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Normal acute behavioral responses to moderate/high dose ethanol in GABA_A receptor $\alpha 4$ subunit knockout mice

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Abstract

Background— γ -Aminobutyric acid type A receptors (GABA_A-Rs) have been implicated in mediating some of the behavioral effects of ethanol (EtOH), but the contribution of specific GABA_A-R subunits is not fully understood. The GABA_A-R $\alpha 4$ subunit often partners with $\beta 2/3$ and δ subunits to form extrasynaptic GABA_A-Rs that mediate tonic inhibition. Several in vitro studies have suggested that these extrasynaptic GABA_A-Rs may be particularly relevant to the intoxicating effects of low doses of EtOH. In $\alpha 4$ subunit knockout mice tonic inhibition was greatly reduced, as were the potentiating effects of EtOH. We therefore hypothesized that those behavioral responses to EtOH that are mediated by $\alpha 4$ -containing GABA_A-Rs would be diminished in $\alpha 4$ knockout mice.

Methods—We investigated behavioral responses to acute administration of moderate/high dose EtOH or pentylenetetrazol in $\alpha 4$ subunit knockout mice. We compared behavioral responses to EtOH in $\alpha 4$ knockout and wild type littermates in the elevated plus maze (0.0, 1.0 g/kg EtOH), screen test (1.5, 2.0 g/kg), hypothermia (1.5, 2.0 g/kg), fixed speed rotarod (1.5, 2.0, 2.5 g/kg), open field (0.0, 1.0, 2.0 g/kg), radiant tail flick (2.0 g/kg), loss of righting reflex (3.5 g/kg), and EtOH metabolism and clearance assays. Sensitivity to pentylenetetrazol-induced seizures was also analyzed.

Results—No differences were observed between $\alpha 4$ knockout mice and wild type controls in terms of the baseline behavior in the absence of EtOH treatment or in the behavioral effects of EtOH in the assays tested. In contrast, $\alpha 4$ knockout mice were significantly more sensitive to pentylenetetrazol-induced seizures.

Conclusions—We conclude that GABA_A-Rs containing the $\alpha 4$ subunit are not absolutely required for the acute behavioral responses to moderate/high dose EtOH that were assessed with the elevated plus maze, screen test, hypothermia, fixed speed rotarod, open field, radiant tail flick, and loss of right reflex assays. We further suggest that these findings are complicated by the demonstrated compensatory alterations in synaptic GABA_A-R EtOH sensitivity and function in $\alpha 4$ knockout mice.

Keywords

GABA; GABA type A receptor; gene knockout mice; alcohol; extrasynaptic receptors

Introduction

Despite the use of EtOH throughout human history for its anxiolytic and intoxicating effects, the precise molecular targets through which EtOH exerts these effects are not well understood. Many different brain circuits and signaling systems have been implicated in the behavioral actions of EtOH in rodents (Harris, 1999). One likely set of targets is the family of γ -aminobutyric acid type A receptors (GABA_A-R), which mediate inhibition throughout the mammalian brain.

GABA_A-Rs are pentameric complexes that function as ligand-gated chloride ion channels. There are a variety of subunit families that make up GABA_A-Rs; a total of nineteen distinct subunits have been cloned, α 1–6, β 1–3, γ 1–3, δ , ϵ , π , θ , and ρ 1–3 (Barnard et al., 1998). This diversity in GABA_A-R subunit composition results in substantial anatomical, functional and pharmacological heterogeneity. For example, GABA_A-Rs containing α 1, α 2, or α 3, with β 2/3 and γ 2 are typically found at sub-synaptic sites, where they mediate fast synaptic inhibition. In contrast, GABA_A-Rs containing α 4 or α 6, with β 2/3 and δ are typically found at extrasynaptic locations, where they mediate a tonic form of inhibition by virtue of their ability to respond to low concentrations of GABA. Recent studies suggest that these extrasynaptic GABA_A-R populations are likely targets for the behavioral effects of EtOH.

A large number of studies have shown that GABA_A-Rs are sensitive to EtOH (e.g., Mihic et al., 1994; Sigel et al., 1993), but in most studies the concentration required to potentiate those receptors was often greater than 60 mM, a concentration much higher than that achieved during social drinking. In contrast, in vitro studies of recombinant GABA_A-Rs of the extrasynaptic subtypes showed that these receptors were potentiated by concentrations of EtOH that approximate those achieved by social drinking (<30 mM) (Hancher et al., 2005). In another set of studies, recombinant GABA_A-Rs containing α 4 and δ were potentiated by 1–3 (Sundstrom-Poromaa et al., 2002) or 3–10 (Wallner et al., 2003) mM EtOH, concentrations achieved by about half a glass to one glass of wine, although different results have been reported by other groups working with the same subunits (Borghese et al., 2006). In native tissues, tonic inhibitory currents thought to be mediated by α 4/ δ receptors were potentiated by 30 mM EtOH (Wei et al., 2004). Finally, the EtOH behavioral antagonist Ro15-4513 inhibited EtOH action at α 4/ δ receptors (Wallner et al., 2006) and EtOH blocked binding of [³H]Ro15-4513 to α 4 β 3 δ receptors (Hancher et al., 2006), although other groups have been unable to observe similar effects (Korpi et al., 2007; Mehta et al., 2007).

In vivo behavioral experiments also suggest that α 6/ δ and α 4/ δ extrasynaptic GABA_A-Rs mediate behavioral effects of EtOH. Rats with the Q100 allelic variation of the α 6 subunit of the GABA_A-R were more sensitive to the motor-incoordinating effects of EtOH compared to rats with α 6 (R100) (Hancher et al., 2005). The same study found that 1) potentiation of tonic current by EtOH was significantly greater in the α 6 expressing cerebellar granule cells of α 6(Q100) rats than α 6(R100) rats and 2) EtOH more robustly potentiated in vitro receptors containing α 6(Q100) β 3 δ than α 6(R100) β 3 δ (Hancher et al., 2005). In another study, progesterone-withdrawn rats that exhibited increased α 4 δ expression were more sensitive to low concentrations of EtOH both at the cellular and behavioral levels (Sundstrom-Poromaa et al., 2002). Finally, mice lacking the δ subunit of the GABA_A-R showed reduced potentiation of tonic inhibition by EtOH (Liang et al., 2006; Wei et al., 2004) and exhibited multiple defects in behavioral responses to EtOH (Mihalek et al., 2001).

GABA_A-Rs containing $\alpha 4$ and δ subunits mediate tonic inhibition in the dentate gyrus and in thalamic relay neurons (Belelli et al., 2005; Chandra et al., 2006; Jia et al., 2005; Nusser and Mody, 2002; Peng et al., 2004). The $\alpha 4$ subunit is also expressed in the cortex, striatum and other brain areas (Khan et al., 1996; Pirker et al., 2000; Wisden et al., 1992). Thus, there is reason to suspect that these receptors might be involved in a variety of behaviors that are influenced by alcohol.

In the present study, we investigated the behavioral effects of EtOH in a recently created strain of $\alpha 4$ subunit knockout (KO) mice. These mice were largely insensitive to some behavioral effects of the GABA_A-R agonist, gaboxadol, also known as THIP (Chandra et al., 2006), previously shown to be relatively selective for extrasynaptic $\alpha 4\beta\delta$ GABA_AR's (Brown et al., 2002; Liang et al., 2004). The $\alpha 4$ KO mice also showed a substantial deficit in tonic inhibition in dentate gyrus and thalamus (Chandra et al., 2006), and the enhancement of tonic current by EtOH was greatly reduced in dentate gyrus granule cells in these mice (Glykys et al., 2007; Liang et al., 2007). We therefore hypothesized that those behavioral responses to EtOH that are mediated by $\alpha 4$ -containing GABA_A-Rs would be diminished in $\alpha 4$ KO mice.

Methods and Materials

Mice

Breeding pairs of $\alpha 4$ heterozygous mice (Chandra et al., 2006) were used to produce $\alpha 4$ KO and WT littermate controls. All mice were of a mixed C57BL/6J and Strain 129S1/X1 genetic background from the F2-F6 generations. Mice were genotyped at weaning using Southern Blot analysis of tail DNA as previously described (Chandra et al., 2006). 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 7:30 AM. For all experiments, both male and female mice were used, but no gender differences were observed and data was collapsed across gender except as noted in the results. Each mouse was used for only a single behavioral assay, except as noted below. EtOH was purchased from Pharmco (Brookfield, CT) and was administered into the peritoneal cavity (i.p.). The Institutional Animal Care and Use Committees at the University of Pittsburgh and the University of California, Los Angeles approved all protocols.

Elevated Plus-Maze

The elevated plus maze was used to test baseline anxiety and activity, as well as the anxiolytic and locomotor stimulatory effect of EtOH, using previously described methods (Homanics et al., 1999). Briefly, 8–10 week old mice were weighed and transported to the mouse behavioral room one day prior to testing. All testing occurred between 10:00 and 13:00 under ambient room light. Mice were injected with normal saline or 1.0 g/kg EtOH 10 min prior to testing. Each mouse was placed on the central platform of the maze, facing an open arm and allowed to freely explore the maze for 5 min. Open-arm and closed-arm entries and the cumulative time spent on the open and closed arms were recorded. A mouse was considered to be on the central platform or on an arm when all four paws were within its perimeter. The percent open-arm entries, total number of entries and percent time in open-arms were determined. Data were analyzed using two-way ANOVA, with genotype and dose as the between-subject factors.

Open Field Assay

The open field assay was used to measure baseline activity and EtOH -induced locomotor stimulation and sedation. Mice (8–11 weeks of age) were weighed and transported to the mouse behavioral room one day prior to testing. All testing occurred between 12:00 and

17:00. Mice were injected with normal saline, 1.0 g/kg EtOH, or 2.0 g/kg EtOH 10 min prior to testing. Each mouse was placed in the center of a walled arena (43.2 cm × 43.2 cm × 30.5 cm) that was located in a sound attenuating cubicle (Med Associates, St. Albans, VT) and allowed to freely explore the activity chamber for 10 min. Distance traveled and the number of rearings were automatically recorded using an activity monitor. The number of rearings was defined as the number of breaks of a photobeam 8 cm above the floor of the arena. Data were analyzed using two-way ANOVA with genotype and dose as the between-subject factors and Fisher's post-hoc test where appropriate.

Fixed Speed Rotarod

Mice were trained on a fixed speed rotarod (Ugo Basile, Model 7650 with rod diameter of 6 cm, Stoelting Co., Wood Dale, IL) and training was considered complete when mice were able to remain on the rotarod for 180 s. After EtOH administration, each mouse was placed back on the rotarod and time spent on the rotarod was measured for up to 180 s at intervals listed below for 60 min post-injection. Three independent experiments were conducted at three different dosages of EtOH, 1.5 g/kg, 2.0 g/kg, and 2.5 g/kg. In one experiment, 8–11 week old drug naïve mice were trained on the rotarod spinning at 14 rpm, and then injected with 1.5 g/kg EtOH. Performance on the rotarod was measured every 15 min. In another experiment, 8–12 week old EtOH naïve mice were trained on the rotarod spinning at 6 rpm and then injected with 2.0 g/kg EtOH. Performance on the rotarod was measured every 10 min. In the final experiment, the same mice injected with 2.0 g/kg were re-trained two weeks later on the rotarod spinning at 6 rpm and injected with 2.5 g/kg EtOH. Performance was measured every 15 min. Data were analyzed within each experiment using repeated measures ANOVA.

Loss of Righting Reflex Assay

Mice were tested for the sedative/hypnotic response induced by EtOH using the loss of righting reflex (LORR) assay. Mice (10–15 weeks of age) were injected with 3.5 g/kg EtOH and then monitored for LORR. Once this occurred, mice were placed on their backs in v-shaped troughs. Mice were monitored until they were able to right themselves three times in 30 s. The duration of LORR was the time elapsed between when they were placed in a supine position and when they were able to right themselves three times. A heat lamp and monitoring of rectal temperatures were used to ensure normothermia. Data were analyzed using an unpaired Student's t test.

Radiant Tail Flick Assay

A radiant tail-flick assay was used as described (Lariviere et al., 2002). Mice that had been tested in the open field assay more than two weeks prior were used for this assay. Briefly, mice (age 10–14 weeks) were lightly restrained by placing them in a soft cloth pouch with the tail extended from one end. Focused light from a tail-flick analgesia meter (IITC Life Sciences, Woodland Hills, CA) was applied directly to a spot ~1 cm from the tip of the tail. Tail-flick latency was measured using a digital timer contained within the experimental apparatus. Baseline measurements were made using a moderate light intensity that would yield ~10 s basal response based on prior experiments. The possibility of tissue damage was avoided by automatic shutoff of the light after 30 s if the mouse did not respond. On the first day, mice were weighed and tested for basal nociception. One day later, mice were injected with 2 g/kg EtOH and tested for latency to tail flick 20 min after injection. At least two measurements were taken and the mean value calculated for each mouse. Data were analyzed using repeated measures ANOVA.

Screen Test and Hypothermia Test

The screen test and EtOH-induced hypothermia were assayed together on the same mice. Mice (age 10–16 weeks) that were tested on the open field assay two weeks prior were used for these studies. The screen test was carried out using an apparatus that was constructed very similar to that described previously (Crabbe et al., 2003). The screen test apparatus was a 5 mm² grid mounted in a plastic frame and positioned 60 cm above a padded table. The grid was supported by two vertical arms in such a manner that allowed the grid to rotate around an axis perpendicular to the supporting arms. Mice were weighed, injected with saline, and then placed on the screen while the screen was horizontal. The screen was then rotated 90 degrees over 3 s. Mice were given two trials to pass the criterion latency of remaining on the screen for 240 s. Three days later, the rectal temperature of each mouse was measured. Five min after temperature measurement, each mouse was injected with either 1.5 or 2.0 g/kg EtOH. Twenty five min after EtOH injection each mouse was assayed on the screen test apparatus for latency to fall. Animals that did not fall after 240 s were given a latency score of 240. Thirty min after injection, rectal temperature was again measured. For both the screen and hypothermia tests, data were analyzed using ANOVA and Fisher's post hoc test.

EtOH Metabolism and Clearance

Mice (13–16 weeks of age) were injected with EtOH (3.5 g/kg) and blood samples were collected from the retro-orbital sinus at 30, 60, 90, and 120 min following injection. Blood EtOH concentration (BEC) was determined as described previously (Harris et al., 1995). Briefly, blood was collected in heparinized capillary tubes, then mixed with 3% perchloric acid, and centrifuged for 10 min at 10,000g at 4 °C. The EtOH concentration in supernatants was measured using spectrophotometry via an alcohol dehydrogenase assay. Clearance was calculated as the average slope of a linear regression of BEC versus time. An unpaired Student's t-test was used to make comparisons between genotypes.

Pentylenetetrazol (PTZ)-Induced Seizure Activity

Seizure thresholds were determined by injection (i.p.) of PTZ at doses of 2.5, 5, 10, 30, 45 and 60 mg/kg in six-month-old male (28–38 g) C57/BL6, $\alpha 4$ WT and $\alpha 4$ KO mice. Each mouse received only one PTZ dose. After slow injection of PTZ, animals were continuously monitored for 60 min for signs of motor convulsions, and the fraction of animals exhibiting frank tonic-clonic seizures was determined. Seizures were terminated in all animals by injection of diazepam (10 mg/kg, i.p.). Chi square analysis was used to determine significant differences in seizure susceptibility between dose and genotype group means.

Results

Elevated Plus Maze

The elevated plus maze test was administered to examine baseline anxiety-like behavior and the anxiolytic and locomotor stimulatory effects of EtOH. There were no differences between WT and KO mice in basal performance (i.e., following saline injection) on the elevated plus maze. Saline treated WT and KO mice did not differ in total arm entries, percentage of entries onto open arms, or percentage of time spent on open arms (Figure 1). The locomotor stimulatory effect of EtOH was assessed by comparing the total number of arm entries in mice treated with saline versus those treated with EtOH (Figure 1A). Although a 1.0 g/kg dose of EtOH produced a significant increase in total entries [ANOVA: $F_{(1,52)} = 10.3$, $p < 0.005$], there were no significant differences between KO and WT mice. The anxiolytic response to EtOH was assessed by comparing the percentage of open arm entries (Figure 1B) and percentage of time spent on open arms (Figure 1C) between mice

treated with saline versus EtOH. A 1.0 g/kg dose of EtOH produced significant anxiolytic responses; EtOH treated mice showed an increase in open arm entries [$F_{(1,52)} = 12.1$, $p < 0.005$] and time spent on open arms [$F_{(1,52)} = 12.7$, $p < 0.001$]. However, KO mice did not differ from WT mice in their sensitivity to the anxiolytic effects of EtOH.

Open Field Assay

The open field assay was used to measure baseline activity as well as the locomotor stimulatory and sedative effects of EtOH.

Analysis of total distance traveled revealed a significant main effect of EtOH treatment [$F_{(2,98)} = 17.4$, $p < 0.0001$] but there were no significant effects of genotype or the interaction of genotype and treatment (Figure 2A). Distance traveled was increased by ~55% by 1.0 g/kg EtOH compared to mice treated with saline ($p < 0.0001$). Mice treated with 2.0 g/kg EtOH did not differ in distance traveled compared to saline controls.

Analysis of the number of rearings observed in the open field revealed a significant effect of gender [$F_{(1,102)} = 5.2$, $p < 0.05$], and so the data from male and female mice were analyzed separately. For both genders, there were significant main effects of EtOH treatment with respect to number of rearings observed [ANOVA: male, $F_{(2,48)} = 19.6$, $p < 0.0001$; female, $F_{(2,44)} = 11$, $p < 0.0001$]. In males, 1.0 g/kg ($p < 0.05$) and 2.0 g/kg ($p < 0.0001$) doses reduced the number of rearings compared to saline-treated mice. In females, only 2.0 g/kg EtOH reduced the number of rearings ($p < 0.0001$). There was no significant effect of genotype or interaction of genotype with dose on the number of rearings for either gender.

Fixed Speed Rotarod

Recovery from ataxia induced by three different doses of EtOH (1.5, 2.0 or 2.5 g/kg) was measured using a fixed speed rotarod in three separate experiments (Figure 3). In all experiments, there was a significant effect of time [1.5 g/kg, $F_{(3,48)} = 21.2$, $p < 0.0001$; 2.0 g/kg, $F_{(4,96)} = 20.2$, $p < 0.0001$; 2.5 g/kg, $F_{(3,54)} = 40.6$, $p < 0.0001$]. However, there were no significant effects of genotype or interaction of genotype with time for any of the doses tested.

Loss of Righting Reflex (LORR) Assay

The sedative/hypnotic effect of a 3.5g/kg dose of EtOH was determined using the LORR assay (Figure 4A). WT and KO mice did not differ in the duration of LORR.

Radiant Tail Flick Assay

The radiant tail flick assay was used to measure thermal pain sensitivity and to study the analgesic effect of 2.0 g/kg EtOH (Figure 4B). WT and KO mice did not differ in their basal thermal pain sensitivity, as measured by their latency to tail flick in the absence of drug. There was a significant main effect of EtOH treatment on the latency to tail flick [ANOVA: $F_{(1,21)} = 196$, $p < 0.0001$]. However, there was no significant effect of genotype or interaction of genotype with EtOH. EtOH significantly prolonged the latency to tail flick compared with basal responses in both WT ($p < 0.0001$) and KO ($p < 0.0001$) mice.

Screen test

Results for the screen test are shown in Figure 5A. Prior to being tested with EtOH, all mice were trained until they were able to remain on the screen for 240 s. There was a significant main effect of EtOH treatment on latency to fall [$F_{(1,76)} = 10.6$, $p < 0.005$]. In contrast, there were no significant effects of genotype or interaction of genotype with EtOH on latency to fall from the screen. This demonstrated that both 1.5 and 2.0 g/kg EtOH impaired the ability

to stay on the vertical screen equally in both genotypes. This effect was dose-dependent as mice treated with 2.0 g/kg were more impaired than those treated with 1.5 g/kg ($p < 0.005$).

Hypothermia test

EtOH -induced reductions in body temperature are displayed in Figure 5B. There were no differences in baseline body temperature between WT and KO mice (data not shown). There was a significant main effect of EtOH treatment on change in body temperature [$F_{(1,76)} = 34.0$, $p < 0.0001$] indicating that EtOH injection induced a hypothermic response. However, there was no significant main effect of genotype or interaction of genotype with dose in the hypothermic effect of EtOH. The effect of EtOH was dose-dependent as 2.0 g/kg EtOH had a greater hypothermic effect than 1.5 g/kg ($p < 0.0001$).

EtOH Metabolism and Clearance

To determine if KO mice differed from their WT littermates with respect to EtOH pharmacokinetics, BEC was measured every 30 min following 3.5 g/kg injection of EtOH. BECs did not differ significantly between genotypes at any timepoint measured. For example, at 90 min postinjection, BEC was 317 ± 27 mg/dl ($n = 5$) in WT animals compared to 333 ± 43 mg/dl ($n = 5$) in $\alpha 4$ KO animals. The rate of clearance of EtOH from the blood also did not differ between genotypes (WT, 2.5 ± 0.5 mg/dl/min, $n = 5$; KO, 2.4 ± 0.2 , mg/dl/min, $n = 5$).

PTZ-induced seizure activity test

Results for the PTZ-induced tonic-clonic seizures are illustrated in Figure 6. The KO mice were significantly ($p < 0.05$) more sensitive to 10 and 30 mg/kg PTZ than the WT controls. In other experiments, the PTZ-sensitivity of $\alpha 4$ WT mice was indistinguishable from that of C57/BL6 mice (data not shown).

Discussion

This study examined the effects of targeted inactivation of the gene encoding the $\alpha 4$ subunit of the GABA_A-R on the acute behavioral effects of moderate/high dose EtOH. $\alpha 4$ KO and WT littermate mice were tested on a wide-ranging battery of behavioral assays. Deletion of the $\alpha 4$ subunit of the GABA_A-R did not influence the behavioral responses to acute administration of EtOH in the elevated plus maze, open field, fixed speed rotarod, LORR, radiant tail flick, screen test, or hypothermia assays. In contrast, $\alpha 4$ KO mice were much more sensitive to the seizure-inducing effects of PTZ.

Thalamocortical and hippocampal circuits are implicated in seizure propagation (Gale, 1990). PTZ-induced blockade of GABA_A-Rs (Macdonald and Barker, 1977) in these circuits likely accounts for PTZ-induced tonic-clonic seizures in rodents (Miller and Ferrendelli, 1990; Mirski and Ferrendelli, 1986; Rock and Taylor, 1986). We recently demonstrated greatly reduced tonic GABA_A-R currents in the dentate gyrus and in thalamic relay neurons of $\alpha 4$ KO mice (Chandra et al., 2006; Liang et al., 2007). It is reasonable to conclude that this loss of tonic inhibition mediated by extrasynaptic $\alpha 4/\delta$ -containing GABA_A-Rs contributes to increased seizure susceptibility of $\alpha 4$ KO mice. This is supported by our previous finding of not only a hyperexcitable hippocampus (Spigelman et al., 2002; Spigelman et al., 2003) (confirmed by Maguire et al., 2005), but also increased PTZ-induced seizure susceptibility of mice with a targeted deletion of the δ subunit (Spigelman et al., 2002). It should also be noted that mice with targeted disruptions of other GABA_A-R subunits also exhibit increased seizure susceptibility (e.g., Kralic et al., 2002) underscoring the critical role of GABA_A-R inhibition in brain homeostasis.

The lack of an EtOH-induced behavioral phenotype in the $\alpha 4$ KO mice is surprising given the following. Recombinant GABA_A-Rs containing $\alpha 4$ and δ subunits are reported to be sensitive to low concentrations (<30 mM) of EtOH associated with social intoxication (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003); however see: (Borghese et al., 2006). In vivo these receptors are located extrasynaptically where they mediate tonic inhibition that is also sensitive to moderate concentrations of EtOH (Liang et al., 2006; Wei et al., 2004). This tonic current is greatly reduced in $\alpha 4$ KO mice and is not potentiated by EtOH (Glykys et al., 2007; Liang et al., 2007). Mutation of the closely related $\alpha 6/\delta$ -containing GABA_A-Rs leads to EtOH-induced behavioral changes (Hancher et al., 2005). The EtOH antagonist, Ro15-4513, binds to $\alpha 4/\delta$ -containing receptors and reverses the effects of EtOH (Hancher et al., 2006; Wallner et al., 2006); however see: (Korpi et al., 2007; Mehta et al., 2007). Lastly, $\alpha 4$ KO mice are largely insensitive to gaboxadol, a GABAergic drug whose effects are primarily mediated by $\alpha 4/\delta$ containing GABA_A-Rs (Chandra et al., 2006). Despite these previous studies that strongly implicate $\alpha 4$ in EtOH action, our current results would argue against a key role for $\alpha 4$ subunit-containing GABA_A-Rs in mediating acute EtOH-induced behavioral effects.

However, there are a number of possible explanations for the lack of an EtOH-induced acute behavioral phenotype in the $\alpha 4$ KO mice that must be considered. As with all knockout studies, compensatory mechanisms may mask the normal endogenous effect of a gene that has been knocked out. As mentioned above, $\alpha 4$ KO mice were less sensitive to EtOH at the cellular level; two independent studies demonstrated that EtOH potentiation of tonic inhibition was reduced in dentate gyrus granule cells of $\alpha 4$ KO mice (Glykys et al., 2007; Liang et al., 2007). However, Liang et al. also unexpectedly observed a compensatory increase in synaptic sensitivity to EtOH. This increase in synaptic potentiation by EtOH in $\alpha 4$ KO mice may mask the true contribution of the $\alpha 4$ subunit in mediating EtOH-induced behaviors. Compensation is not unprecedented in GABA_A-R subunit KO mice as numerous compensatory changes have been observed in other GABA_A-R subunit mutants. Compensatory mechanisms have included alterations in other GABA_A-R subunits (Korpi et al., 2002; Kralic et al., 2002; Ogris et al., 2006; Peng et al., 2002; Tretter et al., 2001), organization of GABAergic circuits (Kralic et al., 2006), neuronal architecture (Heinen et al., 2003), and genes and proteins outside the GABA_A-R system (Brickley et al., 2001; Ponomarev et al., 2006). The molecular compensation in $\alpha 4$ KO mice is only beginning to be understood. For example, we have demonstrated brain-region selective compensatory increases in $\gamma 2$ subunit protein in $\alpha 4$ KO mice (Liang et al., 2007).

It is also conceivable that no genotypic differences in EtOH-induced behaviors between $\alpha 4$ WT and KO mice were observed because the EtOH doses used were too high. The $\alpha 4/\delta$ -containing receptors may be selective targets for only very low dose effects of EtOH. In this study, we gave doses of EtOH of 1.0 – 3.5 g/kg that likely produce peak blood EtOH levels between 15–100 mM (Pastino et al., 1996). In contrast, recombinant receptors containing $\alpha 4$ and δ subunits can be potentiated in vitro by EtOH concentrations as low as 1–3 mM (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). To achieve low millimolar EtOH concentrations in mice might require injection of no more than 0.25 g/kg EtOH. Unfortunately, no behavioral tests in mice have been developed where there is a measurable response to such a low dose of EtOH. Therefore, understanding the role of $\alpha 4$ containing GABA_A-Rs in EtOH action may be dependent on the development of new EtOH-induced behavioral paradigms in rodents that are sensitive to BEC in the low millimolar range.

A third possible explanation is that $\alpha 4$ mediates the effects of EtOH on behaviors that were not assessed by our study. The behavioral effects of EtOH reported here involved anxiety, locomotor activity, motor coordination, thermal pain sensitivity, and hypothermia. It is possible that $\alpha 4$ may be involved in other effects of EtOH such as impairment of cognition

(e.g., executive decision making, or learning and memory), habit formation, control of EtOH drinking behavior, development of tolerance or dependence, or seizure protection. These behavioral endpoints will be examined in future studies in the $\alpha 4$ KO mice as some of these EtOH effects were altered by knockout of the δ subunit (Mihalek et al., 2001). Examination of the role of $\alpha 4$ in mediating alcohol tolerance and dependence will be particularly interesting as $\alpha 4$ expression is robustly increased following chronic exposure to EtOH (e.g., Cagetti et al., 2003; Devaud et al., 1997; Mahmoudi et al., 1997; Mhatre and Ticku, 1992). Studies using $\alpha 4$ KO mice may therefore lead to an understanding of the cause and effect relationships between increased $\alpha 4$ expression and EtOH tolerance and dependence.

We conclude that in contrast to an obligatory role of $\alpha 4$ -containing GABA_A-Rs in gaboxadol action (Chandra et al., 2006), these receptors are not essential for the behavioral effects of acutely administered moderate/high dose EtOH that were tested in this study. However, further study is required to completely understand the true contribution of $\alpha 4$ containing GABA_A-Rs to the entire spectrum of EtOH-induced behaviors.

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Figure 1.

Evaluation of anxiety-like behavior and locomotor activity using the elevated plus maze. Total arm entries (A), percentage of open arm entries (B), and percentage of total time spent on open arms (C) are displayed. Saline or 1.0 g/kg EtOH was injected 10 min prior to testing. EtOH increased total arm entries ($p < 0.005$), percentage of open arm entries ($p < 0.005$), and percentage of time on open arms ($p < 0.001$). However, there was no effect of genotype or interaction of genotype with treatment. Data represent mean $\pm$ SEM. $n = 12$ – 16 mice / genotype / treatment.

Figure 2.

Evaluation of locomotor activity and rearing using the open field assay. Mice were injected with saline or EtOH 10 min prior to testing. (A) Total distance traveled by mice treated with 1.0 g/kg EtOH was greater than saline treated mice ($p < 0.0001$), but there was no effect of genotype or interaction of genotype with treatment. Sample size was 16–18 mice / genotype / treatment. Analysis of the number of rearings revealed a significant effect of gender ($p < 0.05$) that warranted analysis of males and females separately. (B) The number of rearings in male mice was influenced by treatment ($p < 0.0001$) but not by genotype. Both 1.0 g/kg ($p < 0.05$) and 2.0 g/kg ($p < 0.0001$) reduced the number of rearings compared to saline treatment. (C) The number of rearings in female mice was influenced by treatment

($p < 0.0001$) but not by genotype. The 2.0 g/kg ($p < 0.0001$) reduced the number of rearings compared to saline treatment. For panels B and C, $n = 7-10$ mice / genotype / treatment. Data represent mean $\pm$ SEM.

Figure 3.

Fixed speed rotarod measured EtOH's ataxic effects at (A) 14 RPM, 1.5 g/kg ($n = 9$ KO and 9 WT), (B) 6 RPM, 2.0 g/kg ($n = 15$ KO and 11 WT), and (C) 6 RPM, 2.5 g/kg ($n = 12$ KO and 8 WT). Black circles represent KO mice and white circles represent WT mice. Data points are mean $\pm$ SEM. There was a significant effect of time at all doses tested ($p < 0.0001$) but no genotypic effects were observed in EtOH-induced ataxia at any dose. Genotypes did not differ in the rate of learning the task during training sessions (data not shown).

Figure 4.

EtOH-induced sedative/hypnotic and analgesic effects. (A) EtOH-induced (3.5 g/kg) sedative/hypnotic effects measured by the duration of the LORR (n= 13 WT and 11 KO) was not different between genotypes. (B) EtOH-induced (2.0 g/kg) analgesia measured by a radiant tail flick assay (n = 13 WT and 10 KO). EtOH increased latency to tail flick over baseline (BSL) for both WT and KO mice. However, no significant effect of genotype was observed. All data represent mean $\pm$ SEM. *, $p < 0.0001$

Figure 5.

Screen test and EtOH-induced hypothermia. (A) All animals used achieved a baseline performance criterion of 240 sec. Injection of EtOH reduced latency to fall from screen ($p < 0.005$); this effect was dose dependent as 2.0 g/kg had a greater effect than 1.5 g/kg ($p < 0.005$). No effect of genotype was observed. (B) EtOH reduced body temperature ($p < 0.0001$), but no difference between genotype was observed. All data represent mean $\pm$ SEM. $n = 20$ /genotype/treatment.

Figure 6.

Increased pentylenetetrazol (PTZ)-induced seizure susceptibility of $\alpha 4$ KO mice.

Comparison of PTZ-induced seizure incidence in $\alpha 4$ WT and $\alpha 4$ KO mice. Each data point represents the % of mice exhibiting seizures within 60 min after PTZ injection ($n = 4-6$ mice/data point). †, significant effect of PTZ dose; *, significant difference between $\alpha 4$ KO and WT ($p < 0.05$, Chi Square).