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## Knockout of alpha 5 nicotinic acetylcholine receptors subunit alters ethanol-mediated behavioral effects and reward in mice.

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### Abstract

Evidence suggests that there is an association between polymorphisms in the  $\alpha 5$  nicotinic acetylcholine receptor (nAChR) subunit and risk of developing alcohol dependence in humans. The  $\alpha 5$  nAChR subunit has also recently been shown to modulate some of the acute response to ethanol in mice. The aim of the current study was to further characterize the role of  $\alpha 5$ -containing ( $\alpha 5^*$ ) nAChRs in acute ethanol responsive behaviors, ethanol consumption and ethanol preference in mice. We conducted a battery of tests in male  $\alpha 5$  knockout (KO) mice for a range of ethanol-induced behaviors including hypothermia, hypnosis, and anxiolysis. We also investigated the effects of  $\alpha 5^*$  nAChR on ethanol reward using the Conditioned Place Preference (CPP) assay. Further, we tested the effects of gene deletion on drinking behaviors using the voluntary ethanol consumption in a two-bottle choice assay and Drinking in the Dark (DID, with or without stress) paradigm. We found that deletion of the  $\alpha 5$  nAChR subunit enhanced ethanol-induced hypothermia, hypnosis, and an anxiolytic-like response in comparison to wild-type controls. The  $\alpha 5$  KO mice showed reduced CPP for ethanol, suggesting that the rewarding properties of ethanol are decreased in mutant mice. Interestingly, *Chrna5* gene deletion had no effect on basal ethanol drinking behavior, or ethanol metabolism, but did decrease ethanol intake in the DID paradigm following restraint stress. Taken together, we provide new evidence that  $\alpha 5$  nAChRs are involved in some but not all of the behavioral effects of ethanol. Our results highlight the importance of nAChRs as a possible target for the treatment of alcohol dependence.

### Keywords

ethanol; alpha 5 nAChRs; nicotinic receptors; reward; mice

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Conflict of Interest statement

All of the authors declare no conflict of interest.

## 1. Introduction

Alcohol and nicotine are two of the most commonly abused drugs, contributing to over 500,000 deaths annually in the United States, with associated medical costs in excess of \$500 billion per year (Centers for Disease Control and Prevention, 2014; 2015). Multiple studies have observed that up to 90% of individuals diagnosed with alcohol use disorder are cigarette smokers, with between 20–40% of them being heavy smokers, defined by 40+ cigarettes per day (Istvan and Matarazzo, 1984; DiFranza and Guerrero, 1990; Batel et al., 1995; Falk et al., 2006). Interestingly, increased alcohol abstinence rates have been reported in alcoholics who attempt to quit smoking and smoking cessation was shown not to disrupt alcohol abstinence (Gulliver et al., 2007; for review, see Hughes & Kalman, 2006). Evidence from both human and animal studies supports the notion that there are common genetic factors underlying these disorders (Schlaepfer et al., 2008; Cross et al., 2017). Here, we investigate the role of the alpha 5 subunit of nicotinic acetylcholine receptor, *Chrna5*, in mouse models of ethanol addiction-related behaviors (for review, see Bühler et al., 2015).

Investigations of the *CHRNA5-CHRNA3-CHRNA4* gene cluster in humans have suggested a role for  $\alpha 5^*$  nicotinic subunits in nicotine and alcohol dependence (Joslyn et al., 2008; Schlaepfer et al., 2008), as well as in the level of response to alcohol, which may impact liability to develop alcohol dependence. These studies have implicated single nucleotide polymorphisms (SNPs) in the *CHRNA5* gene, encoding the  $\alpha 5$  nAChR subunit, to be associated with an increased risk to develop alcohol dependence (Joslyn et al., 2008; Schlaepfer et al., 2008; Choquet et al., 2013). Furthermore, human genome-wide association studies have associated a promoter single nucleotide polymorphism (SNP) in *CHRNA5*, rs588765, with alcohol dependence in European Americans. Individuals homozygous for this SNP had significantly higher mRNA levels of *CHRNA5* in the frontal cortex than heterozygotes or those without the SNP (Wang et al., 2009). Mouse genetic studies have reported that there is an allelic difference in *Chrna5* between C57BL/6J and DBA/2J (alcohol-avoiding mice) that can be associated with alcohol preference in the strains (Symons et al., 2010). Furthermore, these researchers reported that whole brain *Chrna5* mRNA levels were significantly increased in C57BL/6J mice compared to DBA/2J mice following ethanol treatment (Symons et al., 2010). Finally, using  $\alpha 5$  knockout (KO) mice, Santos et al., (2013) study suggest that the  $\alpha 5$  nAChR subunit is important for the sedative effects of ethanol but does not play a role in ethanol oral intake in mice. Overall, these human and mouse studies have suggested an important role for the  $\alpha 5$  nicotinic subunit in alcohol responses and alcohol dependence.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that form pentamers containing  $\alpha$  ( $\alpha 2$ - $\alpha 10$ ) and/or  $\beta$  ( $\beta 2$ - $\beta 4$ ) subunits. Alpha 5 is an accessory subunit that forms pentamers with either  $\alpha 4\beta 2$ ,  $\alpha 3\beta 4$ , or  $\alpha 3\beta 2$  subunits, and together, these constitute 10–37% of all nAChRs (Kuryatov et al., 2008; Baddick and Marks, 2011). Although high levels of expression are found only in few brain areas (Salas et al., 2003a), *Chrna5* mRNA has been found in the majority of brain regions (Brown et al., 2007), suggesting that  $\alpha 5^*$  nAChRs could have a substantial impact on brain function. For example,  $\alpha 5^*$  nAChRs play a key role in nicotine intake, reward, and withdrawal in rodents (Salas et

al., 2003a; Jackson et al., 2010; Jackson et al., 2008; Fowler et al., 2011; Morel et al., 2014). For example,  $\alpha 5$  KO mice showed an enhancement of nicotine reward and intake (Jackson et al., 2010; Fowler et al., 2011) and a reduction in some nicotine withdrawal signs (Salas et al., 2003; Jackson et al., 2008). Expression studies in *Xenopus* oocytes also found that ethanol potentiated currents produced by acetylcholine in certain nAChR subunit combinations ( $\alpha 4\beta 2^*$ ,  $\alpha 4\beta 4^*$ ,  $\alpha 2\beta 4^*$ , and  $\alpha 2\beta 2^*$ ), had no effect on receptors containing  $\alpha 3\beta 4$  or  $\alpha 3\beta 2$  subunits, and inhibited the function of  $\alpha 7$  receptors, demonstrating that the effects of ethanol depend on the nAChR subtype considered (Cardoso et al., 1999; Borghese et al., 2003). A variety of ethanol's behavioral effects can be modulated by altered nAChR function (Kuzmin et al., 2008; Kamens et al., 2010, 2012; Santos et al., 2012; Sajja and Rahman, 2012). Importantly, Santos and colleagues (Santos et al., 2012), observed that mice lacking *Chrna5* demonstrated prolonged sleep time following administration of a sedative dose of ethanol, showed greater impairment of locomotion by ethanol evidenced by a decreased latency to fall off of a rotarod, but consumed similar amounts of ethanol compared to wild-type mice in the Drinking in the Dark (DID) forced drinking model (Santos et al., 2012). These results indicate that more work is needed to fully understand the role  $\alpha 5^*$  nAChRs plays in mediating phenotypes associated with alcohol dependence.

In this study, we seek to further characterize the role of the  $\alpha 5$  nAChR subunit on ethanol responsive behaviors and rewarding effects in mice. We hypothesized that deletion of the *Chrna5* gene would result in altered responses to ethanol in a battery of behavioral tests with emphasis on phenotypes associated with risk for alcoholism and across a range of alcohol doses. Using  $\alpha 5$  knockout (KO) and wild-type (WT) mice, we explored the role of  $\alpha 5^*$  nAChRs in three acute ethanol-responsive behaviors: hypothermia, loss of righting reflex, and anxiolysis. We also examined ethanol consumption and reward in multiple mouse paradigms of oral drinking and conditioned place preference (CPP).

## 2. Materials and methods

### 2.1. Animals

The  $\alpha 5$  KO breeding pairs were procured from The Jackson Laboratory (Bar Harbor, ME) and bred in the animal facility at Virginia Commonwealth University (Richmond, VA). They were originally reported by Salas et al. (2003a). All mice used in each experiment were backcrossed for at least 12 generations on C57BL/6J background. Heterozygote KO/+ mice were crossed to generate homozygous mutant and WT control littermates. Male mice were group-housed in a temperature and humidity controlled animal care facility approved by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and had free access to food and water under a 12-h light/dark cycle (lights on at 6:00 am) schedule. All experiments were performed during the light cycle. Mice were 8–10 weeks old at the start of the experiments. The study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. Behavioral and pharmacological tests were performed on different cohorts of mice.

## 2.2. Drugs

200-proof ethanol (Pharmco-AAPER, USA) was dissolved in 0.9% saline to yield a 20% (v/v) solution and injected intraperitoneally (i.p) for acute experiments. For drinking studies, ethanol (3–30%) was administered orally (p.o.) in the drinking water. Ketamine HCl (100 mg/mL solution, KetaVed™), obtained from Vedco Inc (Saint Joseph, MO), was diluted to 10 mg/mL in saline. Sodium pentobarbital, USP (50 mg/mL), purchased from Virginia Commonwealth University Hospital Pharmacy, was diluted to 3 mg/mL in saline. Ketamine HCl and sodium pentobarbital were each administered i.p. at a volume of 0.1 mL per 10 g of mouse mass for doses of 100 mg/kg and 30 mg/kg, respectively. The doses of alcohol (2 – 4g/kg) used in acute behavioral studies and conditioned place preference test were based on previously published studies (Alkana et al., 1992, Browman et al., 2000; Tanchuck-Nipper et al., 2015; Putman et al., 2016; Slater et al., 2016; Guildford et al., 2016).

## 2.3. Body Temperature Measurement

Hypothermia was measured by rectally inserting a standard thermometer probe ~24mm (Fischer Scientific, Pittsburgh, PA). Baseline temperatures were recorded and five minutes later, mice were administered 2.5 g/kg ethanol or saline (i.p.). Body temperature was recorded 15, 30, 60, and 120 minutes following ethanol injection. Data were expressed as the mean temperature change (°C) ± SEM from baseline after ethanol treatment. The room temperature of the laboratory varied from 21–24°C.

## 2.4. Loss of Righting Reflex (LORR)

The LORR assay was used to assess the sedative-hypnotic effects of ethanol, pentobarbital, and ketamine. The  $\alpha 5$  KO and WT mice were administered i.p. injections of ethanol (3.8 g/kg), pentobarbital (30 mg/kg) or ketamine (100 mg/kg), then placed into a supine position in a V-shaped trough. A mouse was confirmed to have achieved LORR only after it remained on its back for at least 30 sec. The time from ethanol, pentobarbital or ketamine injection until initial LORR (latency to LORR) was recorded. The time for a mouse to recover the ability to right itself three times within 30 sec was measured as the duration of LORR. Mice taking longer than five minutes for LORR onset were eliminated from the study due to the possibility of misplaced injection. Data were expressed as latency to LORR and LORR duration (in sec).

## 2.5. Elevated Plus Maze (EPM)

Anxiolytic-like behavior was assessed in the EPM, an elevated platform consisting of four black arms in a cross shape raised from the ground. Two arms have walls (closed arms) and the other two arms do not (open arms). Mice innately avoid open, elevated spaces, therefore, increased time spent in the open arms of the EPM is interpreted as anxiolytic-like behavior (Pellow et al. 1985). Prior to testing, mice were acclimated to the room for at least 1–2 hours. On test day,  $\alpha 5$  WT and KO mice were administered 0 or 2.0 g/kg ethanol (i.p.) and returned to their home cage for 15 min to minimize interference from the hyperlocomotion produced by acute ethanol treatment. Each mouse was placed briefly into a plastic container, transferred to the center of the EPM, and allowed to roam freely for five minutes. (Data were

expressed as the total time (sec) spent in the open arms. To control for locomotor effects, the total number of crossovers across the center of the maze was recorded.

## 2.6. Conditioned Place Preference (CPP)

An unbiased CPP paradigm was performed, as we previously described (Kota et al., 2007; Harenza et al., 2014). The experimenter handled each mouse for approximately 2 minutes for each of three days prior to the start of CPP. The apparatus (Med-Associates, St. Albans, VT, USA, ENV3013) consists of two conditioning chambers (20 × 20 × 20 cm each) that differ in color (black and white) and floor texture (white mesh and black rod) to provide two distinct conditioning environments. There is a smaller, gray holding chamber with a smooth PVC floor with partitions that allow closure from or access to the larger chambers. On day 1 (Pre-Test), animals were confined to the middle chamber for 5 min, then allowed to move freely explore the entire apparatus for 15 min. Time spent in each conditioning chamber was recorded and used to separate the animals into groups of approximately equal initial bias for the drug-paired chambers. For conditioning, days 2–4, the control group received saline (i.p.) in both chambers and the drug group received ethanol (2 g/kg, i.p., 10 min pretreatment) in one chamber and saline in the other chamber for 15 min. Treatments were counterbalanced equally in order to ensure that some mice received the unconditioned stimulus in the morning while others received it in the afternoon. The alcohol-paired chamber was randomized among all groups. Sessions were 4 hours apart and were conducted by the same investigator. On day 5 (Post-Test), mice were allowed to freely explore and time spent in each chamber was recorded. Preference scores (sec) were calculated as the difference in time spent in the drug-paired chamber, Post-Test time minus Pre-Test time.

## 2.7. Drinking in the Dark (DID)

DID is a limited-access drinking procedure used to model binge-drinking behavior in rodents (Rhodes et al., 2005; Thiele et al., 2014). The  $\alpha 5$  KO and WT mice were single-housed in a reverse light-dark cycle (7:00 am – 7:00 pm) one week prior to testing with *ad libitum* access to food and water. At the end of the acclimation period, three hours into the dark cycle (10:00 am), the water bottle from each cage was replaced with one drinking tube containing 20% ethanol for four hours. Baseline alcohol intake was calculated using the average consumption across four days of ethanol presentation.

On day 5, we investigated the impact of restraint stress on ethanol intake. Alpha 5 KO and WT mice were assigned to either the experimental (Stress) or control (No Stress) groups. The stress procedure was performed in an adjacent room to avoid disrupting the control group. Mice were removed from their home cage and placed in a Plexiglas restraint tube (diameter: 25.4 mm; length: 83 mm) for 15 min. Restraint stress was performed on all mice at the same time each day (between 09:00 and 10:00 am) for 4 consecutive days. At the end of each restraint period, mice were returned to the DID procedure room for 4 hours drinking session. The No Stress group remained undisturbed in their home cages in the DID procedure room during this phase of the experiment except for routine handling.

## 2.8. Blood Ethanol Concentration (BEC) Analysis

To rule out the possibility of the  $\alpha 5$  gene deletion directly altering ethanol metabolism, we assessed BEC in drug-naïve  $\alpha 5$  KO and WT mice over a two-hour time course following administration of a single high dose of ethanol (4.0 g/kg, i.p.). Blood was collected into microtainers containing dipotassium EDTA (BD Biosciences, San Jose, CA, USA) via submandibular cheek punch at 15-, 30-, 60- or 120 min time-points after injection. Samples were prepared for analysis by aliquoting 20  $\mu$ l of whole blood into 20 ml GC vials (Autosampler Guys, Alexandria, VA) containing 960  $\mu$ l deionized (DI) water and 20  $\mu$ l of 0.1 mg/ml 1-propanol standard. Vials were capped and stored at  $-20^{\circ}\text{C}$  until BECs were analyzed using headspace gas chromatography as described (Wolstenholme et al., 2011).

## 2.9. Statistical Analysis

Data were tested at an alpha level of 0.05 using either Student's t-test, one-way or multi-way analysis of variance (ANOVA), using GraphPad Prism (GraphPad Inc., San Diego, CA). When multiple measurements were taken from individual mice, repeated measures ANOVAs were used. Significant ANOVAs were followed with Tukey *post-hoc* tests and results are reported with corrected p-values.

## 3. Results

### 3.1. Ethanol-induced hypothermia accentuated in $\alpha 5$ null mice

We first tested whether  $\alpha 5$  nAChRs influence ethanol's hypothermic effect. Treatment with 2.5 g/kg ethanol reduced body temperature up to 120 min in both WT and KO mice (Fig. 1A). Alpha 5 KO mice had a significantly larger decrease in body temperature as compared to WT mice. A three-way ANOVA with repeated measures (genotype x treatment x time) resulted in a significant genotype x treatment interaction,  $F_{(1,19)} = 5.057$ ;  $p = 0.031$ . A significant main effect of genotype [ $F_{(1,19)} = 5.376$ ;  $p = 0.0317$ ] and treatment [ $F_{(1,19)} = 66.028$ ;  $p < 0.0001$ ] was also found. Ethanol's hypothermic effect was long lasting, as body temperature changes were relatively stable for the first hour of observation [ $F_{\text{time}(1,19)} = 3.300$ ;  $p = 0.0851$ ]. Body temperature did not differ between saline treated WT and  $\alpha 5$  KO at any time point. These results suggest that the  $\alpha 5$  subunit plays an important role for hypothermic response of ethanol. In addition, the increased hypothermic effect in  $\alpha 5$  KO mice is not due to changes in alcohol metabolism, as BEC levels were the same in  $\alpha 5$  KO and WT mice following a 4 mg/kg ethanol injection [Fig 1B,  $F_{\text{treatment} \times \text{time}(3,18)} = 1.16$ ,  $p = 0.35$ ].

### 3.2. Deletion of *Chrna5* enhances the duration of both ethanol- and pentobarbital-induced LORR

Our lab has previously highlighted the role of  $\beta 2$  nAChRs in the induction on LORR after ethanol treatment (Dawson et al., 2013). Given that ethanol has been shown to have distinct effects depending on the subunit composition of nAChRs (Cardoso et al., 1999; Borghese et al., 2003), we tested the latency and duration of ethanol-induced LORR after treatment with 3.8 g/kg ethanol in WT and  $\alpha 5$  null mice. There was no significant difference between WT and  $\alpha 5$  KO for the onset of LORR (Fig 2A), but there was a significant increase in the



duration of LORR in the  $\alpha 5$  KO (Fig 2B, [t=2.636, df=13; p= 0.02]. Our findings are similar to those reported by Santos and colleagues (Santos et al., 2012).

We tested whether the interaction between  $\alpha 5$ \*nAChRs and LORR effect was specific to ethanol or could be generalized to other sedative drugs, such as pentobarbital and ketamine. The  $\alpha 5$  genotype did not alter the latency to LORR for either pentobarbital or ketamine (Fig. 3). In contrast,  $\alpha 5$  KO mice exhibited a significantly longer duration of pentobarbital-induced LORR compared to WT mice (Fig 3, [t<sub>barb</sub>=8.755 df=5; p < 0.001]), similar to what was observed in ethanol-treated mice. However, the  $\alpha 5$  genotype had no effect on the duration of ketamine-induced LORR (Fig. 3). Given that both ethanol and pentobarbital modulate GABA<sub>A</sub> receptors, this suggests a possible potentiation of GABA<sub>A</sub> inhibitory tone in  $\alpha 5$  KO mice.

### 3.3. Deletion of *Chrna5* enhances ethanol-induced anxiolytic-like behavior

Ethanol is known to have anxiolytic-like effect in the EPM. As expected, ethanol increased the time spent in the open arms in both genotypes (anxiolytic-like effect) but had a significantly larger effect in the  $\alpha 5$  KO mice relative to the WT mice (Fig. 4A). A two-way ANOVA (genotype x treatment) resulted in significant main effects of treatment [ $F_{(1,36)} = 55.99$ ; p< 0.0001] and genotype [ $F_{(1,36)} = 9.88$ ; p= 0.003], as well as a significant interaction [ $F_{(1,36)} = 10.89$ ; p= 0.002]. These differences were not due to changes in locomotor activity since the number of crossovers between the arms was the same for all groups (Figure 4B). Our EPM results suggest that  $\alpha 5$  nAChR subunit plays an important role in the anxiolytic effect of acute ethanol.

### 3.4. Deletion of *Chrna5* reduced ethanol CPP

CPP was used to measure the influence of  $\alpha 5$ \*nAChRs in the rewarding effects of ethanol. While WT animals displayed a robust development of ethanol CPP, as measured by a significant increase in the amount of time spent in the drug-paired chamber, there was no CPP produced in the  $\alpha 5$  KO mice (Fig. 5, [ $F_{\text{genotype} \times \text{treatment} (1,40)} = 13.36$ , p < 0.001]). These data suggest that  $\alpha 5$  nAChR subunit expression may be required for acquisition of ethanol CPP in mice.

### 3.5. Deletion of *Chrna5* reduces ethanol consumption following stress in mice

Given that  $\alpha 5$  null mice display altered acute ethanol-induced responses and ethanol CPP, we hypothesized that  $\alpha 5$ \* nAChRs could also influence alcohol consumption. We tested mice in two distinct drinking paradigms: 1) standard two-bottle choice, which measures ethanol preference; and 2) DID, which models binge-like ethanol consumption. Unlike the standard two-bottle choice test which allows for continuous access to ethanol, DID is thought to motivate compulsive-like drinking by limiting the amount of time mice have access to ethanol. Overall, the DID paradigm has been found to produce increased ethanol consumption and BEC than the standard two-bottle choice paradigm (Fritz and Boehm, 2016).

We assessed ethanol consumption (3–30% v/v) in the two-bottle choice paradigm over the course of 2 weeks (see supplemental methods for the procedure). We found that deletion of

the  $\alpha 5$  subunit did not affect ethanol consumption or preference at any concentration of ethanol (Fig. 1 Supplement). A two-way ANOVA with repeated measure revealed a significant main effect of ethanol concentration ( $F_{(4,117)} = 86.390$ ,  $p < 0.0001$ ), but not to genotype ( $F_{(1,117)} = 1.795$ ,  $p = 0.194$ ) or the interaction between genotype and ethanol concentrations ( $F_{(4,117)} = 0.852$ ,  $p = 0.496$ ).

Similar to what has been reported by Santos and colleagues (Santos et al., 2012), we found no differences in the average ethanol intake between WT and KO mice after 4 days of DID ( $p = 0.529$ , t-test; Fig. 6, BL). Since ethanol reduces anxiety-like behavior in  $\alpha 5$  KO mice, we explored the influence of stress on drinking behavior.  $\alpha 5$  KO and WT mice were subjected to daily restraint stress once/day for 4 days before each DID session. We found that daily ethanol significantly decreased in  $\alpha 5$  KO mice compared to WT counterparts in all stress days ( $F_{(7,48)} = 9.545$ ,  $p < 0.001$ ) (Fig. 6).

#### 4. Discussion

The goal of this study was to characterize the role of the  $\alpha 5$  nAChR subunit (*Chrna5*) in several ethanol-responsive behaviors using  $\alpha 5$  KO mice and their WT littermates. We found that deletion of *Chrna5* significantly enhanced ethanol's effects on body temperature, increased LORR duration, and accentuated ethanol's anxiolytic effects in the EPM in comparison to WT controls. Further, we investigated the involvement of  $\alpha 5$  nAChRs in ethanol reward in the CPP, voluntary drinking in continuous access two-bottle choice and binge-like ethanol drinking in the DID paradigm. Deletion of *Chrna5* attenuated ethanol CPP and reduced ethanol intake in the DID following restraint stress. However, drinking behavior was not changed in the continuous access two-bottle choice assay or DID without restraint stress. Taken together, our findings indicate that  $\alpha 5$  nAChRs are involved in acute ethanol-responsive behaviors. Furthermore, the effects of  $\alpha 5$  on ethanol drinking behaviors are more prominent under stressful conditions.

The LORR test is used to assess sensitivity to ethanol's hypnotic effects. Our results are in agreement with a previous report where  $\alpha 5$  KO mice showed increased ethanol-induced LORR duration without affecting LORR onset (Santos et al., 2012). One possibility for the effect of  $\alpha 5$  nAChRs on ethanol-induced sedation could be due to changes in ethanol metabolism. This is unlikely, given that we found no difference in BEC between KO and WT mice during the exact time frame that we measured LORR behavior. The sedative effects of ethanol are known to be associated with hypoactivity of NMDA receptors and hyperactivity of GABA receptors (Beleslin et al., 1997). To understand the mechanism of  $\alpha 5$  nAChRs effect on the hypnotic effects of ethanol, we used two different types of sedative/hypnotics. Pentobarbital is a GABAA receptor potentiator (Loscher and Rogawski, 2012), while sedative effects of ketamine are attributed to its inhibitory action on NMDA receptors and HC1 channels (MacDonald et al., 1987; Chen et al., 2009). Like ethanol, we found that pentobarbital significantly increased LORR duration without affecting LORR onset in  $\alpha 5$  KO mice, suggesting that  $\alpha 5$  plays a role in GABA<sub>A</sub> receptor function. In contrast, ketamine had no genotype-related changes in LORR duration or onset. Together, these results suggest that deletion of *Chrna5* enhances ethanol-induced LORR, at least in part, by increasing the



activity of GABAA receptor function without altering NMDA receptor or HC1 channel function.

The  $\alpha 5$  subunit is co-expressed with  $\alpha 4\beta 2^*$  and  $\alpha 3\beta 4^*$  nAChRs subtypes to form functional receptor complexes (Ramirez-Latorre et al., 1996; Gotti et al., 2007). While the present study did not examine the exact composition of  $\alpha 5^*$  nAChRs, work in the field using nAChR specific antagonist and nAChR knockout mice give insight into the possible receptor composition. Work in our lab has highlighted the role of  $\alpha 4\beta 2^*$  nAChRs in modulating acute-ethanol behavioral responses. Drugs that specifically block this receptor complex amplify LORR duration and changes in body temperature. Similar results were also found when testing the  $\beta 2$  KO mice (Dawson et al., 2013). Human genetic studies have highlighted the influence of the *CHRNA5*, *CHRNA3* and *CHRNA4* gene cluster that encodes  $\alpha 5$ ,  $\alpha 3$ , and  $\beta 4$  nAChR subunits respectively, in alcohol-related behaviors (Schlaepfer et al., 2008). Mice overexpressing the human  $\alpha 5$ ,  $\alpha 3$ ,  $\beta 4$  gene cluster, display normal ethanol-induced sedative-hypnotic effects, changes in body temperature and ataxia as measured via the balance beam and rotarod test (Gallego et al., 2012). Studies using  $\beta 4$  KO mice also found no significant difference between WT littermates in ethanol LORR and locomotor behavior (Kamens et al., 2017). Not surprisingly, neither overexpression of the human gene cluster or  $\beta 4$  null mutation affected ethanol metabolism. Overall, this suggests that the influence of  $\alpha 5^*$  nAChRs in modulating the sedative, locomotor and temperature changes associated with acute ethanol are likely due to  $\alpha 4\beta 2\alpha 5^*$  and not  $\alpha 3\beta 4\alpha 5^*$  receptor subtypes.

One of the key findings is that  $\alpha 5$  KO mice demonstrated an increased response to ethanol's anxiolytic-like effects during the EPM test. There was no difference in the total number of crossovers in  $\alpha 5$  KO compared to WT mice, indicating that the effect of  $\alpha 5$  KO in the EPM is not due to changes in the locomotor activity. Our results are in line with a previous study where  $\alpha 5$  KO mice demonstrated an enhanced anxiolytic phenotype in the EPM (Gangitano et al., 2009). It is possible that ethanol's effect is mediated by  $\alpha 4\beta 2\alpha 5$  nAChRs, since  $\alpha 4\beta 2^*$  nAChR antagonist and  $\beta 2$  KO mice accentuate alcohol-induced anxiolytic behavior in the EPM (Dawson et al., 2013). At this point, the role of  $\alpha 5\alpha 3\beta 4$  nAChRs in modulating ethanol-induced anxiolysis cannot be excluded given that drugs targeting  $\alpha 3\beta 4^*$  nAChRs and  $\beta 4$  KO mice have not been tested. In addition, it is known that  $\beta 4^*$  nAChRs play a role in regulating anxiety-like behavior in the EPM during basal conditions (Salas et al., 2003b).

Furthermore, we found that deficiency of  $\alpha 5$  nAChRs did not increase ethanol intake in two-bottle choice or in the DID paradigm under basal conditions, suggesting that the effect of  $\alpha 5$  is more complex than simple enhancement of ethanol-induced anxiolysis. Given that stress elevates ethanol drinking (Anderson et al., 2016), we further examined voluntary ethanol consumption in the DID assay following restraint-induced stress. Here, we found a profound effect of  $\alpha 5$ : While the WT mice showed a modest increase of ethanol consumption following stress, the  $\alpha 5$  KO mice showed a significant reduction of ethanol intake following stress. These results suggest that deletion of *Chrna5* may alter the sensitivity to stress and/or loss of  $\alpha 5$  may decrease the effects of ethanol experienced by animals in stressful situations. Overall, the results from the EPM and drinking behaviors suggest that there could be additional aspects of anxiety or emotional behaviors regulated by  $\alpha 5$  nAChRs. To further understand the nature of the role of  $\alpha 5$  nAChR subunits in anxiolytic-like behaviors induced

by acute ethanol, different tests for more aspects of anxiety-like behaviors may prove useful in future studies.

The results of our DID study confirms previously published data where deletion of *Chrna5* did not change ethanol intake in this binge drinking assay (Santos et al., 2012). The fact that *Chrna5* deletion did not change ethanol consumption in either the DID or two-bottle choice procedures during basal conditions suggests that  $\alpha 5^*$  nAChRs do not have a strong role in ethanol consumption under non-stress conditions.

We used the CPP assay to examine the role of  $\alpha 5$  in ethanol reward-like effect. We found for the first time that ethanol preference was absent in  $\alpha 5$  KO mice in the CPP assay. This finding compliments our DID stress data where deletion of *Chrna5* reduced ethanol consumption in response to restraint stress in a voluntary drinking assay. Interestingly, our observation of a decrease in the rewarding properties of ethanol in  $\alpha 5$  KO mice is in contrast to our previous observation that they demonstrate increased preference for higher doses of nicotine in the CPP assay (Jackson et al., 2010). While unlikely, it is possible that emergent differences in ethanol metabolism between WT and KO mice occurring after 120 min post-alcohol injection could explain the difference in CPP scores since the interval between conditioning session was 4 hours.

In contrast to our observations with ethanol, in which we found that loss of the  $\alpha 5$  subunit enhanced the hypnotic effects of ethanol, the  $\alpha 5$  gene deletion confers decreased sensitivity to the locomotor depressant effect of nicotine (Jackson et al., 2010, Fowler et al., 2011). While the exact mechanisms underlying these results remain unknown, these results support the significance of the role of  $\alpha 5$  nAChR in the early responses to both nicotine and alcohol exposure. Finally, it is possible that the effects observed in the  $\alpha 5$  KO mice could be due to compensatory changes in the expression levels of other genes resulting from deletion of the *Chrna5* gene. While the expression of major nAChR subunits is not affected by *Chrna5* gene deletion (Salas et al., 2003a), compensatory mechanisms with other nicotinic or non-nicotinic pathways in the brain cannot be entirely ruled out. Despite this limitation, our data collectively confirm that *Chrna5* gene plays a significant role in ethanol responsive behaviors and reward.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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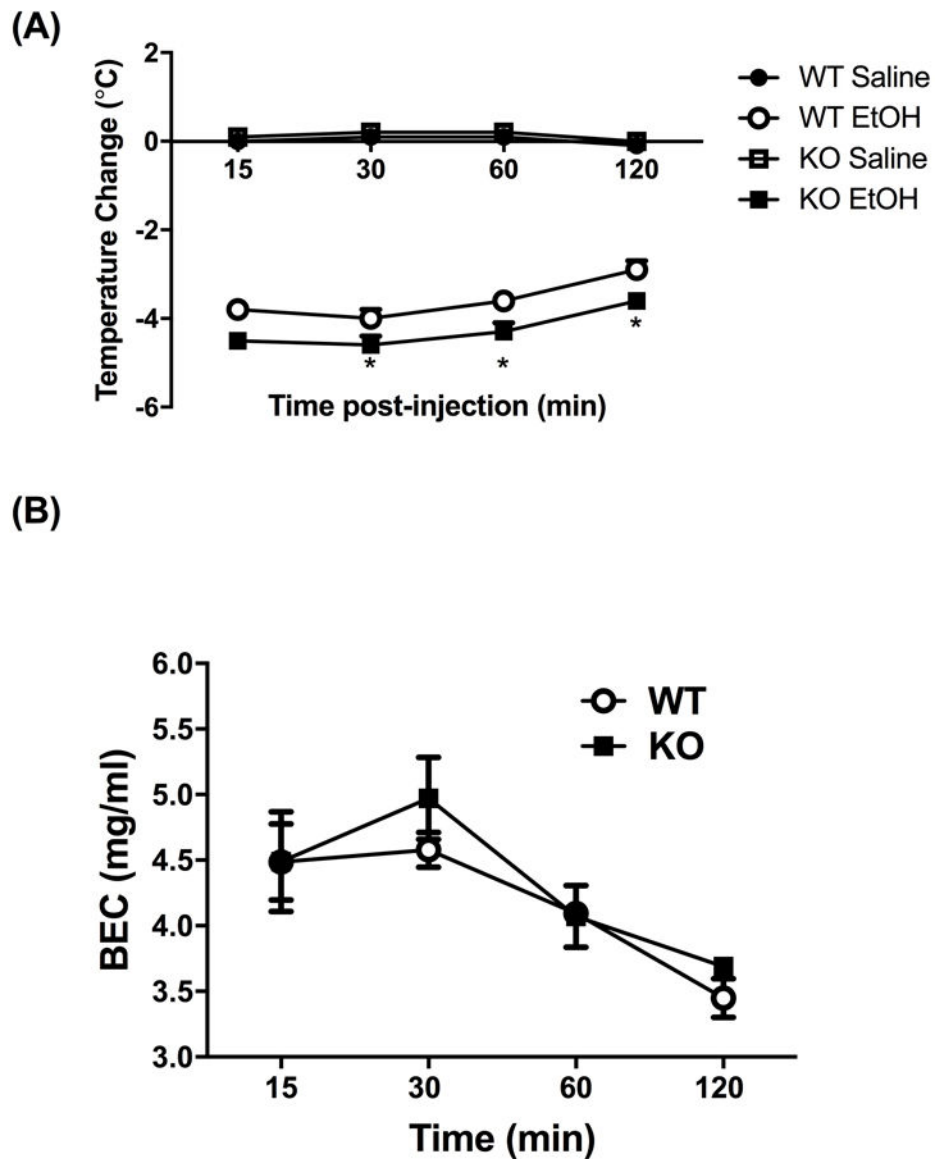
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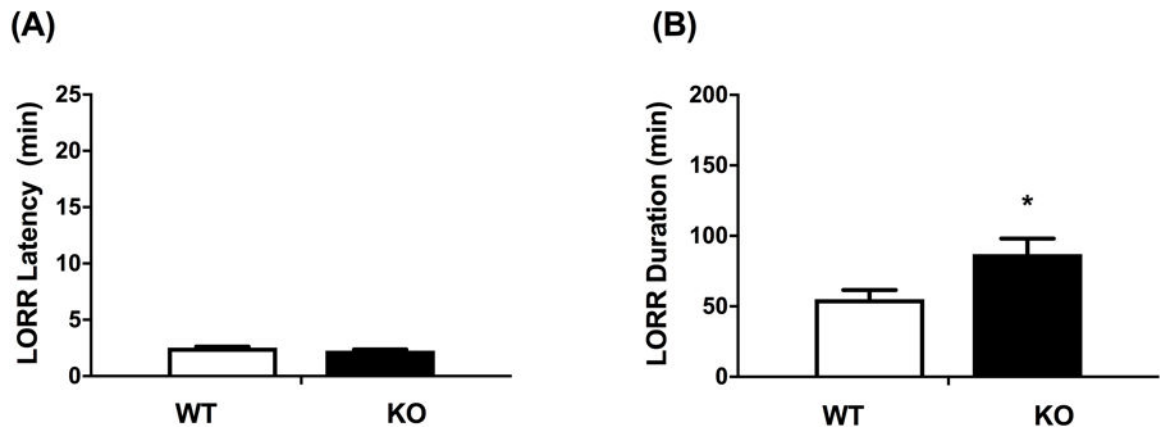
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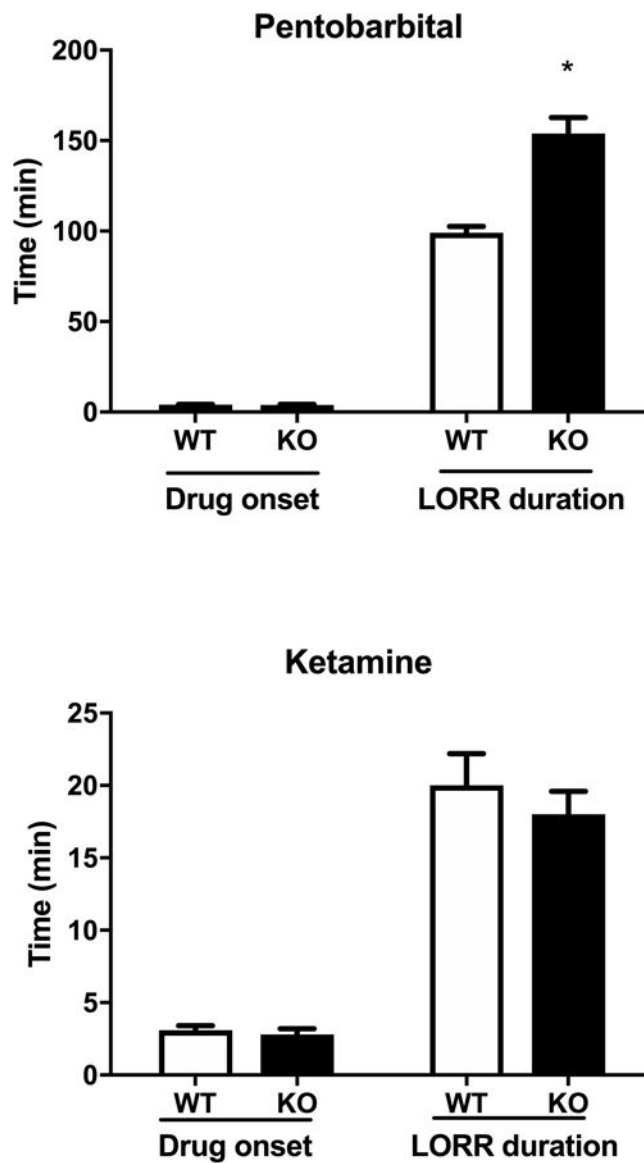
**Figure 1.**

(A) Deletion of the *Chrna5* gene enhances ethanol-induced hypothermia in mice. Data (mean  $\pm$  SEM) represent the change in body temperature from baseline in degrees Celsius of  $\alpha 5$  WT and KO mice at time points following an injection of 2.5 g/kg ethanol. Ethanol-induced hypothermia was significantly enhanced in  $\alpha 5$  KO mice compared to WT mice at the 30-, 60-, and 120-min timepoints (\* $p < 0.05$ ,  $n = 8$  per group). (B) Deletion of the *Chrna5* gene does not alter BEC levels in mice. Data (mean  $\pm$  SEM) represent BEC (mg/mL) at 15, 30, 60, and 120 min after receiving an injection of 4.0 g/kg ethanol ( $n = 5$  per group).

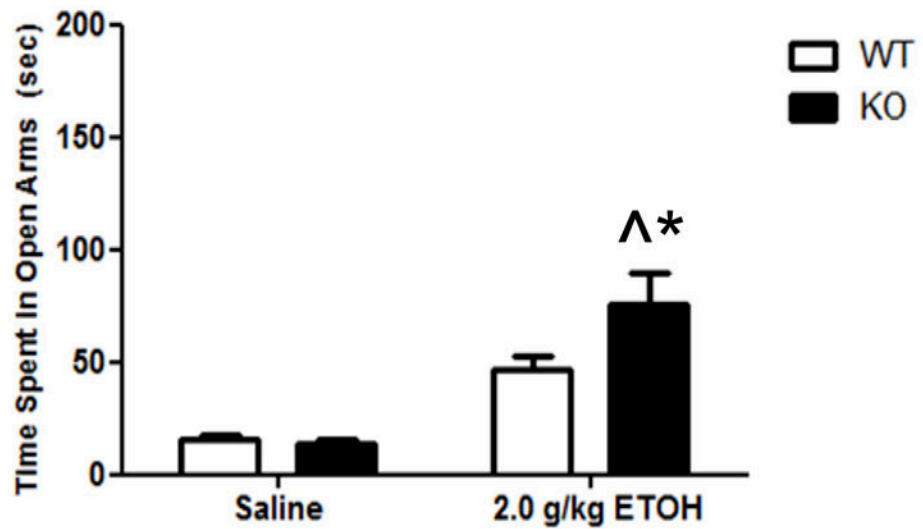
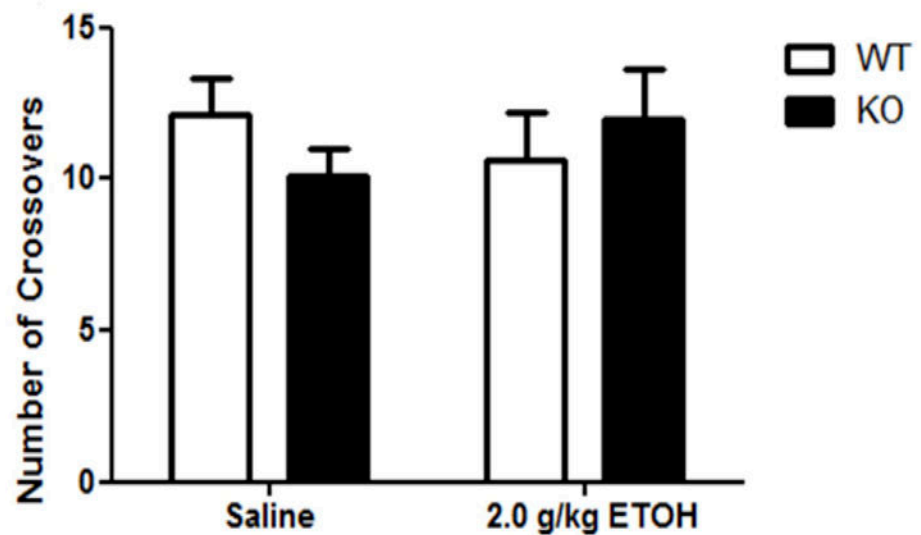




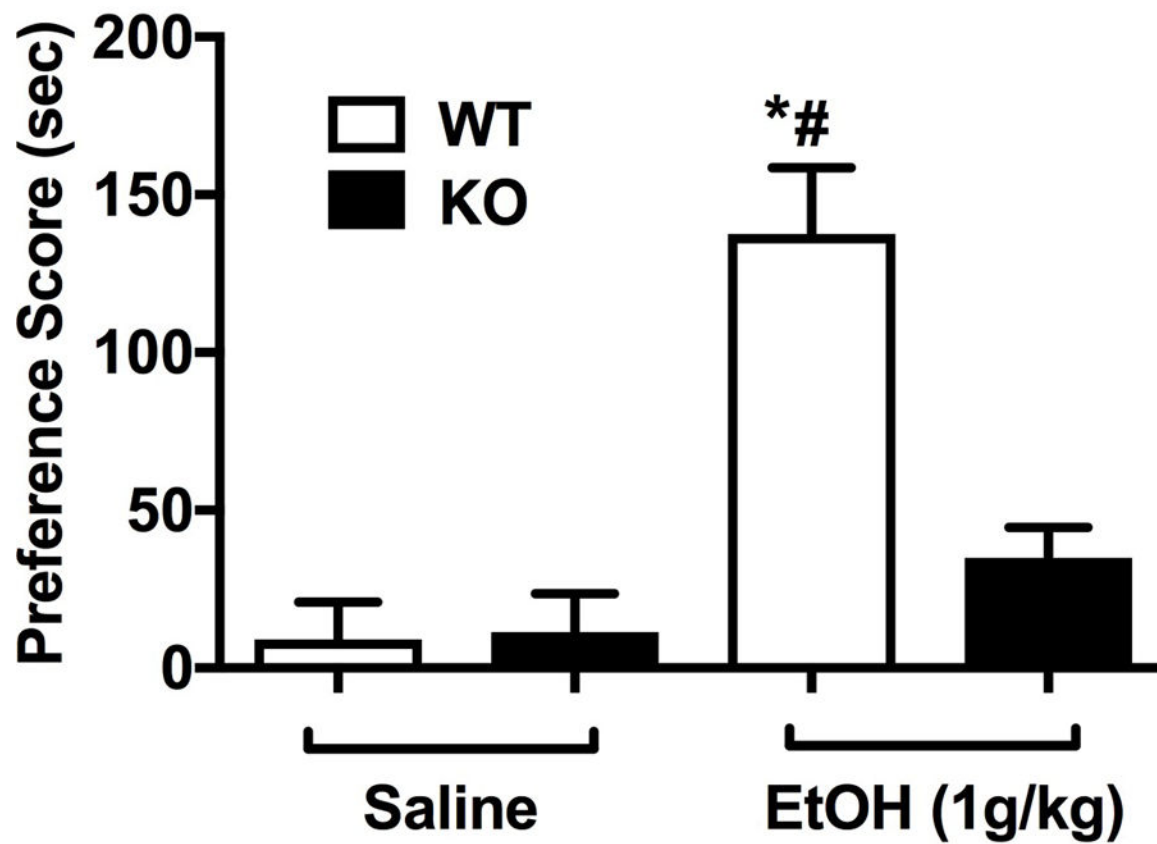
**Figure 2.** Deletion of the *Chrna5* gene had no effect on latency to loss of righting reflex (LORR) (A) but enhanced ethanol-induced LORR duration (B). Data represent the mean  $\pm$  SEM for  $\alpha 5$  WT and KO mice after receiving 3.8 g/kg i.p. ethanol (\* $p < 0.05$ ,  $n = 7$  per group).



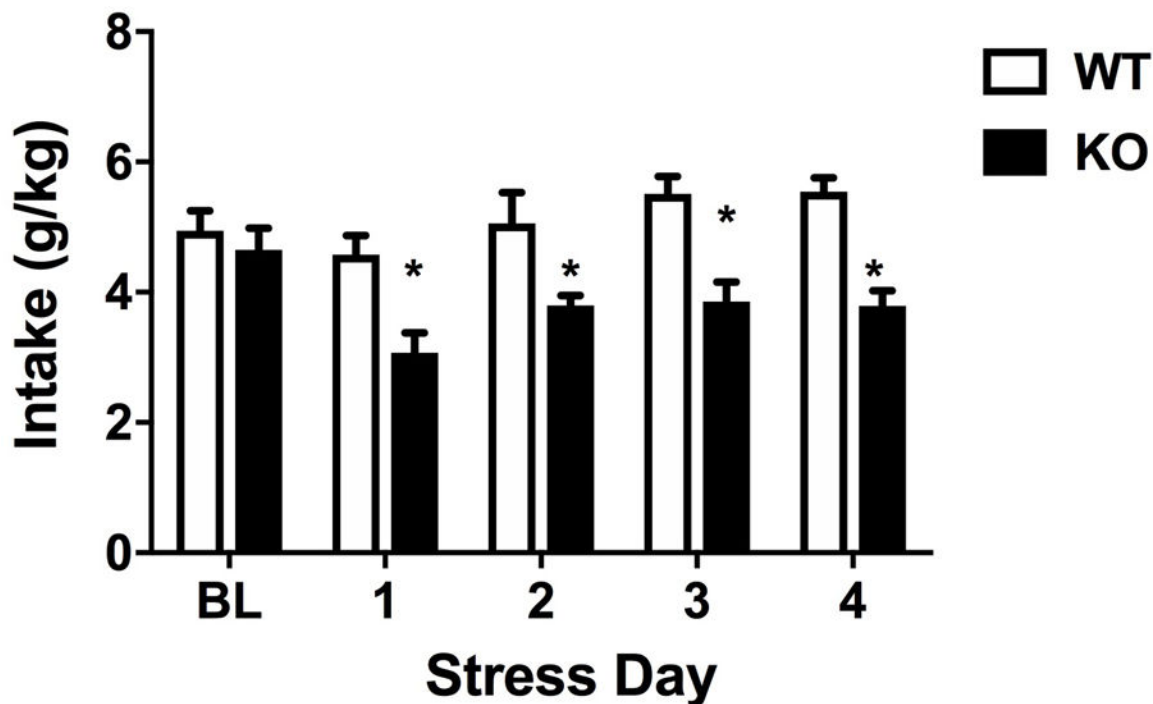
**Figure 3.** Deletion of *Chrna5* enhances loss of righting reflex (LORR) duration following pentobarbital, but not ketamine. Data (mean  $\pm$  SEM) represent Pentobarbital (30 mg/kg, i.p.) and Ketamine (100 mg/kg, i.p.) LORR latency and LORR duration in WT and  $\alpha$ 5 KO mice (\* $p$ <0.05 compared to correspondent WT mice,  $n$ = 8 per group).

**A****B****Figure 4.**

Deletion of the *Chrna5* gene enhances ethanol-induced anxiolytic-like behavior. Data (mean  $\pm$  SEM) represent (A) time spent in open arms and (B) total number of crossovers in WT and  $\alpha 5$  KO mice following an i.p. injection of 2.0 g/kg ethanol ( $^{\wedge}p < 0.05$  compared to correspondent saline-treated group;  $*p < 0.05$  compared to correspondent WT mice,  $n = 8-13$ ).



**Figure 5.** Deletion of the *Chrna5* gene reduces ethanol conditional place preference in mice. Data (mean  $\pm$  SEM) represent preference scores (sec) in WT and  $\alpha 5$  KO mice conditioned with saline or ethanol (\* $p < 0.05$  compared to the correspondent saline-treated group; # $p < 0.05$  compared to correspondent WT mice,  $n = 11$ ).



**Figure 6.** Comparison of ethanol consumption before and during repeated stress in  $\alpha 5$  WT and KO mice using the DID paradigm. Deletion of the *Chrna5* gene reduces DID drinking behavior after stress in mice. The baseline (BL) EtOH intake for  $\alpha 5$  WT and KO mice is similar. Data (mean  $\pm$  SEM) represent daily DID ethanol intake in g/kg for 4 hours in WT and  $\alpha 5$  KO mice that underwent daily restraint stress for 15 min for 4 days (\* $p < 0.05$  compared to correspondent WT group,  $n = 8$ ).