecutive days. Saline was administered i.p ten minutes before the trial on day 8 in order to

habituate the mice to injection. On the final day (trial 9), each mouse was injected with ethanol (1.5 g/kg; ip) and tested 10 min later on the rotarod. All trials were conducted for a total of 180 seconds, during which the rotarod increased in speed from 5-40 rpm. The time each mouse remained on the rod was recorded. Data were analyzed with SigmaStat using repeated measures two-way ANOVA with Tukey's post hoc test.

Acute Functional Tolerance. Acute functional tolerance assays the rapid development of tolerance to ethanol and is based on the ataxic effects of ethanol (Erwin and Deitrich, 1996). Mice were acclimated to a stationary 2.5 cm diameter rod for one minute (Rotarod, model 7650, Ugo Basile, Italy). The mice were then injected with ethanol (1.75 g/kg; i.p.) and tested again on the stationary rod for balance. Once the mice could remain on the rod for 60 seconds (t1), a blood sample was collected retro-orbitally to determine the first blood-ethanol concentration (BEC1) and then immediately injected with 2.0 g/kg ethanol. The mice were subsequently tested every five minutes for ability to remain on the rod for 60 seconds. Once this was achieved (t2), a second blood sample was drawn for blood ethanol determination (BEC2). Acute functional tolerance (AFT) was determined as the difference between BEC2 and BEC1. Parameters AFT, BEC1, BEC2, t1 and t2 were statistically analyzed by Student's test using SigmaStat.

Bicuculline-Induced Seizure Threshold Test. Seizure thresholds were determined at the beginning of the light cycle from 8:00 to 12:00 hours in a room adjacent to the colony room under normal lighting conditions with low level white background noise as previously described (Devaud et al., 1995). Mice were injected i.p. with ethanol (2 or 3 g/kg), allopregnanolone (4, 8 or 16 mg/kg) or respective vehicles (saline or 20% 2-hydroxypropyl-□-cylcodextrin/saline) in a 10 ml/kg volume 60 minutes and 15 minutes, respectively, prior to seizure threshold determination. Mice were restrained in a Plexiglas plunger-style mouse restraint (Braintree

Scientific, Inc., Braintree, MA). Threshold determination was made by constant lateral tail vein infusion via a 28 gauge butterfly needle of bicuculline (Sigma Aldrich) dissolved in 0.1 N HCl and diluted with isotonic saline to a final concentration of 0.05 mg/ml, pH 7. The solution was infused at a constant rate of 0.5 ml/min; the endpoint taken as the first myoclonic jerk of the head and neck. This time point precedes forepaw clonus and generalized tonic/clonic convulsions. Each animal was tested once. Seizure thresholds were determined by experienced observers who were blind to the experimental conditions. Seizure thresholds were calculated from the time of infusion x dose of bicuculline/body weight and presented as mg/kg bicuculline. Data were analyzed using SigmaStat using two-way ANOVA with Tukey's post hoc test.

Loss of Righting Reflex. Mice were tested for duration of the loss of the righting reflex (i.e., sleep time) in response to ethanol (Pharmco, Brookfield, CT; 3.0 g/kg & 3.5 g/kg); the BZD, midazolam (ESI Lederle, Philadelphia, PA; 75 mg/kg); the neurosteroid, pregnanolone (Sigma, St. Louis, MO; 8 mg/kg); the barbiturate, pentobarbital (Abbott, Chicago, IL; 45 mg/kg); and the anesthetics, propofol (AstraZeneca, Wilmington, DE; 4 mg/kg), ketamine (Fort Dodge, Fort Dodge, IA; 150 mg/kg) and etomidate (Bedford Laboratories, Bedford, OH; 20 mg/kg) as previously described (Mihalek et al., 1999). Doses were chosen based on their capacity to induce hypnosis. All agents were injected i.p. except for pregnanolone and propofol which were injected intravenously via the retro-orbital sinus. Upon becoming ataxic, mice were placed on their backs in a v-shaped trough, and the time 'down' was noted. Mice were monitored until they could right themselves three times in thirty seconds, and the time was recorded when they passed this criteria. A heat lamp and random rectal temperature measurements were used to ensure normothermia. Mice were tested in 3 groups with a seven day period between each drug: Group #1 received midazolam and etomidate; Group #2 received diazepam, propofol,

pregnanolone, and ketamine; Group #3 received ethanol only. All assays were performed by an investigator blinded to the genotypes of the animals being tested. Mice in the ethanol, ketamine, and etomidate experiments were determined to have a misplaced injection and excluded from data analysis if the time to lose the righting reflex after injection was greater than 4 min (Ponomarev and Crabbe, 2002). Mice in all other sleep time experiments were determined to have a misplaced injection and excluded from data analysis if the time to lose the righting reflex following injection was greater than two standard deviations from the group mean. Data were evaluated using a two-way ANOVA with sex and genotype as factors. If no effect of sex was observed, data were collapsed within genotype and reanalyzed by ANOVA.

Results

Ethanol and THDOC Potentiation of Muscimol-stimulated Chloride Uptake. Previous results demonstrated that the potency and efficacy of muscimol-stimulated Cl^- uptake was reduced in $\alpha1^{-/-}$ mice (Kralic et al., 2002a). Ethanol and THDOC potentiation of muscimol-stimulated chloride uptake was measured in synaptoneurosomal preparations of cerebral cortex from $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice using respective EC_{30} concentrations of muscimol. Ethanol and THDOC potentiated the effects of muscimol on chloride uptake in $\alpha1^{+/+}$ mice confirming previous reports (Suzdak et al., 1986b; Allan and Harris, 1987; Morrow et al., 1988b). Net potentiation by ethanol (30 mM) over EC_{30} muscimol-stimulated chloride uptake was similar between $\alpha1^{+/+}$ and $\alpha1^{-/-}$ (3.2 ± 0.6 and 3.8 ± 0.8 nmol Cl^-/mg protein), respectively (Figure 1A). THDOC (1 nM – 10 μM) potentiated chloride uptake in both $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice in a dose-dependent manner resulting in similar potency of THDOC (522 ± 76 and 486 ± 59 nM) and maximal efficacy (15 ± 1 and 14 ± 1 nmol Cl^-/mg protein) (Figure 1B).

Ethanol Metabolism and Clearance. To determine if $\alpha1^{-/-}$ mice differed from their $\alpha1^{+/+}$ littermates with respect to ethanol pharmacokinetics, the BEC at 60 and 180 min was measured after i.p. injection of 3.5 g/kg ethanol. Female mice exhibited significantly higher BECs than male mice (235 ± 9 and 186 ± 10 mg/dl, respectively) at the 180 min time point only [two-way ANOVA, gender: $F_{(1,22)} = 14.3$, $p<0.001$]. However, there was no effect of genotype on the BEC at 60 min ($\alpha1^{+/+}$, 362 ± 16 and $\alpha1^{-/-}$, 341 ± 16 mg/dl) or 180 min ($\alpha1^{+/+}$, 203 ± 9 and $\alpha1^{-/-}$, 218 ± 10 mg/dl), thereby allowing valid comparisons between genotypes for behavioral responses to ethanol.

Elevated Plus Maze. Baseline performance on the elevated plus maze did not vary significantly with genotype as previously reported (Kralic et al., 2002a). Total arm entries, a measure of locomotor activity, were increased by ethanol in both $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice (Fig. 2A) [two-way ANOVA, Dose: $F(3, 155) = 9.3$, $p<0.001$]. However, $\alpha1^{-/-}$ mice were more sensitive to the locomotor-activating effects of ethanol at the 1.0 g/kg dose compared to $\alpha1^{+/+}$ mice (Fig. 2A) (Tukey's post hoc, $p<0.05$). To determine if deletion of $\alpha1$ subunits altered the anxiolytic effect of ethanol, the behavior of $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice was evaluated following ethanol administration (0.75, 1.0 and 1.5 g/kg) in the elevated plus maze. As presented in Figure 2, ethanol administration produced an anxiolytic effect in both $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice. Ethanol increased both the percentage of open arm entries (Fig. 2B) [two-way ANOVA, GT: $F(1,155) = 14.3$, $p<0.001$; Dose: $F(3, 155) = 13.7$, $p<0.001$] and percentage of time spent in open arms (Fig. 2C) [two-way ANOVA, Dose: $F(3, 155) = 13.5$, $p<0.001$]. Since no effect of gender was detected on any parameter, data from both genders was collapsed for these analyses.

Exploratory Activity. Baseline exploratory activity in the open field did not vary significantly with genotype. However, sensitivity to the locomotor stimulating effect of ethanol was enhanced in $\alpha1^{-/-}$ mice. Ethanol (0 -1.5 g/kg) administration produced a 65 -125% increase in total square entries (Fig. 3A) [two-way ANOVA, GT: $F_{(1,149)} = 10.0$, $p<0.01$; Dose: $F_{(3,149)} = 6.7$, $p<0.001$; GT x Dose: $F_{(3,149)} = 4.0$, $p<0.01$] in $\alpha1^{-/-}$ with no change in $\alpha1^{+/+}$ mice. The anxiolytic effect of ethanol assessed as the percentage of center square entries did not significantly vary with dose or genotype (Fig. 3B). Since no effect of gender was detected in this test, data from both genders was collapsed for these analyses.

Accelerating Rotarod. Since motor coordination involves proper cerebellar $GABA_A$ receptor function and cerebellum contains a relatively high proportion of $\alpha1$-containing

receptors, the rotarod test was employed to determine whether deletion of α1 subunit-containing receptors affected baseline motor coordination and the ataxic effects of acute ethanol administration. Over the first seven trials of baseline measurements, the ability of the mice to remain on the rod increased over the trials, but there was no significant difference between $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice with respect to the amount of time able to remain on the rod in 6 of 7 trials [two-way repeated measures ANOVA, Trial: $F(6, 412) = 33.78$, $p<0.001$, Genotype x Trial: $F(6, 412) = 3.24$, $p<0.01$] (data not shown). However, compared to females, males showed reduced ability over all seven trials to remain on the rod although mice of both genders reached similar criterion by trial 7 [two-way repeated measures ANOVA, Gender: $F(1, 412) = 13.56$, $p<0.001$, Trial: $F(6, 412) = 33.0$, $p<0.001$, Gender x Trial: $F(6, 412) = 3.23$, $p<0.01$] (data not shown). Control $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice exhibited a similar performance on the rotarod following saline injection (trial 8) and a similar ataxic effect following ethanol (1.5 g/kg, i.p.) administration (trial 9, Fig. 4) [two-way repeated measures ANOVA, Trial: $F(1, 116) = 25.98$, $p<0.001$]. Since no effect of gender was detected, data from both genders was collapsed for analysis of trials 8 and 9.

Acute Functional Tolerance. To determine if $GABA_A$ α1-containing receptors contribute to the development of tolerance to an acute injection of ethanol, acute functional tolerance to ethanol was measured. The test for acute functional tolerance to ethanol measured four parameters: t1, the time to regain balance on a stationary rod after an initial injection of ethanol; BEC1, blood ethanol concentration at t1; t2, time to regain balance after a second ethanol injection; and BEC2, blood ethanol concentration at t2. Comparison of $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice revealed no difference in the motor ataxic effects of ethanol at t1 or t2 or respective BECs. Furthermore, the AFT parameter was similar between genotypes (Table 1). Since no effect of gender was detected on any parameter, data from both genders was collapsed for these analyses.

Anticonvulsant Effects of Allopregnanolone and Ethanol. The bicuculline-induced seizure threshold assay was employed to determine the anticonvulsant effect of ethanol and allopregnanolone in $\alpha1^{+/+}$, $\alpha1^{+/-}$ and $\alpha1^{-/-}$ mice. Our previous study demonstrated that bicuculline seizure thresholds were reduced in $\alpha1^{-/-}$ mice (Kralic et al., 2002a). Ethanol administration (2 and 3 g/kg) produced an anticonvulsant effect in all genotypes (Fig.5A) [two-way ANOVA, GT: $F_{(2,59)} = 21.0$, $p<0.001$; Dose: $F_{(2,59)} = 21.08$, $p<0.001$]. At the highest dose, ethanol increased the seizure threshold to a similar magnitude; furthermore, the potency of ethanol did not vary with genotype. Allopregnanolone administration (4, 8 and 16 mg/kg) produced a dose-dependent anticonvulsant effect in all genotypes (Fig. 5B) [two-way ANOVA, GT: $F_{(2,85)} = 31.1$, $p<0.001$; Dose: $F_{(3,85)} = 16.81$, $p<0.001$]. At the highest dose, allopregnanolone increased the seizure threshold to a similar magnitude; furthermore, the potency of allopregnanolone did not vary with genotype. Since no effect of gender was detected in either study, data from both genders was collapsed for these analyses.

Drug-induced Hypnosis. The loss of righting reflex (LORR) assay was conducted to determine if the hypnotic effects of ethanol (3 and 3.5 g/kg, i.p.), pregnanolone (8 mg/kg), pentobarbital (45 mg/kg), midazolam (75 mg/kg), etomidate (20 mg/kg), ketamine (150 mg/kg) and propofol (33 mg/kg) were altered following deletion of $\alpha1$ subunit-containing receptors. As shown in Figure 6, the duration of LORR induced by ethanol, pregnanolone and propofol did not differ between $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice. The duration of pentobarbital and midazolam-induced LORR were reduced in $\alpha1^{-/-}$ mice [Student's t test, $p<0.001$ and $p<0.05$, respectively]. The hypnotic effect of etomidate was greater in female $\alpha1^{+/+}$ than male $\alpha1^{+/+}$ mice and reduced by deletion of $\alpha1$ subunits in female $\alpha1^{-/-}$ mice only [two-way ANOVA, GT: $F(1, 30) = 5.8$, $p<0.05$, Gender: $F(1, 30) = 7.6$, $p<0.05$]. The duration of LORR induced by ketamine was greater in $\alpha1^{-/-}$

mice than in $\alpha 1^{+/+}$ mice [Student's t test, $p<0.05$]. Data from both genders was collapsed for all drugs except etomidate for these analyses since no effect of gender was detected.

Discussion

Previous examination of mice with a targeted deletion of the $GABA_A$ receptor α1 subunit revealed loss of ~50% of all $GABA_A$ receptors and altered functional and behavioral responses to BZD site agonists (Kralic et al., 2002a). Surprisingly, deletion of all α1-containing receptors did not alter the ability of ethanol or allopregananolone to potentiate $GABA_A$ receptor function. Moreover, the anxiolytic, ataxic, anticonvulsant or hypnotic effects of ethanol and the development of acute functional tolerance was unaffected by deletion of α1 subunits. The anticonvulsant and hypnotic effects of neurosteroids were unaltered following deletion of α1 subunits as predicted by results of neurosteroid potentiation of $GABA_A$ receptor function. In contrast, alpha-$1^{-/-}$ mice were more sensitive to the locomotor stimulating effects of ethanol. These results suggest that most of the effects of ethanol and neurosteroids are not dependent upon the expression of α1-containing $GABA_A$ receptors. However, low dose ethanol responses such as the stimulating effects may be dependent upon α1-containing receptors. Moreover, the duration of LORR induced by ethanol, pregnanolone and propofol were unaffected by deletion of α1 subunits while the effects of pentobarbital, midazolam, etomidate and ketamine were significantly altered, demonstrating the selectivity of α1 subunit deletion on drug responses.

The absence of any effect of α1 subunit deletion on the ability of ethanol to potentiate $GABA_A$ receptor-mediated chloride uptake suggests that ethanol's actions are not selectively mediated through α1 subunit-containing $GABA_A$ receptors. These results do not support findings in which α1 subunit expression/zolpidem responses predicted the sensitivity of neurons to ethanol (Criswell et al., 1995; Duncan et al., 1995). Furthermore, our results suggest that the reduction in α1 subunit expression following chronic ethanol consumption does not underlie the

decreased sensitivity or tolerance to the functional effects of acute ethanol (Morrow et al., 1988a; Devaud et al., 1995; Devaud et al., 1997).

As with ethanol, neurosteroid potentiation of GABA-mediated chloride uptake was not altered following deletion of α1 subunits. This result supports findings from recombinant receptor systems in which there was no or only a modest contribution of α subunit subtypes to the pharmacological profile of neurosteroids (Puia et al., 1993; Lambert et al., 2001). The δ subunit, whose expression is unaltered in $\alpha1^{-/-}$ mice (Kralic et al., 2002b), appears to be influential in mediating sensitivity to neuroactive steroids *in vivo* as well as sensitivity to the anticonvulsant effects of ethanol (Mihalek et al., 1999; Mihalek et al., 2001). The presence of δ subunits in the receptor have been shown to confer increased neurosteroid efficacy *in vitro* (Brown et al., 2002).

Both ethanol and neurosteroids have been shown to produce anticonvulsant effects in several different seizure paradigms (Kokate et al., 1994; Finn et al., 1995; Devaud et al., 1996). The lack of effect of α1 subunit deletion on the anticonvulsant effects of these compounds suggests that their actions are not selective for α1-containing receptors. Furthermore, the absence of any effect of α1 subunit deletion helps to define which changes in $GABA_A$ receptor subunit expression may underlie the increased sensitivity to neurosteroids following chronic ethanol consumption (Devaud et al., 1996). Therefore, the decrease in α1 subunit expression following chronic ethanol consumption is unlikely to contribute to the increased sensitivity to the anticonvulsant effects of neurosteroids (Devaud et al., 1997). Studies in δ subunit knockout mouse suggest that the anticonvulsant of ethanol is influenced by the presence of the δ subunit (Mihalek et al., 2001). Furthermore, the absence of any effect on the functional and anticonvulsant response of neuroactive steroids, selective modulators of $GABA_A$ receptors,

suggests that the reduction in $GABA_A$ receptor number does not account for changes in the anticonvulsant activity of other compounds (e.g,, BZDs) (Kralic et al., 2002a).

The surprisingly normal behavioral responses to ethanol observed in $\alpha1^{-/-}$ mice suggest that mechanisms involving indirect interaction of ethanol with $GABA_A$ receptors through induction of intermediary proteins, endogenous modulators, or action on non-GABAergic systems altogether may be responsible for many behavioral actions of ethanol. These results are also compatible with other $GABA_A$ receptor subunits being directly involved in ethanol's mechanism of action. Ethanol may also exert its effects by modulating post-translational modification pathways since targeted deletion of PKCγ and PKCε reduced and increased, respectively, the sensitivity to acute ethanol (Harris et al., 1995; Hodge et al., 1999; Bowers et al., 2001). In addition, the ability of systemic ethanol administration to induce physiologically relevant levels of allopregnanolone is required to observe certain behaviors and electrophysiological effects of ethanol (Morrow et al., 1999; Khisti et al., 2000; VanDoren et al., 2000). Since various ethanol effects are postulated to involve neurosteroid intermediates, it is noteworthy that α1 subunit deletion lacked an effect on responses to either modulator. This observation is consistent with the hypothesis that both of these modulators have indirect actions on $GABA_A$ receptors (Morrow et al., 2001). Although adaptations in $GABA_A$ receptor subunit expression, notably α2 and α3, could compensate for the loss of α1 expression in knockout mice masking changes in ethanol sensitivity that would be otherwise observed, this outcome would argue against subunit selectivity of ethanol (Kralic et al., 2002a; Kralic et al., 2002b). Lastly, since ethanol has been shown to affect other neurotransmitter systems, the behavioral effects of ethanol described may be mediated by a non-GABAergic system mechanism.

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The differential effects of α1 subunit deletion on the hypnotic effects of the anesthetics tested may suggest selectivity of anesthetics for α1-containing receptors or the importance of adaptations in $GABA_A$ receptors or other systems. The selective effects of α1 subunit deletion on the hypnotic effects of pentobarbital and etomidate over ethanol, pregnanolone and propofol suggest a contribution of α1-containing receptors to these actions. The enhanced hypnotic effects of ketamine, an NMDA antagonist, suggest a compensatory adaptation of the glutamatergic system in response to the loss of $GABA_A$ receptors or a demonstration of the imbalance in CNS excitability resulting from α1 subunit deletion. The reduced hypnotic effect of midazolam in $\alpha1^{-/-}$ mice was unexpected since it had been previously shown that diazepam, another member of the BZD class, exhibited enhanced effects in the same test (Kralic et al., 2002a). These results suggest that these compounds may have unique pharmacological profiles such as selectivity for Type I and II BZD sites. Indeed, midazolam, rather than diazepam, is prescribed for its anesthetic properties. Since etomidate has been shown to act upon β3-containing receptors for high-dose effects (Jurd et al., 2002), the reduced effect of etomidate in the LORR assay is supported by the reduced expression of β2/3 in $\alpha1^{-/-}$ mice (Kralic et al., 2002a).

The hypnotic effects of some of these same drugs have been studied in an independently generated α1 knockout mouse line (Blednov et al., 2003). Results with zolpidem and some benzodiazepines are consistent between the different mouse lines (Kralic et al., 2002a; Blednov et al., 2003). Surprisingly, the sleep time in response to some drugs appears to differ between the two α1 knockout mouse lines. For example, Blednov et al. report a reduction in ethanol-induced sleep time in males only, whereas we did not detect a difference in either sex. We observed a reduction in etomidate-induced sleep time in female knockouts and in pentobarbital

sleep time in both sexes, whereas Blednov et al. observed no difference in either sex for both of these drugs. The differences between drug responses of the two α1 knockout mouse lines are likely the result of differences in breeding strategies or genetic backgrounds, or less likely the result of differences in experimental conditions.

In conclusion, we have demonstrated that deletion of α1-containing receptors does not significantly alter the functional and most behavioral responses to ethanol or neurosteroids suggesting that the pharmacological effects of these compounds are not selectively mediated by α1-containing receptors. Moreover, loss of over half of $GABA_A$ receptor expression following deletion of α1 subunits does not alter most of the behavioral effects of acute ethanol administration and suggests that the molecular basis of ethanol actions remains elusive. In contrast, the hypnotic effects of midazolam, pentobarbital and etomidate are altered by deletion of α1 subunits, emphasizing that subunit specificity can play a role in the responses of many GABAergic modulators.

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Footnotes

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Legends for Figures

Figure 1. Deletion of □1 subunits does not alter the ability of ethanol or THDOC to potentiate GABA-mediated chloride uptake. Ethanol and THDOC potentiation of muscimol-stimulated Cl^- uptake in pooled cortical synaptoneurosomes of $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice was determined using EC_{30} concentrations of muscimol 2.5 □M and 5 □M, respectively. (A) Ethanol (30 mM) potentiated Cl^- uptake to a similar extent in $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice (3.2 ± 0.6 and 3.8 ± 0.8 nmol Cl^-/mg protein) over EC_{30} muscimol-stimulated uptake, respectively. (B) Chloride uptake was potentiated by THDOC (1 nM – 10 μM) in a dose-dependent manner in both $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice. The potency (522 ± 76 and 486 ± 59 nM) and maximal efficacy (15 ± 1 and 14 ± 1 nmol Cl^-/mg protein) of THDOC was similar in both $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice, respectively. Data represent the mean ± SEM of four independent experiments each conducted in quadruplicate.

Figure 2. Effect of ethanol (0 –1.5 g/kg) on (A) total arm entries, (B) percentage of open arm entries and (C) percentage of time on the open arms of $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice on the elevated plus maze. Ethanol or vehicle was administered i.p. ten minutes before testing. An anxiolytic effect of ethanol was detected in both $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice; however, $\alpha1^{-/-}$ mice were more sensitive to the motor-stimulating effects of ethanol as observed by total arm entries. Data represent the mean ± SEM, n=17-21 mice/group; Tukey's test, **p<0.01, ***p<0.001 within genotype compared to vehicle; #p<0.05, ##p<0.01 within dose compared to $\alpha1^{+/+}$ mice.

Figure 3. Effect of ethanol (0 – 1.5 g/kg) in $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice on (A) total distance traveled and (B) percentage of entries into the center square during a 10 minute trial in an activity box twenty minutes following i.p. injection. Ethanol dose-dependently increased the distance traveled in $\alpha1^{-/-}$ mice, but not the percentage of entries into the center square. Data represent the mean ± SEM, n=15-20/group; Tukey's test, *p<0.05, ***p<0.001 within genotype compared to vehicle, #p<0.05, ###p<0.001 within dose compared to $\alpha1^{+/+}$ mice.

Figure 4. Effect of ethanol (1.5 g/kg, i.p.) on rotarod performance in $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice. Mice were trained over eight trials on the rotarod following vehicle injection and then tested ten minutes after ethanol injection. There was no difference between $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice in baseline rotarod performance (time remaining on rod) on the eighth trial (black bars) following vehicle injection. A motor incoordinating effect of ethanol (grey bars) was observed in both $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice. Data represent the mean ± SEM, n = 28-30; Tukey's test, ***p<0.001.

Figure 5. Anticonvulsant effect of (A.) ethanol and (B.) allopregnanolone following deletion of α1 subunit-containing receptors. Ethanol (2 and 3 g/kg, i.p) and allopregnanolone (4, 8 and 16 mg/kg, i.p.) administration 60 and 15 minutes, respectively, before determination of bicuculline-induced seizure threshold produced a similar anticonvulsant effect in $\alpha1^{+/+}$, $\alpha1^{+/-}$ and $\alpha1^{-/-}$ mice. Data represent the mean ± SEM, n = 6-13 mice/group.

Figure 6. Duration of loss of righting reflex induced by ethanol (3 and 3.5 g/kg, i.p.), pregnanolone (8 mg/kg, i.v.), etomidate (20 mg/kg, i.p.), propofol (4 mg/kg, i.p.), midazolam (75 mg/kg, i.p.), pentobarbital (45 mg/kg, i.p.) and ketamine (150 mg/kg, i.p.) in $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice. In $\alpha1^{-/-}$ mice, there was a significant reduction in the duration of LORR for etomidate in females, midazolam, pentobarbital and an increase in the duration of LORR for ketamine. Data represent the mean ± SEM; Student's t or Tukey's test, *$p<0.05$ and ***$p<0.001$.

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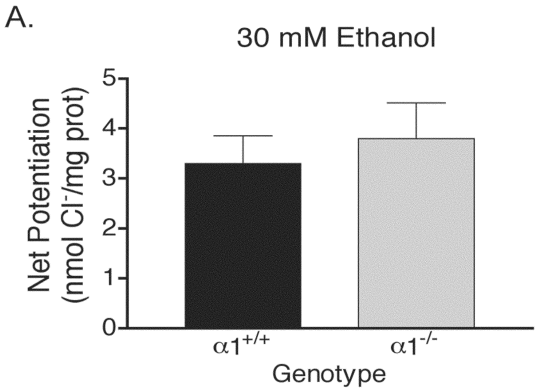
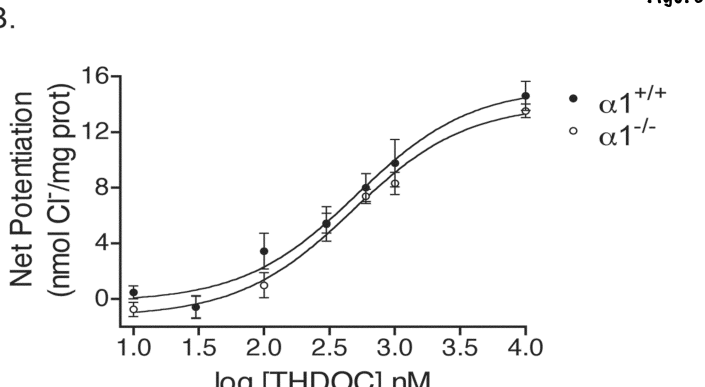
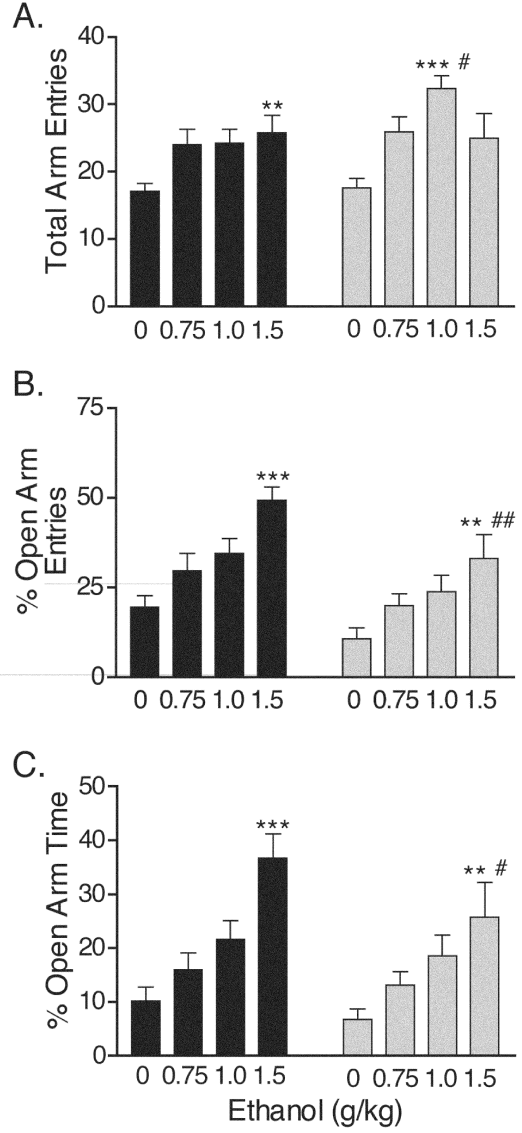
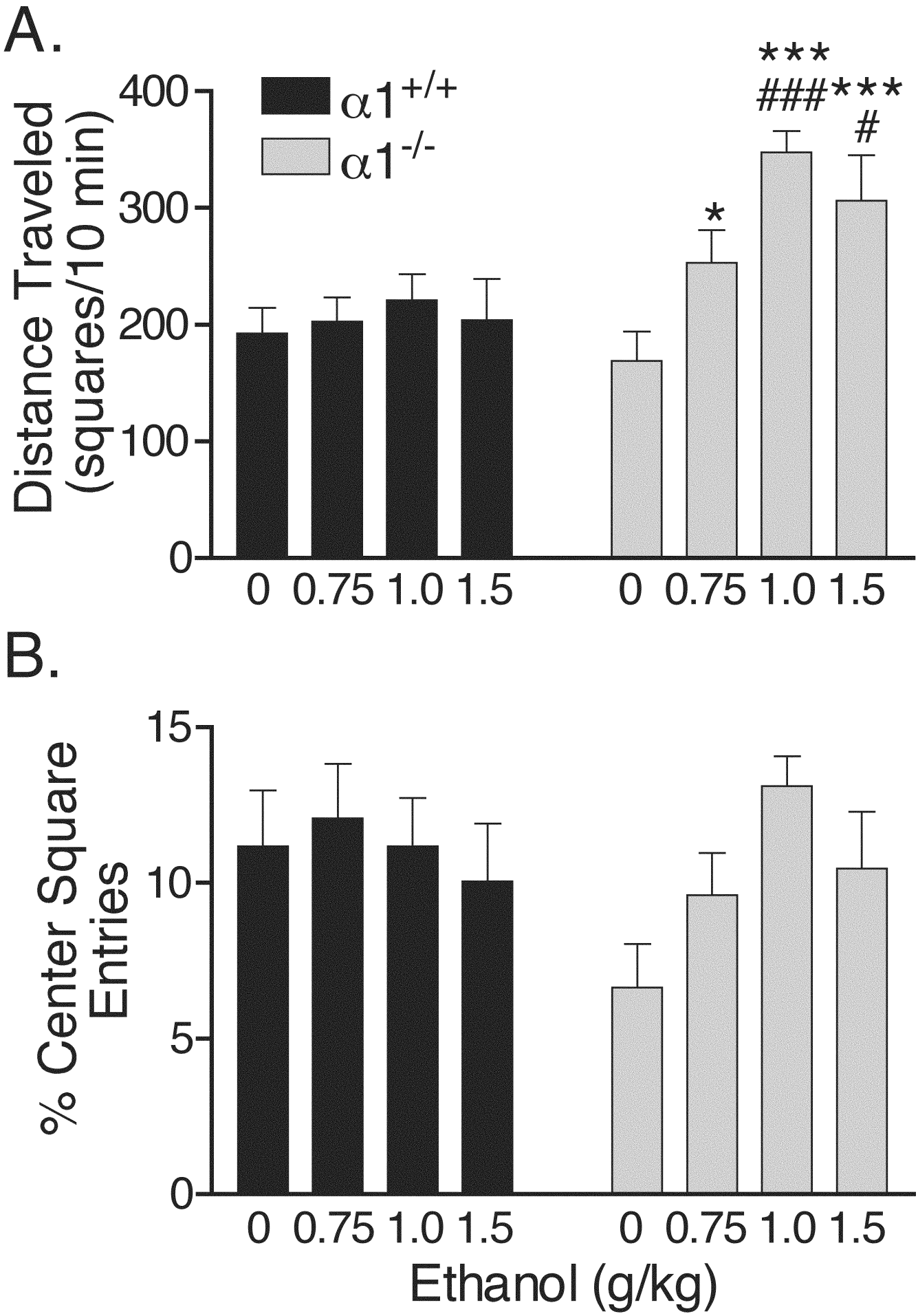
Table 1. Acute Functional Tolerance

Genotype	n	t_1 (min)	BEC_1 (mg/ml)	t_2 (min)	BEC_2 (mg/ml)	AFT (mg/ml)
$\alpha1^{+/+}$	20	6.5 ± 0.3	182 ± 8	93 ± 4	294 ± 20	112 ± 16
$\alpha1^{-/-}$	20	6.0 ± 0.2	191 ± 9	88 ± 4	293 ± 20	102 ± 16


A.
30 mM Ethanol
Net Potentiation
(nmol Cl-/mg prot)
5
4
3
2
1
0
α1+/+
α1-/-
Genotype


B.
Net Potentiation
(nmol Cl-/mg prot)
16
12
8
4
0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
log [THDOC] nM
α1+/+
α1-/-


A.
Total Arm Entries
40
30
20
10
0
**
*** #
α1+/+
α1-/-
0 0.75 1.0 1.5
0 0.75 1.0 1.5
B.
% Open Arm Entries
75
50
25
0
***
** ##
0 0.75 1.0 1.5
0 0.75 1.0 1.5
C.
% Open Arm Time
50
40
30
20
10
0
***
** #
0 0.75 1.0 1.5
0 0.75 1.0 1.5
Ethanol (g/kg)


A.
Distance Traveled (squares/10 min)
400
300
200
100
0
α1+/+
α1-/-
***
###***
#
*
0 0.75 1.0 1.5
0 0.75 1.0 1.5
B.
% Center Square Entries
15
10
5
0
0 0.75 1.0 1.5
0 0.75 1.0 1.5
Ethanol (g/kg)

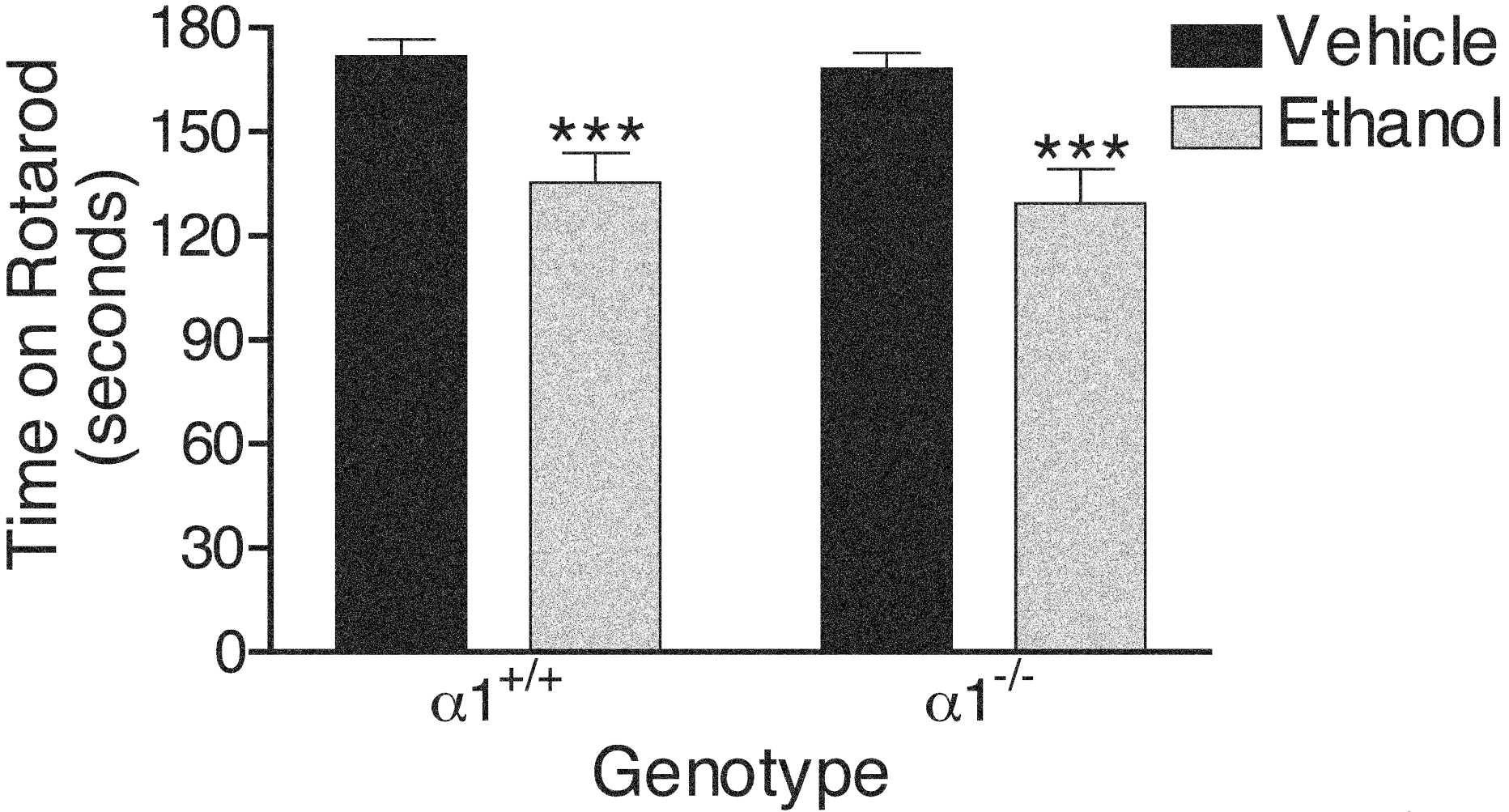

Time on Rotarod (seconds)
180
150
120
90
60
30
0
***
***
Vehicle
Ethanol
α1+/+
α1-/-
Genotype

Figure 4

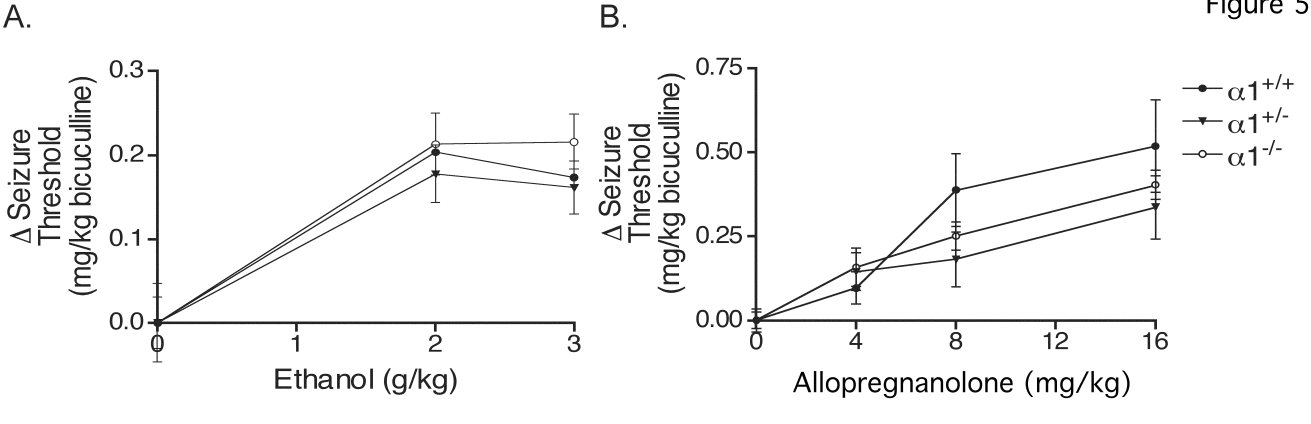
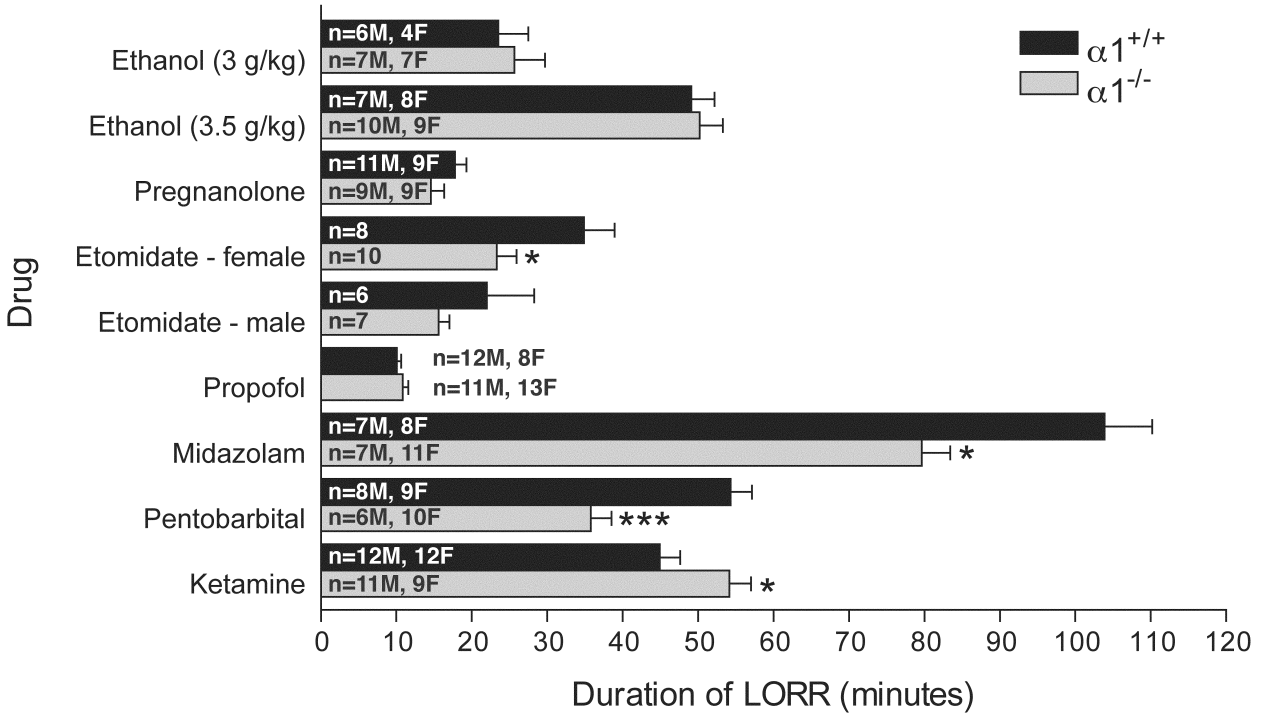

A.
Δ Seizure Threshold (mg/kg bicuculline)
0.3
0.2
0.1
0.0
0
1
2
3
Ethanol (g/kg)
B.
Δ Seizure Threshold (mg/kg bicuculline)
0.75
0.50
0.25
0.00
0
4
8
12
16
Allopregnanolone (mg/kg)
α1+/+
α1+/-
α1-/-

Fig. 6


α1+/+
α1-/-
Drug
Ethanol (3 g/kg)
n=6M, 4F
n=7M, 7F
Ethanol (3.5 g/kg)
n=7M, 8F
n=10M, 9F
Pregnanolone
n=11M, 9F
n=9M, 9F
Etomidate - female
n=8
n=10
*
Etomidate - male
n=6
n=7
Propofol
n=12M, 8F
n=11M, 13F
Midazolam
n=7M, 8F
n=7M, 11F
*
Pentobarbital
n=8M, 9F
n=6M, 10F
***
Ketamine
n=12M, 12F
n=11M, 9F
*
0 10 20 30 40 50 60 70 80 90 100 110 120
Duration of LORR (minutes)