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Alcohol-induced tolerance and physical dependence in mice with ethanol insensitive $\alpha 1$ GABA_A receptors

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Abstract

Background—Although many people consume alcohol (ethanol), it remains unknown why some become addicted. Elucidating the molecular mechanisms of tolerance and physical dependence (withdrawal) may provide insight into alcohol addiction. While the exact molecular mechanisms of ethanol action are unclear, γ -aminobutyric acid type A receptors (GABA_A-Rs) have been extensively implicated in ethanol action. The $\alpha 1$ GABA_A-R subunit is associated with tolerance and physical dependence, but its exact role remains unknown. In this report, we tested the hypothesis that $\alpha 1$ -GABA_A-Rs mediate in part these effects of ethanol.

Methods—Ethanol-induced behavioral responses related to tolerance and physical dependence were investigated in knockin mice that have ethanol-insensitive $\alpha 1$ GABA_A-Rs and wildtype controls. Acute functional tolerance (AFT) was assessed using the stationary dowel and loss of righting reflex assays. Chronic tolerance was assessed on the loss of righting reflex, fixed speed rotarod, hypothermia, and radiant tail flick assays following ten consecutive days of ethanol exposure. Withdrawal-related hyperexcitability was assessed by handling-induced convulsions following 3 cycles of ethanol vapor exposure/withdrawal. Immunoblots were used to assess $\alpha 1$ protein levels.

Results—Compared to controls, knockin mice displayed decreased AFT and chronic tolerance to ethanol-induced motor ataxia, and also displayed heightened ethanol-withdrawal hyperexcitability. No differences between wildtype and knockin mice were seen in other ethanol-induced behavioral measures. Following chronic exposure to ethanol, control mice displayed reductions in $\alpha 1$ protein levels, but knockins did not.

Conclusions—We conclude that $\alpha 1$ -GABA_A-Rs play a role in tolerance to ethanol-induced motor ataxia and withdrawal-related hyperexcitability. However, other aspects of behavioral tolerance and physical dependence do not rely on $\alpha 1$ -containing GABA_A-Rs.

Keywords

GABA; GABA type A receptor; gene knockin mice; alcohol; tolerance; dependence

Introduction

While many people imbibe alcohol (ethanol), the driving force that propels certain individuals toward alcoholism remains unknown. Alcohol intoxication results in well-known behavioral effects such as impaired motor coordination, impaired judgment, and anxiolysis, as well as

tolerance and physical dependence. The latter two are thought to be prognostic indicators of alcoholism according to the DSM-IV (Hasin et al., 2000; Miller et al., 1987). Thus, understanding the mechanism that leads to tolerance and dependence may give valuable insight into alcohol addiction and ultimately result in effective therapeutic intervention to combat this disorder.

Tolerance is defined as a reduced response to a constant amount of ethanol or an increase in the amount necessary to elicit the same effect, while dependence is characterized by a withdrawal syndrome upon cessation of ethanol exposure (DSM-IV TR, 2000). While simple in definition, tolerance and dependence can vary depending on ethanol exposure (e.g., acute vs. chronic) and can impact various ethanol-induced behaviors, through either interrelated or distinct unknown mechanisms.

Although the physical effects of ethanol on behavior are well known, the molecular mechanisms governing these effects remain unclear. However, it is clear that alcoholism involves a significant genetic component (Heath et al., 1997; Prescott and Kendler, 1999) and numerous genes are likely to be involved (Fan et al., 2004; Lewohl et al., 2000). γ -aminobutyric acid (GABA) type A receptors (GABA_A-Rs) have been extensively implicated in ethanol action and alcoholism (see Grobin et al., 1998). GABA_A-Rs mediate the majority of rapid inhibition in the brain. GABA_A-Rs are pentameric ligand-gated chloride channels for which 19 subunits exist – α 1–6, β 1–3, γ 1–3, δ , ϵ , π , θ and ρ 1–3 (Sieghart and Sperk, 2002). Most native receptors are comprised of 2 α , 2 β and γ or δ subunits (Tretter et al., 1997). GABA_A-R agonists such as benzodiazepines or neurosteroids have been shown to accelerate the development of tolerance to ethanol (Barbosa and Morato, 2000; Martijena et al., 2001) and result in cross-tolerance to ethanol (Le et al., 1986; Palmer et al., 2002). Benzodiazepines may also reduce withdrawal symptoms (Becker and Veatch, 2002) and may be used clinically for alcohol withdrawal (Miller and McCurdy, 1984; Newman et al., 1995).

Because of the multitude of GABA_A-R subunits, it is conceivable that specific GABA_A-R subtypes may mediate specific aspects of tolerance and dependence. α 1 is the major alpha subunit and is the most widely expressed, being present in approximately 50% of all GABA_A-Rs (McKernan and Whiting, 1996; Mohler et al., 1992). α 1-containing GABA_A-Rs have been suggested to be involved in tolerance and dependence. Genetic association studies in rodents and humans have suggested α 1 may be involved in tolerance (Crabbe et al., 1996) and withdrawal (Buck et al., 1997; Dick et al., 2006). Pharmacologic studies have implicated α 1 in drug seeking behavior (Harvey et al., 2002) and withdrawal-related seizures (summarized in Faingold et al., 1998). Additionally, studies using zolpidem, an α 1 selective agonist, have implicated α 1 containing receptors in the molecular mechanism of ethanol action (e.g., Criswell et al., 1995).

Additional studies have demonstrated that chronic ethanol decreased α 1 levels (e.g., Buck et al., 1991; Devaud et al., 1997; Lewohl et al., 1997) via receptor internalization (Kumar et al., 2003), lending support to the idea that GABA_A-R plasticity may result in tolerance and withdrawal. However, it is unknown if the ethanol-induced decreases in α 1 subunit levels are due to a direct effect of ethanol on the α 1 subunit or due to an indirect effect on other targets.

Although much work has implicated α 1 GABA_A-Rs in tolerance and dependence, the exact role of α 1 has yet to be determined. Genetically modified mice are a useful tool for addressing the role of individual GABA_A-R subunits in ethanol action. A molecular switch had been identified in GABA_A-Rs that was sufficient to eliminate potentiation of GABA_A-R function by ethanol (Mihic et al., 1997). Mutation of serine(S)270 in the α 1 subunit to histidine(H) abolished enhancement of recombinant GABA_A-R by ethanol (Ueno et al., 2000) but also resulted in enhanced sensitivity to GABA (Nishikawa et al., 2002). Gene targeted mice

possessing this mutation displayed developmental and behavioral abnormalities, probably related to the shift in GABA sensitivity (Homanics et al., 2005). However, combining the S270H mutation with a second mutation of leucine(L)277 to alanine(A) normalized GABA sensitivity while maintaining ethanol insensitivity in recombinant receptors expressed in vitro (Borghese et al., 2006). $\alpha 1$ GABA_A-R knockin mice harboring both of these mutations were recently created (Borghese et al., 2006). Knockin mice were overtly normal. Hippocampal synaptic recordings were less sensitive to ethanol-induced potentiation at high ethanol concentrations (80mM) compared to controls (Werner et al., 2006). Behaviorally, knockin mice recovered more quickly from ethanol-induced motor ataxia compared to wildtype controls, but were normal on numerous additional tests of acute ethanol-induced behaviors (Werner et al., 2006).

Because $\alpha 1$ -containing GABA_A-Rs are very abundant in adult brain and because of converging lines of evidence that implicate these receptors in ethanol action, we hypothesized that $\alpha 1$ GABA_A-Rs mediate tolerance to, and withdrawal from, ethanol exposure. In the current study, the contribution of $\alpha 1$ -containing GABA_A-Rs to ethanol tolerance and dependence was examined by comparing knockin mice with ethanol-insensitive $\alpha 1$ GABA_A-R subunits with control mice on numerous ethanol-induced behaviors related to tolerance and dependence.

Materials and Methods

Mice

GABA_A-R $\alpha 1$ subunit wildtype (SL/SL genotype) and knockin (HA/HA genotype) mice were produced as described elsewhere (Borghese et al., 2006). Briefly, wildtype and knockin littermates were derived from heterozygous matings and genotyped using standard Southern blot techniques of tail biopsies. All mice were of a mixed C57BL/6J $\times$ Strain 129SvJ background of the F4 – F11 generations. Mice were between 8 – 14 weeks of age. For all experiments, male and female mice were used, and all mice were ethanol and experimentally naïve. Minimal handling of the animals occurred during all experimental procedures. Effect of gender was initially analyzed for all experiments. If no overall effect of gender was observed, data were collapsed for further analysis. In some instances, the limited number of animals did not allow for accurate analysis of gender differences. All treatment and experimental procedures were conducted between the hours of 9:00AM and 1:00PM with the exception of the final nociception measurement, which was performed 12 h following the final ethanol exposure. Ethanol was obtained from Pharmco (Brookfield, CT).

Acute Functional Tolerance (AFT)

Acute tolerance to the motor ataxic effects of ethanol was measured using the two-dose method originally designed by Erwin and Deitrich (Erwin and Deitrich, 1996). For motor ataxia, ethanol naïve mice were trained to balance on a stationary dowel (Ugo Basile Rota-Rod, model 7650; Stoelting Co., Wood Dale, IL; rod size = 2.5cm) for a 60 sec period. All mice accomplished this task within 3 trials. Once basal training was accomplished, mice were injected with ethanol (1.75 g/kg, i.p.) and placed back on the dowel until they fell off. Mice were repeatedly tested until they regained the ability to balance on the rod for 60 sec. Once this was achieved (t1), a retro-orbital blood sample was collected for blood ethanol concentration determination (BEC1). Mice were then immediately given a second ethanol injection (2.0 g/kg, i.p.). After losing the ability to remain on the rod, mice were tested repeatedly (5-min intervals) until they regained the ability to balance on the dowel for 60 sec (t2). Once this was achieved, a second blood sample was collected for BEC determination (BEC2). BECs were determined using a standard enzymatic spectrophotometric method (Harris et al., 1995). AFT was defined as the difference in BECs at t2 versus t1 (i.e., BEC2 – BEC1).

Development of AFT to ethanol-induced loss of righting reflex (LORR) was also determined similarly to previous studies (Erwin et al., 2000). Mice received an initial hypnotic dose of ethanol (3.5 g/kg, i.p.). Once the mice lost the ability to right themselves, they were placed on their backs in a v-shaped trough. The duration of time until the animal could turn over (right itself) 3 times in a 30 sec period was recorded and a blood sample taken for BEC1 determination. Mice were then given a second ethanol injection (2.0 g/kg, i.p.), and immediately placed back in the v-shaped troughs. The duration of the second LORR period was recorded and when the mice regained the ability to right themselves (t2), another blood sample taken for BEC determination (BEC2). AFT was calculated as described for the dowel test. All data were analyzed using student's t-test.

Loss of Righting Reflex

Mice were repeatedly administered either saline or ethanol (2.0 g/kg, i.p.) once daily for 10 days. On day 11, all mice were given ethanol (3.5 g/kg, i.p.) and tested for the sedative/hypnotic effects of ethanol. Immediately following injection, mice were observed for LORR as described above. Only mice that lost the righting reflex were included in the analysis. A 3.5 g/kg dose was used to enhance our ability detect differences in tolerance to ethanol's hypnotic effects. Data were analyzed using two-way ANOVA and Fisher's post-hoc test when necessary.

Rotarod and Hypothermia

Mice were repeatedly administered ethanol (2.0 g/kg, i.p.) once daily for 11 days, and motor ataxia and hypothermia were assessed on day 1, day 6 and day 11 as described in Werner et al. (2006). Briefly, to assess the motor ataxic effects of ethanol, mice were trained to stay on a fixed speed rotarod (6.0 rpm, rod size = 6cm) for 60 sec immediately prior to ethanol treatment on each test day. Fifteen min following ethanol injection, each mouse was placed back on the rotarod and the time that they remained on the rotating rod was measured (60 sec maximum). Mice were retested every 15 min for 75 min. Area under the curve (AUC) was calculated as the area between baseline response (60s) and ethanol response curve and numerically integrated.

To assess hypothermic effects of ethanol, rectal body temperatures were measured via a digital thermometer (Thermalert Model TH-8 with probe RET-3; Physiotemp Instruments, Clifton, NJ). Body temperatures were assessed after initial training (i.e., baseline) and following each motor ataxia measurement.

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

Radiant Tail-Flick

The radiant tail-flick assay was used as described (Lariviere et al., 2002). Briefly, mice were lightly restrained in a soft denim cloth pouch which they would readily enter after a few acclimating trials. The mice would enter the pouch 'head first' so that the tail was exposed. A focused beam of light from a tail-flick apparatus (IITC Life Sciences, Woodland Hills, CA) was directed at a spot ~1 cm from the tip of the tail and the latency to remove the tail was recorded via a sensor triggered upon removing the tail. Light intensity was set to a low-moderate intensity (active intensity setting of 15) that yielded average cutoff times of ~10 sec in preliminary experiments. A maximum cutoff time of 30 sec was used to prevent tissue damage. A minimum of 2 readings was taken for each mouse at each test and the mean calculated. Mice were assessed for basal responses 1 day prior to ethanol exposure. Mice were then given ethanol (2.0 g/kg, i.p.) once daily for 11 days. Ethanol-related effects were assessed 30 min after ethanol injection on day 1 and day 11, as well as 10–12 hours following the last

ethanol exposure. Data were analyzed by repeated-measures ANOVA with Bonferroni-coorrected student's t-test for further analysis.

Ethanol Withdrawal-Related Hyperexcitability

To test withdrawal hyperexcitability following cessation of chronic exposure to high concentrations of ethanol, ethanol vapor chambers (Goldstein, 1972; Goldstein and Pal, 1971) were used coupled with the multiple withdrawal paradigm developed by Becker and Hale (1993). Briefly, mice were treated with the alcohol dehydrogenase inhibitor pyrazole (68 mg/kg, i.p., Sigma, St. Louis, MO) and a priming dose of ethanol (1.5 g/kg, i.p.) and placed in chambers for a 16 h ethanol vapor exposure period followed by 8 h of recovery. Ethanol was dripped into a 55°C flask at a flow rate of ~120–140 µl/min by a peristaltic pump (Model 75 23–30; Cole Parmer Instruments, Chicago, IL) where it was vaporized and delivered to a sealed Plexiglas chamber with air at 8 L/min. This procedure was repeated for a total of 3 exposure/recovery cycles. Control mice were also treated with pyrazole but were injected with saline instead of ethanol and were placed in chambers with normal air. Injection volumes for pyrazole, ethanol, and saline were 0.01 ml/g body weight. At the end of the third 16 h ethanol exposure, blood samples (~50 µL) were taken and assessed for BEC using a standard enzymatic spectrophotometric method (Harris et al., 1995). Ethanol-treated mice that had BECs <50 mg/dl or >300 mg/dl were excluded.

Mice were scored for handling-induced convulsions (HIC) starting 1 h following termination of the last cycle of ethanol exposure. The mice were scored using the scale described elsewhere (Becker and Hale, 1993). Briefly, the 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, may be delayed by a few sec; 6 – severe tonic/clonic convulsion on pick up, no delay and often lasting after mouse was released, and; 7 – severe tonic/clonic convulsion prior to pick up, may result in death. Scoring was done hourly for 10 hours and then again the following day at 24 h. A number of knockin mice unexpectedly died during the assay. Therefore, if an animal died during the scoring period, then its scores were excluded from analysis. Scoring was done by two observers blind to genotype and treatment. If scores differed between observers, the mean of the two scores was used. Area under the curve (AUC) was calculated and data were analyzed by ANOVA with Fisher's post-hoc test when appropriate. A Kaplan-Meier (Kaplan and Meier, 1958) estimate was used to look for differences in survival between the WT and KI groups given ethanol. The Mantel-Haenszel log-rank was then performed to assess statistical differences between these groups. The Kaplan-Meier estimate with log-rank test is a nonparametric analysis for survival probability and is used for varied time-related outcomes (i.e., death). BECs and body weights were analyzed by ANOVA.

α1-GABA_A-R Immunoblotting Analysis

Mice were repeatedly administered ethanol (2.0 g/kg, i.p.) or saline once per day for 10 days. On the eleventh day, mice were euthanized with an overdose of isoflurane and brains were rapidly dissected and flash-frozen on dry ice, and stored at –80°C. P2 membrane fractions from individual cortices were processed and protein was quantified using a bicinchoninic acid method. Aliquots of 25 µg of protein from each sample were separated by electrophoresis on 10%-SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Wildtype and knockin samples were run on separate gels with investigator blinded to treatment. Detection of α1 was with a polyclonal specific rabbit anti-GABA_A-R α1 antibody (1:10,000; 06-868, Upstate, Lake Placid, NY; now with Millipore, Billerica, MA) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (1:5000; NB-730H, Novus, Littleton, CO). Bands were visualized by

enhanced chemiluminescence (Western Lightning; PerkinElmer Life and Analytical Sciences, Boston, MA). For normalization, blots were stripped using Re-blot (Chemicon International, Temecula, CA) and re probed with a rabbit anti- β -actin polyclonal antibody (1:10,000, ab8227-50; Abcam). Multiple exposures of each membrane were made to ensure the signal was within the linear range of the film. Band intensity was measured densitometrically (Kodak 1D software; Eastman Kodak, Rochester, NY). Saline and ethanol values within genotype were analyzed using Student's t-test.

Results

Knockin mice have reduced AFT to ethanol-induced motor ataxia, but not LORR

To assess acute/rapid tolerance, AFT to the ataxic effects of ethanol was measured using a stationary dowel (Figure 1A). Time to recover from the first ethanol exposure (t1) and BEC at t1 (BEC1) did not differ between genotypes. Knockin (KI) mice displayed a significantly longer time to recover from the second ethanol exposure (t2) and also had reduced BEC at t2 (BEC2) compared to controls ($p < 0.01$). This resulted in an overall decrease in AFT (defined as BEC2-BEC1) with respect to ataxia in knockins compared to controls (64.2 ± 7.9 versus 138.5 ± 11.8 , respectively, $p < 0.01$).

To determine if reduced tolerance generalized to other behavioral measures, AFT to the hypnotic effects of ethanol was assessed (Figure 1B). Wildtype and knockin mice did not differ on any parameter measured (i.e., t1, t2, BEC1, BEC2, AFT). AFT for wildtype and knockin mice was 54.6 ± 23.4 and 44.4 ± 15.5 mg/dL, respectively.

Knockin mice have normal chronic tolerance to ethanol-induced LORR

LORR was assessed to determine $\alpha 1$ -GABA_A-R involvement in tolerance to ethanol's hypnotic effects after chronic ethanol exposure. Mice were repeatedly treated with moderate doses of ethanol or saline for 10 days and LORR to a hypnotic dose (3.5 g/kg) of ethanol was assessed on day 11. Repeated ethanol exposure resulted in tolerance to ethanol's hypnotic effects (Figure 2) as evidenced by a reduced duration of LORR in ethanol treated mice compared to saline treated mice ($F_{1, 73} = 25.33$, $p < 0.0001$). No significant effect of genotype, or genotype by treatment interaction was found. Additionally, the number of chronic ethanol treated mice failing to respond to the hypnotic effects of ethanol (i.e., sleeptime = 0 min) did not appear to differ between genotypes (5/23 control and 3/22 knockins). The total number of mice not responding to ethanol's hypnotic effects was much higher in all ethanol treated animals (8/45) than saline treated animals (1/37), thereby also suggesting that tolerance was observed. Thus, although tolerance to the hypnotic effect of ethanol was observed, the $\alpha 1$ subunit knockin mutation did not affect the development of tolerance to this behavior.

Knockin mice have reduced tolerance to ethanol-induced ataxia following chronic ethanol

To determine if $\alpha 1$ -containing GABA_A-R are involved in tolerance to the motor ataxic effects of ethanol, mice were tested using the fixed speed rotarod. Mice were treated every day with ethanol (2.0 g/kg, i.p.) for 11 days and tested on days 1, 6 and 11 for motor ataxia. AUC was used to compare ethanol-induced ataxia following ethanol injection. Analysis revealed a significant main effect of day ($F_{2, 76} = 36.9$, $p < 0.0001$) and the interaction of day $\times$ genotype ($F_{2, 76} = 4.7$, $p < 0.05$), but no effect of genotype (Figure 3A). Both genotypes had reduced motor ataxia on day 6 and day 11 compared to day 1. Further analysis to determine where the interaction occurred revealed that genotypes differed in ethanol-induced motor ataxia on day 1. Therefore, knockin mice had less ethanol-induced motor ataxia than controls on day 1.

Because genotypes initially differed on recovery from ethanol-induced ataxia (day 1), to better compare the effects of ethanol within and between genotypes, all mice were normalized to the

ethanol-induced ataxia observed on day 1 (i.e., Motor ataxia on day 1 was set to 100%; Figure 3B). Analysis of day 6 and day 11 indicated a trend toward significance with respect to a main effect of genotype ($F_{1,38} = 3.9$, $p = 0.056$). While not significant, the suggestive difference for genotype hints that knockin mice developed less tolerance than wildtype mice. To further explore this possibility, genotypes were compared. Knockins were more impaired on day 11 as evidenced by the greater AUC compared to controls ($p < 0.01$). Overall, these data indicate that knockin mice had decreased sensitivity to ethanol's motor ataxic effects on day 1 and displayed decreased tolerance after 11 days of chronic ethanol exposure.

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

Knockin mice have normal chronic tolerance to ethanol-induced hypothermia

The same mice were also tested for ethanol-induced hypothermia concurrently with rotarod. Preliminary analysis of the data revealed that ethanol produced a maximum decrease in body temperature at 30 min post ethanol injection (not shown). Therefore, this time point was selected for detailed analysis. Compared to basal body temperatures, a significant main effect of ethanol-induced hypothermia was observed on day 1 ($F_{1,38} = 222.2$, $p < 0.0001$), but no effect of genotype was detected. Ethanol treatment decreased body temperatures compared to baseline ($p < 0.0001$, student's t-test). A significant main effect was also detected for day after repeated ethanol exposure ($F_{2,76} = 70.1$, $p < 0.0001$), but no significant effect of genotype or interaction was observed (Figure 3C). Repeated ethanol exposure decreased ethanol-induced hypothermia on day 6 and day 11 compared to day 1. Thus, tolerance developed to the hypothermic effects of ethanol, but this effect was not influenced by the gene knockin.

Knockin mice have normal ethanol-induced nociceptive responses

To determine if $\alpha 1$ -GABA_A-Rs are involved in ethanol-related nociceptive effects, mice were assessed using the radiant tail-flick assay. Genotypes did not differ in basal thermal nociceptive responses (Baseline, Figure 4). Mice were then assessed with respect to ethanol after a single exposure (day 1), repeated exposure (day 11), and during withdrawal (day 11.5). A significant main effect was observed for day ($F_{3,135} = 50.9$, $p < 0.0001$), but not for genotype or interaction of day and genotype. Further analysis indicated acute ethanol treatment increased latency to tail-flick compared to baseline ($p < 0.001$). Chronic ethanol treatment did not reveal any difference from baseline, but mice had a slightly reduced latency to tail-flick during withdrawal from chronic ethanol exposure (i.e., day 11.5, $p < 0.01$). Thus, while acute ethanol resulted in an analgesic effect, repeated ethanol exposure resulted in tolerance to this effect, and an enhanced sensitivity was observed during withdrawal following repeated ethanol exposure. However, these responses did not differ with respect to genotype.

Knockin mice have enhanced chronic ethanol withdrawal-related hyperexcitability

The role of the $\alpha 1$ subunit in ethanol dependence was tested following multiple cycles of exposure and withdrawal from high concentrations of ethanol vapor by assessing ethanol withdrawal-related hyperexcitability as quantified by the intensity of HICs. BECs did not differ with respect to gender or genotypes at the end of the third cycle of ethanol exposure. BEC's were not detectable in air-exposed mice, but were readily measurable in ethanol vapor exposed groups. Ethanol administration resulted in weight loss (Table 1, $p < 0.05$), but this also was not significant between genotypes.

For HICs, a main effect of gender was observed ($F_{1, 55} = 6.8$, $p < 0.05$) for AUCs; therefore males and females were analyzed separately. Analysis of AUCs did not include animals that died. A time course of ethanol withdrawal hyperexcitability for both genders is shown in Figures 5A and 5D.

For male mice, a two-way ANOVA of AUCs indicated a main effect of treatment ($F_{1, 23} = 19.3$, $p < 0.001$), and a trend towards an interaction of treatment $\times$ genotype ($F_{1, 23} = 4.0$, $p = 0.057$) but no effect of genotype. Because of the suggestive interaction further analysis was done to determine the observed effect. No differences in genotype were detected in air-exposed mice (Figure 5B). Ethanol treated male mice displayed greater AUCs compared to control treated male mice ($p < 0.01$). Ethanol treated knockin mice displayed greater AUCs compared to ethanol treated wildtype mice ($p < 0.05$). Interestingly, an ~40% mortality rate ($\chi^2_1 = 6.04$, $p < 0.05$) occurred in ethanol treated male knockin mice whereas none was observed in ethanol treated male wildtype mice (Figure 5C). No mortalities were seen in control treated wildtype mice, but it should be noted that one control treated knockin male died (not shown) toward the end of the HIC assay. It should also be noted that the observed deaths were the result of severe tonic-clonic seizures, with the animals often ending in a tonic-extensor position as noted elsewhere (Freund, 1973).

For female mice (Figure 5E) two-way ANOVA of AUCs indicated no effect of treatment, genotype or the interaction of treatment and genotype for AUC. However, female ethanol-exposed knockin mice had a substantially higher mortality rate than ethanol-exposed wildtypes (Figure 5F, $\chi^2_1 = 12.28$, $p < 0.001$) due to seizure-like activity. No deaths were observed in ethanol treated female wildtype mice. No deaths were observed in air-exposed mice of either genotype (not shown).

Knockin mice do not have reduced $\alpha 1$ levels following repeated ethanol exposure

To examine ethanol-induced reduction in $\alpha 1$ levels, the amount of $\alpha 1$ protein was assessed in the cortex of wildtype and knockin mice following repeated ethanol exposure (2.0 g/kg, i.p. per day for 11 days) or saline. In wildtype mice (Figure 6A), chronic ethanol resulted in ~30% reduction of $\alpha 1$ subunit levels compared to saline treated mice (saline $100 \pm 9.1\%$, ethanol $68.8 \pm 7.9\%$; $p < 0.05$). In contrast, knockin mice (Figure 6B) repeatedly administered ethanol did not differ from saline treated mice (saline $100.0 \pm 11.9\%$, ethanol $89.2 \pm 7.5\%$). This indicates that while chronic ethanol reduced $\alpha 1$ GABA_A-R subunit levels in wildtype mice, $\alpha 1$ in knockins were unaffected.

Discussion

$\alpha 1$ -containing GABA_A-Rs have been suggested to be involved in the mechanism of action of alcohol and alcoholism. In this report, knockin mice harboring ethanol-insensitive $\alpha 1$ GABA_A-R subunits were assessed on a variety of measures of behavioral tolerance and dependence – two prognostic behaviors of alcoholism. Knockin mice displayed reduced AFT and chronic tolerance to motor ataxia and increased withdrawal-related hyperexcitability compared to controls. Additionally, knockin mice did not display ethanol-related decreases in $\alpha 1$ protein levels compared to saline controls. In contrast, tolerance and dependence on several other ethanol-related behaviors were not altered in knockins compared to controls, such as righting reflex, hypothermia and nociception. These results suggest that $\alpha 1$ -containing GABA_A-Rs are involved in certain aspects of ethanol-induced tolerance and physical dependence.

Knockin mice displayed decreased AFT – i.e., in other words, knockin mice did not recover from a second ethanol exposure as readily as controls. To determine whether decreased acute tolerance was observed in other ethanol-related behaviors, acute/rapid tolerance was also

assessed with respect to the righting reflex. No differences were observed between genotypes. Therefore, it is possible that $\alpha 1$ -containing GABA_A-Rs may mediate in part the development of acute/rapid tolerance to some behavioral effects, but this did not generalize to other measures of acute/rapid tolerance. Because of this effect, tolerance to motor ataxia was assessed after repeated ethanol exposure. Knockin mice were initially less sensitive to ethanol's acute motor ataxic effects. This agrees with our earlier report (Werner et al., 2006). However, in parallel to AFT, knockins also displayed decreased development of tolerance to ethanol-induced motor ataxia after repeated ethanol exposure compared to controls. These parallel changes in sensitivity and tolerance are similar to those seen in other experiments. For instance, in selectively bred lines of rats and mice, those which had decreased initial ethanol sensitivity developed little to no tolerance whereas those lines which were more sensitive developed greater tolerance (Radcliffe et al., 2005; San-Marina et al., 1989; Tabakoff and Culp, 1984). Additionally, greater sensitivity may be related to increases in tolerance (Kalant et al., 1971). Indeed, the ethanol-induced motor ataxia results presented here also support a relationship between sensitivity and tolerance. $\alpha 1$ GABA_A-R knockin animals had decreased initial sensitivity as well as decreased tolerance. It is interesting that ethanol-related behaviors that were similar for genotypes such as loss of righting reflex also did not differ after chronic ethanol. Taken together, these results support the suggestion that the development of tolerance is directly related to initial sensitivity.

While some studies suggest that sensitivity and tolerance are not directly related and where animals become more sensitized in some instances (Gallaher and Gionet, 1988; Kurtz et al., 1996; Waller et al., 1983), the discrepancy between these results and ours may be due to the assay(s) employed, and the initial sensitivity of the animals being used. The differences may also be due in part to genetic dissociation among inbred strains (Radcliffe et al., 2005), although the complex interplay of multiple mechanisms of sensitivity and tolerance may account for this observation as suggested elsewhere (Rustay and Crabbe, 2004). The possibility exists that these phenotypes may be due to the mixed backgrounds commonly used to create genetically modified mouse models. However, C57BL/6J and 129/SvJ mice did not differ in AFT (Homanics et al., 1998).

Although these knockin mice displayed decreased response to motor ataxia compared to controls (i.e., faster recovery, Werner et al., 2006), it is possible that the necessary molecular adaptations did not occur thereby leaving knockin mice more susceptible to the next intoxicating state compared to controls. Indeed, repeated ethanol exposure decreased $\alpha 1$ subunit levels in the cortex of wildtype mice as observed previously in numerous studies (e.g., Devaud et al., 1997; Kumar et al., 2003). In stark contrast, $\alpha 1$ levels in knockins were unaffected in the current study. While it is possible that the previously observed decrease in ethanol-induced potentiation of mutant $\alpha 1$ -GABA_A-Rs may result in decreased receptor regulation, this needs to be explored further. Nonetheless, the absence of a decrease in $\alpha 1$ subunit levels parallels the decrease in tolerance observed in ethanol's motor ataxic effects. Alternatively, a floor effect may have occurred. Previous experiments indicate that $\alpha 1$ subunit levels in knockins were decreased by ~50 percent in cortex compared to controls (Borghese et al., 2006). It is possible that this may be the minimum physiologically required level of $\alpha 1$ for 'normal' brain function, although this would also need to be further evaluated experimentally.

The lack of a decrease in $\alpha 1$ subunits in cortical tissue may correlate with some of the observed behaviors. Given that the cortex is involved in numerous behaviors such as sensory and motor effects, it is possible that this brain region may play role in ethanol's ataxic effects as well as other ethanol-related behaviors assessed here. However, behaviors such as motor coordination are complex and likely involve the interplay of multiple brain regions. Although previous results have shown that $\alpha 1$ levels were unaltered in cerebellum of knockin mice (Borghese et al., 2006), it is not known whether $\alpha 1$ levels were altered in other brain regions or whether

these regions were altered after chronic ethanol exposure in these mice. Compensatory changes in other GABA_A-R subunits may account for some of the observed behavioral effects by either masking or augmenting behavioral responses. However, compensatory effects may be less likely for other behaviors. The $\alpha 1$ knockins assessed here and global $\alpha 1$ knockouts both displayed compensatory changes such as increased $\alpha 2$ and $\alpha 3$. However, decreased AFT was not observed in $\alpha 1$ knockout mice (Kralic et al., 2003), but it is unknown whether knockout mice differed in recovery from ataxia as this was not assessed.

Interestingly, knockin mice also displayed more severe ethanol withdrawal-related phenotypes. Withdrawal-related hyperexcitability was severe enough to result in fatal seizures in 40–60% of knockin mice. Due to the unexpected number of withdrawal-related mortalities in knockins, no further experiments were conducted. While gender differences were noted for ethanol withdrawal severity, this was not unexpected. Studies elsewhere had noted similar differences in ethanol withdrawal-related responses with respect to gender (Veatch et al., 2007). Blood ethanol concentrations did not appear to play a role in gender or genotype differences during ethanol withdrawal. While blood ethanol concentrations were not different between genotypes, it is possible that differences in blood alcohol levels after the first and second ethanol exposures may account for the difference in withdrawal sensitivity between genotypes. This is less likely, however, given the fact that previous studies indicated similar ethanol metabolism and clearance between genotypes (Werner et al., 2006).

Because the knockin mutation eliminated ethanol action at $\alpha 1$ -GABA_A-Rs (Borghese et al., 2006), it was postulated that knockins would have less withdrawal-related behavior than controls, but the results indicate the opposite. One distinct possibility may be that the knockin mice are in a pre-kindled state and are therefore pre-disposed to more severe hyperexcitable states. It is possible the reduction in $\alpha 1$ subunit levels observed previously may relate to this pre-kindled state. Knockins also previously exhibited ~10% mortality rate where some seizures have been observed. Interestingly, in all control treated knockin mice in the HIC study, 1 (10%) died whereas no control wildtype animals died. This is in line with the observed mortality rate seen in this colony. Alternatively, seizure-protective effects may be eliminated in the knockin. Because $\alpha 1$ is expressed throughout the brain, it remains to be seen which neuroanatomic region is responsible for ethanol withdrawal-related seizure activity. However, it's tempting to speculate that regions such as the hippocampus and dentate gyrus which have been implicated in spontaneous seizures and temporal lobe epilepsy may play a role (Laschet et al., 2007; Peng and Houser, 2005). Further experiments such as the use of conditional tissue-specific gene knockin of this mutation (Skvorak et al., 2006) could be used to determine if these regions are involved in ethanol withdrawal-related seizure activity.

$\alpha 1$ knockout mice did not display increased withdrawal related hyperexcitability (Blednov et al., 2003). The discrepancy between this study and ours may be due to differences in methodology. In the $\alpha 1$ knockouts, an ethanol diet was used to assess withdrawal whereas ethanol vapor was used in the current studies. $\alpha 1$ knockouts consumed less ethanol than controls, which may have contributed to the observed effects. However, in other studies $\alpha 1$ knockout mice had increased seizure susceptibility to bicuculline-induced seizures and knockouts exhibited more severe seizures and death (Kralic et al., 2002). These results are in line with what was observed in the current studies.

Knockin mice did not differ from controls in tolerance to ethanol's hypnotic effects. This is somewhat surprising as $\alpha 1$ -GABA_A-Rs have been shown to mediate the sedative effects of benzodiazepines (Rudolph et al., 1999). However, previous studies indicated no difference in ethanol-induced hypnosis (Werner et al., 2006). Recent work has suggested that sedation and hypnosis are mechanistically distinct for intravenous anesthetics (Jurd et al., 2003; Reynolds et al., 2003). Therefore, it is possible that ethanol-related hypnosis may be mediated by other

GABA_A-R subtypes. In fact, other studies have suggested that the hypnotic effects of ethanol may be mediated by $\alpha 2$ -GABA_A-Rs (Tauber et al., 2003). It would be interesting to assess the hypnotic effects of ethanol as well as tolerance to these effects in mice harboring $\alpha 2$ ethanol-insensitive GABA_A-R subunits. Knockin mice also did not differ from controls in tolerance to ethanol-induced hypothermia. Nonetheless, these results are consistent with other studies in that they noted a negative genetic correlation with withdrawal hyperexcitability and tolerance to hypothermia (Crabbe and Kosobud, 1986; Metten and Crabbe, 2005).

Lastly, knockin mice did not differ from controls in nociceptive responses to ethanol. Acute, chronic and withdrawal-related effects of ethanol on nociceptive responses were observed as reported elsewhere (e.g., Gatch and Lal, 1999). The withdrawal-related responses, while significant, did not have as severe a magnitude as seen elsewhere – although this could be related to the dose of ethanol used and/or length of ethanol exposure. While a moderate concentration of ethanol was employed here, other studies have employed higher doses that may precipitate withdrawal-related seizure effects (Gameiro et al., 2003; Gatch and Lal, 1999). However, recent work has suggested that lower doses paradoxically increased ethanol withdrawal hyperalgesia (Dina et al., 2006). While GABA_A-Rs and cell signaling proteins that regulate GABA_A-R function have been suggested to play a role in ethanol-nociceptive effects (Dina et al., 2000; Gatch, 1999), these results suggest $\alpha 1$ has little involvement. It is possible that other GABA_A-R subunits or non-GABAergic targets mediate this effect [e.g., adenosine receptors (Gatch and Selvig, 2002), calcium channels (Gatch, 2002)].

In summary, $\alpha 1$ -GABA_A-Rs play a role in the development of tolerance to ethanol's ataxic effects as well as ethanol withdrawal-related hyperexcitability. More importantly, the experiments conducted here support the hypothesis that $\alpha 1$ GABA_A-Rs mediate in part ethanol tolerance and dependence, but further work is necessary to conclusively establish the role played by this GABA_A-R subunit.

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Figure 1.

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

Figure 2.

Hypnotic effects of ethanol following repeated ethanol exposure. Mice were treated with saline or ethanol (2.0 g/kg) once daily for 10 days and LORR in response to a hypnotic dose of ethanol (3.5 g/kg) was assessed on day 11. Tolerance to the hypnotic effects of ethanol was observed after repeated ethanol exposure compared to saline as evidenced by the decreased duration of LORR. A significant main effect of treatment was observed ($p < 0.0001$), but genotype or genotype $\times$ treatment interaction was not significant. Data are presented as mean $\pm$ SEM. $n = 18 - 21$ per group.

Figure 3.

Ethanol-induced motor ataxia and hypothermia. Mice were treated with ethanol (2.0 g/kg) once daily for 11 days and motor coordination and body temperatures were assessed following ethanol exposure on day 1, 6 and 11. AUC of ethanol-induced motor ataxia (A). Knockin mice had decreased ethanol-induced motor ataxia on day 1 (##, $p < 0.01$) compared to wildtype controls. Both genotypes had reduced AUCs on day 6 and day 11 of repeated ethanol exposure compared to day 1 ($p < 0.0001$). AUC's normalized to percent ataxia based on day 1 (B). Knockin mice were more impaired than wildtype controls on day 11 (##, $p < 0.01$). Peak ethanol-induced hypothermia (C). Ethanol exposure resulted in hypothermia on day 1 compared to basal body temperature ($p < 0.0001$), but hypothermia did not differ between

genotypes. Tolerance to ethanol's hypothermic effects was seen on day 6 and day 11 compared to day 1 ($p < 0.0001$), but no differences between genotypes were observed. Data represent mean $\pm$ SEM. $n = 19$ – 21 per group.

Figure 4.

Ethanol effects on nociception. Mice were tested for basal and ethanol-related nociceptive responses using the radiant tail-flick. Mice did not differ in basal responses to thermal pain stimuli. Acute ethanol increased tail-flick latencies compared to baseline ($p < 0.001$). Mice tested immediately after the last ethanol exposure (day11) did not differ from basal responses. Mice had decreased latencies 12h following the last ethanol exposure (i.e., during withdrawal) compared to baseline (day11.5, $p < 0.01$). Data represent mean $\pm$ SEM. $n = 13-16$ per genotype.

Figure 5.

Ethanol withdrawal-related hyperexcitability. Male mice exhibited more severe withdrawal-related hyperexcitability than females ($F_{1,55} = 6.8$, $p < 0.05$). Therefore, male and female mice were analyzed separately (Figs. A–C and D–F, respectively). Time-course of mean handling-induced convulsions (HIC) scores in male and female mice, (A and D, respectively) after completion of three cycles of 16h per day exposure to ethanol vapor. Area under the curves for HIC time-course in males and females (B and E, respectively). A trend was observed for ethanol treated male mice having more severe withdrawal-related behavior than controls (B, ANOVA, $p = 0.057$). Knockin mice exhibited more severe withdrawal-related behavior than ethanol treated wildtype mice (B, $p < 0.05$). No differences were detected in female mice (E).

Survival curves following cessation of ethanol treatment in male and female mice (C and F, respectively). Male and female knockins were prone to withdrawal-related mortality (*, $p < 0.05$; ***, $p < 0.001$).

Figure 6. GABA_A-R $\alpha 1$ subunit protein levels following repeated ethanol exposure. Representative western blots of $\alpha 1$ levels in wildtype (A) and knockin (B) mice following saline or repeated ethanol exposure. Repeated ethanol exposure decreased $\alpha 1$ levels by ~30% in wildtype mice compared to saline treated controls ($p < 0.05$). In contrast, knockin mice did not display any alterations in $\alpha 1$ levels following repeated ethanol exposure. $n = 7-9$ mice per treatment, per group.

Table 1

BECs and percent weight loss from HIC assay. BECs and percent weight loss are shown for controls and ethanol exposed wildtype and knockin mice.

Genotype and Treatment	BEC (mg/dL)	Weight Loss (%) [*]
Wildtype Air	n.d.	1.7 ± 0.6
Knockin Air	n.d.	1.3 ± 1.2
Wildtype Ethanol	143.7 ± 10.0	2.8 ± 0.5
Knockin Ethanol	165.5 ± 14.8	4.1 ± 0.8

^{*} Ethanol exposed mice had higher percent weight loss (ANOVA, $F_{1, 62} = 6.8$, $p < 0.05$).

No effect of gender or genotype was observed on either measure. n.d. – not detectable.