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LINKING $GABA_A$ RECEPTOR SUBUNITS TO ALCOHOL-INDUCED CONDITIONED TASTE AVERSION AND RECOVERY FROM ACUTE ALCOHOL INTOXICATION

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Abstract

GABA type A receptors ($GABA_A$-R) are important for ethanol actions and it is of interest to link individual subunits with specific ethanol behaviors. We studied null mutant mice for six different $GABA_A$-R subunits (α1, α2, α3, α4, α5 and δ). Only mice lacking the α2 subunit showed reduction of conditioned taste aversion (CTA) to ethanol. These results are in agreement with data from knock-in mice with mutation of the ethanol-sensitive site in the α2-subunit (Blednov et al., 2011) and indicate this aversive property of ethanol is dependent on ethanol action on α2-containing $GABA_A$-R. Deletion of the α2-subunit led to faster recovery whereas absence of the α3-subunit slowed recovery from ethanol-induced incoordination (rotarod). Deletion of the other four subunits did not affect this behavior. Similar changes in this behavior for the α2 and α3 null mutants were found for flurazepam motor-incoordination. However, no differences in recovery were found in motor-incoordinating effects of an α1-selective modulator (zolpidem) or an α4-selective agonist (gaboxadol). Therefore, recovery of rotarod incoordination is under control of two $GABA_A$-R subunits: α2 and α3. For motor activity, α3 null mice demonstrated higher activation by ethanol (1 g/kg) whereas both α2 and α3 (-/-) knockout mice were less sensitive to ethanol-induced reduction of motor activity (1.5 g/kg). These studies demonstrate that the effects of ethanol at GABAergic synapses containing α2 subunit are important for specific behavioral effects of ethanol which may be relevant to the genetic linkage of the α2 subunit with human alcoholism.

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Keywords

gamma-aminobutyric acid; mutant mice; alcohol; conditioned taste aversion; ataxia; GABA receptors

1. Introduction

γ-Aminobutyric acid A receptors ($GABA_A$-Rs) represent the major inhibitory neurotransmitter receptors in the mammalian brain. $GABA_A$-Rs mediate a number of pharmacological effects, including sedation/hypnosis, anxiolysis, and anesthesia for drugs such as barbiturates, benzodiazepines, neuroactive steroids, and intravenous anesthetics. A number of behavioral effects of ethanol have been also attributed to actions at the $GABA_A$-R (for reviews see: Kumar et al., 2009; Chester and Cunningham, 2002; Enoch, 2008; Lobo and Harris, 2008). Some of the early evidence implicating the GABA receptor system in ethanol's motivational effects came from studies showing that $GABA_A$ receptor antagonists (Hyytia and Koob, 1995) and benzodiazepine partial inverse agonists (Balakleevsky et al., 1990) consistently reduced ethanol self-administration in rats. Other work proposes a role for $GABA_A$ receptors in the discriminative stimulus effects of ethanol (Hodge and Cox, 1998) as well as in its motor stimulatory or sedative activities (Hinko and Rozanov, 1990; Koechling et al., 1991). Acute ethanol treatments can enhance the function of $GABA_A$ receptors by at least three different mechanisms: direct actions on the receptor, increased presynaptic release of GABA and increased production of neuroactive steroids (Kumar et al., 2009; Lobo and Harris, 2008). Increased GABAergic function is sufficiently important for alcohol action that activators of $GABA_A$ receptors have even been suggested as ‘substitution’ therapy for treatment of alcohol use disorders (Chick and Nutt, 2012).

Studies relating human allelic variation to alcoholism and other alcohol phenotypes have found linkage with $GABA_A$-R clusters. Thus, a polymorphism of the γ2 subunit of the $GABA_A$ receptor has been associated with genetic susceptibility to ethanol-induced motor incoordination and hypothermia, conditioned taste aversion, and withdrawal (Buck and Hood, 1998). Human genetic association studies suggest that the $GABA_A$ β2, α6, α1 and γ2 subunit genes have a role in the development of alcohol dependence, although their contributions may vary between ethnic group and phenotype (for review see Enoch, 2008). The Collaborative Studies on Genetics of Alcoholism (COGA), and other groups, have identified a region of chomosome 4p associated with alcoholism, which includes a cluster of four $GABA_A$-R subunits, wherein the strongest linkage lies with α2 (Edenberg et al., 2004; Enoch et al., 2006; Soyka et al., 2008). Moreover, single nucleotide polymorphisms near the human α2 gene modulate the amount of α2 mRNA and protein in human brain as well as behavioral sensitivity to alcohol (Haughey et al., 2008). Two independent studies of gene expression profiles in humans and rodents with high alcohol intake found changes in genes related to GABAergic synaptic function (Tabakoff et al., 2009; Enoch et al., 2012).

$GABA_A$ receptor is pentameric in structure, with five subunits forming an ion pore. Seven classes of $GABA_A$ receptor subunits have been described to date (α1–6, β1–3, γ1–3, δ, ε, θ1–3, π, ρ1–3), allowing for extensive heterogeneity in receptor subunit composition across neuronal cell types and brain regions. However, most native $GABA_A$ receptors are thought to consist of two α, two β, and one γ or δ subunit.

As indicated above, there is also considerable evidence that ethanol enhances the function of $GABA_A$ receptors, although we are only beginning to elucidate the specific roles of each receptor subtype in ethanol induced behaviors (Lobo and Harris, 2008, Harris et al., 2008; Boehm et al., 2004; Wallner et al., 2006; Korpi et al., 2007). Evidence supporting subunit

specific pharmacological and behavioral roles comes from knock-in mice which possess a point mutation that alters one aspect of protein function (e.g., response to a drug), leaving all other aspects of protein function intact. These studies show that whereas α1 subunit containing receptors mediate the sedative, amnestic, and anticonvulsant actions of diazepam (Rudolph et al., 1999; McKernan et al., 2000), α2 subunit containing receptors mediate its anxiolytic actions (Low et al., 2000; Rudolph and Möhler , 2004) and α3/α2 the muscle relaxant actions (Crestani et al., 2001). Polymorphisms in the α2 subunit also implicated this subunit in human cocaine addiction (Dixon et al., 2010). To evaluate the importance of alcohol action on specific $GABA_A$ receptor subunits, mutations were introduced with the goal of producing receptors with normal responses to GABA but lacking modulation by ethanol. The mutated genes can then be used to replace the normal $GABA_A$ receptor subunits in knock-in mice. This was accomplished for the α1 and the α2 subunits (Werner et al., 2006; Blednov et al., 2011). Knock-in mice with mutations (serine 270 to histidine and leucine 277 to alanine) making the α1 subunit of the $GABA_A$ receptor resistant to ethanol, showed quicker recovery from the motor-impairing effects of ethanol and increased anxiolytic effects of ethanol (Werner et al., 2006). Mice with the same mutations in the α2 subunit of $GABA_A$ receptors did not develop conditioned taste aversion in response to ethanol and showed loss of the motor stimulant effects of ethanol (Blednov et al., 2011). This suggests that specific behavioral effects of ethanol result from direct action of ethanol on these subunit proteins. This interpretation assumes that these mutations do not alter other functions of the receptor or produce other changes in brain circuitry. However, these mutations are not completely ‘silent’ as they result in changes in gene expression in brain, especially in the case of α1 subunit (Harris et al., 2011).

To better define the role of $GABA_A$ subunits we studied two behaviors, conditioned taste aversion to ethanol (CTA) and recovery from ethanol-induced intoxication using the rotarod, in knockout mice for six $GABA_A$ receptor subunits: α1, α2, α3, α4, α5 and δ.

2. Materials and Methods

2.1 Animals

Knockout mice were generated as described previously: α1 (-/-) (Sur et al., 2001), α2 (-/-) (Dixon et al., 2008), α4 (-/-) (Chandra et al., 2006), α5 (-/-) (Collinson et al., 2002), δ (-/-) (Mihalek et al., 1999), α3 (-/Y) (Yee et al., 2005). All mice used in the experiments were produced from heterozygous breeding, with the exception that due the localization of the *Gabra*3 gene on the X chromosome α3 null and wild type control mice were produced by breeding female heterozygotes with male wild type mice. α1 (-/-), α4 (-/-), α5 (-/-) and δ (-/-) mutant colonies were maintained on the original mixed genetic background. Mice from α2 knockout colony were backcrossed on to C57BL/6J background twice and mice from the α3 knockout colony were backcrossed on to C57Bl/6J background three times (starting from a 129X1/SvJ background). After weaning, mice were housed in the conventional facility in University of Texas with *ad libitum* access to rodent chow and water with 12-h light/dark cycles (lights on at 7:00 AM). All mice used for behavioral experiments were between 8 and 12 weeks of age. Only male mice were used. Each mouse was used for only one experiment, and all mice were ethanol naive at the start of each experiment. All experiments were approved by Institutional Animal Care and Use Committees and were conducted in accordance with National Institutes of Health guidelines with regard to the use of animals in research. All efforts were made to minimize animal suffering and reduce the number of animals used.

2.2 Rationale for the behavioral tests

Published data suggest that the rewarding and aversive effects of EtOH play an important role in determining whether people who drink will continue to consume alcohol (Cunningham et al., 2000). Conditioned taste aversion is used as the index of motivational properties of ethanol (mostly aversive in doses higher than 1 g/kg) (Liu et al., 2009) and the response in this test is negatively correlated with voluntary ethanol intake (Green and Grahame, 2008). It should be noted that this one of the few behaviors which shows stable and substantial correlation with ethanol consumption in animal studies (Blednov et al, 2012). The rotarod test measures an aspect of motor incoordination as well as recovery from acute ethanol intoxication. Examples from human research indicate that resistance or low response to the physiological effects of ethanol may predict the future development of alcoholism (Schuckit, 1986, 1988, 1994). Another reason for the choice of this behavior was its high sensitivity to ethanol intake. Thus, C57Bl/6 mice after one month of ethanol consumption with limited access to alcohol showed significantly faster recovery from acute ethanol intoxication compare with isolated for the same period of time (Blednov, unpublished data). Because the ataxia is a complex behavior (Crabbe et al., 2005; 2010), we measured also some behaviors related to ataxia such as missteps and grip strength. The behavior in the elevated-plus maze serves as an indicator for the level of anxiety.

2.3. Conditioned Taste Aversion (CTA)

Subjects were adapted to a water-restriction schedule (2 hr of water per day) over a 7-day period. At 48-hr intervals over the next 10 days (days 1, 3, 5, 7, 9 and 11), all mice received 1-hr access to a solution of saccharin (0.15% w/v sodium saccharin in tap water). Immediately after 1-hr access to saccharin, mice received injections of saline or ethanol (2.5 g/kg) (days 1, 3, 5, 7 and 9). All mice also received 30-min access to tap water 5 hr after each saccharin access period to prevent dehydration (days 1, 3, 5, 7 and 9). On intervening days, mice had 2 hr continuous access to water at standard times in the morning (days 2, 4, 6, 8 and 10). Reduced consumption of the saccharin solution is used as a measure of CTA.

2.4. Rotarod

Mice were trained on a fixed speed rotarod (Economex; Columbus Instruments (Columbus, OH); speed of rod, 5.0 rpm), and training was considered complete when mice were able to remain on the rotarod for 60 s. Most mice were able to perform this task within first two or three trials. After completion of this training period, mice were injected with ethanol (2 g/kg i.p.) and every 15 min after injection each mouse was placed back on the rotarod and latency to fall was measured until the mouse was able to stay on the rotarod for 60 s. Overall, each experiment was completed within several hours on the same day.

2.5. Elevated Plus Maze

Mice were evaluated for basal anxiety as well as ethanol-induced anxiolysis using the elevated plus maze and a between groups design as was described (Blednov et al., 2001). Detailed description of experimental procedure is presented in Supplemental materials.

2.6. Motor activity testing

Locomotor activity was measured in standard mouse cages in Opto-microvarimex (Columbus Instruments, Ohio, USA) after three days of pre-habituation to handling, stress of transfer to experimental cage and to saline injection. Detailed description of experimental procedure is presented in Supplemental materials.

2.7. Ethanol metabolism

Animals were given a single dose of ethanol (4 g/kg i.p.), and blood samples were taken from the retro-orbital sinus 30, 60, 120, 180 and 240 min after injection. Blood ethanol concentration (BEC) values, expressed as mg ethanol per ml blood, were determined spectrophotometrically by an enzyme assay (Lundquist, 1959).

2.8. Missteps (foot-fault) test

The number of missteps was counted automatically using Columbus Instruments' foot misplacement apparatus (Columbus Instruments, Columbus, OH, USA). Detailed description of experimental procedure is presented in Supplemental materials.

2.9. Grip strength test

Grip Strength was assessed using a grip strength meter consisting of horizontal forelimb mesh (Columbus Instruments, Columbus, OH). Three successful forelimb strength measurements within 2 minutes were recorded and normalized to body weight as previously described (Spurney et al., 2009).

2.10. Hypothermia

Each mouse was weighed and placed into an individual plastic ventilated chamber 1 h before the start of testing; no food or water was available. Its core body temperature was then recorded with a rectal thermometer probe (2 mm ball on 2 cm shaft) and digital thermometer (Sensortek TH-8, Suffolk, UK) shortly before an ethanol injection (2.0 g/kg, i.p.) and then 30 min, 60 min, 90 min and 120 min post-injection.

2.11. Drug Injection

All ethanol (Aaper Alcohol and Chemical, Shelbyville, KY) solutions were made in 0.9% saline (20%, v/v) and injected i.p.). Flurazepam hydrochloride (Sigma-Aldrich, St. Louis, MO; 35.0 mg/kg, i.p), 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol (THIP hydrochloride or gab**o**xadol, Sigma-Aldrich, St. Louis, MO; 10 mg/kg i.p.) were dissolved in 0.9% saline and zolpidem (Sigma-Aldrich, St. Louis, MO; 5.0 mg/kg) was dissolved in 0.9% saline with few drops of Tween-80 and injected i.p. at a volume of 0.1 ml/10g body weight.

2.12. Statistical analysis

Mixed (between and within groups) experimental design was used for the all experiments except elevated plus-maze which was been designed as a between-groups comparison. In all other experiments between group design was used for comparison of genotypes and a within group design (i.e., repeated testing of same mice for each genotype) was used for calculation effect of trial (CTA experiment), dose (locomotion, grip strength and mis-steps) or time (rotarod). Different groups of mice were used in all behavioral experiments.

Data were reported as the mean ± S.E.M. The statistics software program GraphPad Pri**s**m (Jandel Scientific, Costa Madre, CA) was used. Analysis of variance (two-way ANOVA or one-way ANOVA with repeated measurements with Bonferroni or Dunnett's post hoc tests respectively) and Student's *t*-test were carried out to evaluate differences between groups. Statistics (three-way ANOVA) for the analysis of data obtained in CTA experiments was performed using Statistica version 6 (StatSoft, Tulsa, Oklahoma).

3. RESULTS

3.1. Conditioned taste aversion

Consumption of saccharin during the 1-hr period varied with genetic background in both wild type and mutant mice (Suppl. Table 1). To correct for these initial differences in tastant consumption, intake was calculated as a percentage of the trial 0 consumption for each subject by dividing the amount of saccharin solution consumed on subsequent conditioning trials by the amount of saccharin solution consumed on trial 0 (before conditioning). Figure 1 depicts saccharin intake (as a percentage of trial 0) for each mouse colony over the course of the five saccharin access periods. Ethanol administration produced trial-dependent reductions in saccharin intake over trials, indicating the development of conditioned taste aversion in all groups (statistically significant dependence on trial and trial × treatment interaction) (Fig. 1; Suppl. Table 2). Two mutant colonies – α2 (-/-) and δ (-/-) - showed effects of gene deletion but only α2 null mice showed a genotype × treatment × trial interaction indicating different development of CTA between (-/-) knockout and wild type mice (Fig.1 b,f; Suppl. Table 2).

3.2. Ethanol-induced motor incoordination

Acute administration of ethanol (2 g/kg) produced motor incoordination followed by time-dependent recovery in all genotypes (Fig.2; Suppl. Table 3). However, α2 (-/-) knockout mutant mice recovered from this impairment faster than wild type mice (Fig.2 b; Supplemental Table 3). On the contrary, α3 (-/Y) knockout mice recovered slower than wild type (Fig.2 c; Supplemental Table 3). No differences in recovery from motor impairment induced by ethanol were found for four other mutant mouse colonies (Fig.2 a,d,e,f; Suppl. Table 3). To determine if deletion of α2 or α3 subunits affected the sensitivity of other $GABA_A$ receptors, we tested motor impairment in rotarod by three drugs with different $GABA_AR$ subunit specificity: flurazepam – specific for γ-containing $GABA_AR$ (Sigel, 2002; Ernst et al., 2003); low doses of zolpidem - specific for α1 subunit of $GABA_AR$ (Sieghart, 1995) and gaboxadol – selective for α4 and δ containing $GABA_AR$ (Chandra et al., 2006; Herd et al., 2009). Because genotype-dependent differences in recovery from motor incoordination induced by ethanol were found only in two null colonies, α2 and α3, only these mutant mice were used in subsequent experiments.

Administration of gaboxadol (10 mg/kg) produced incoordination in wild type as well as in the knockout mice (Fig. 3 e,f; Suppl. Table 4). Administration of zolpidem (5 mg/kg) also produced incoordination in wild type as well as in knockout mice (Fig. 3 c,d; Suppl. Table 4). However, no differences between null and wild type mice were found for either drug. Similar to the results from ethanol, α2 (-/-) knockout mice showed faster recovery from motor impairment induced by flurazepam (35 mg/kg) whereas α3 (-/Y) knockout mice recovered slower compare with wild type mice (Fig.3. a,b; Suppl. Table 4).

It is well known that ethanol impairs thermoregulation and produces hypothermia in mice. Prior studies have shown that body temperature during intoxication can affect both the sensitivity of the animals to the behavioral effects of ethanol and the rate of ethanol metabolism. To rule out this possibility we studied the effect of ethanol (2.0 g/kg) on body temperature in α2 (-/-) and α3 (-/Y) knockout mice. Ethanol produced clear hypothermic effect in mice of all genotypes. However, no differences in body temperature between knockout mice and wild type mice were found during two hrs of observation (Supplemental Fig.1).

The differences in recovery from acute ethanol intoxication could be due to differences in effects of ethanol on anxiety, motor activation, sedation or myorelaxation. To test these possibilities, effects of low doses of ethanol were studied on several behaviors that might

influence rotarod performance. Low doses of ethanol were used because differences in the rotarod motor-incoordination were not seen for the initial effect of the 2 g/kg dose, but rather during recovery when the blood ethanol concentration is lower than the initial value. In addition, the metabolism of ethanol was measured for all genotypes to assure that differences in blood ethanol were not influencing the behavioral results.

3.3. Spontaneous locomotion

Ethanol dose dependently reduced motor activity in α2 mutant colony [$F_{(1,39)} = 16.9$, $p < 0.001$ main effect of genotype; $F_{(2,78)} = 24.5$, $p < 0.001$ main effect of dose; $F_{(2,78)} = 10.2$, $p < 0.001$ genotype × dose interaction] (Fig.4 a). Post-hoc analyses showed that motor activity of α2 (-/-) knockout mice after administration of saline or ethanol (1 g/kg) was lower that motor activity of wild type mice. Additional analyses by one-way ANOVA within each genotype showed that ethanol in doses 1 g/kg and 1.5 g/kg significantly reduced motor activity in wild type mice whereas no effect of ethanol was found in α2 (-/-) knockout mice.

Effects of ethanol on locomotor activity were modest in α3 wild-type and knockout mice [$F_{(2,60)} = 7.9$, $p < 0.001$ main effect of dose; $F_{(1,30)} = 0.18$, $p > 0.05$ main effect of genotype; $F_{(2,60)} = 5.5$, $p < 0.01$ genotype × dose interaction] (Fig. 4 b). No difference in basal (saline injection) motor activity between α3 (-/Y) knockout mice and wild type mice were found. Analyses by one-way ANOVA within each genotype showed that 1.5 g/kg ethanol reduced motor activity in wild type mice whereas 1 g/kg ethanol increased motor activity in α3 (-/Y) knockout mice.

3.4. Missteps and grip strength

Ethanol dose-dependently reduced the grip strength [$F_{(2,32)} = 13.7$, $p < 0.001$ main effect of dose] and increased the number of missteps [$F_{(2,32)} = 5.4$, $p < 0.01$ main effect of dose] (Fig. 5 a,c) in α2 mouse colony. However, no differences in effect of ethanol on grip strength [$F_{(1,16)} = 1.5$, $p > 0.05$ main effect of genotype; $F_{(2,32)} = 0.1$, $p > 0.05$ genotype × dose interaction] or on number of missteps [$F_{(1,16)} = 0.1$, $p > 0.05$ main effect of genotype; $F_{(2,32)} = 1.1$, $p > 0.05$ genotype × dose interaction] was found between α2 (-/-) knockout and wild type mice (Fig.5 a,c).

Reduction of grip strength [$F_{(2,64)} = 7.3$, $p < 0.001$ main effect of dose; $F_{(1,32)} = 0.2$, $p > 0.05$ main effect of genotype; $F_{(2,64)} = 0.4$, $p > 0.05$ genotype × treatment interaction] and increase the number of missteps [$F_{(2,64)} = 20.3$ $p < 0.001$ main effect of dose; $F_{(1,32)} = 1.1$, $p > 0.05$ main effect of genotype; $F_{(2,64)} = 1.6$, $p > 0.05$ genotype × treatment interaction] after ethanol administration was found in α3 mouse colony (Fig. 5 b,d). No differences between α3 (-/Y) knockout and wild type mice were found.

3.5. Elevated Plus Maze

Locomotor activity was assessed by number of entries into the closed arms and total numbers of entries, whereas anxiety was measured by percentage of time spent in open arm entries and percentage of open arm entries. In α2 mutant mice and their wild-type controls, ethanol affected the percent of time spent in open arms [$F_{(1,37)} = 4.3$, $p < 0.05$ main effect of dose; $F_{(1,37)} = 0.6$, $p > 0.05$ main effect of genotype; $F_{(1,37)} = 2.2$, $p > 0.05$ genotype × dose interaction]. However, the percentage of open arm entries showed only a genotype × treatment interaction [$F_{(1,37)} = 0.5$, $p > 0.05$ main effect of dose; $F_{(1,37)} = 0.1$, $p > 0.05$ main effect of genotype; $F_{(1,37)} = 4.6$, $p < 0.05$ genotype × dose interaction] (Fig. 6 a,b). Number of entries into the closed arms [$F_{(1,37)} = 14.8$, $p < 0.001$ main effect of dose; $F_{(1,37)} = 0.1$, $p > 0.05$ main effect of genotype; $F_{(1,37)} = 0.8$, $p > 0.05$ genotype × dose interaction] as well as number of total entries [$F_{(1,37)} = 20.2$, $p < 0.001$ main effect of dose; $F_{(1,37)} = 0.1$, $p > 0.05$ main effect of genotype; $F_{(1,37)} = 1.9$, $p > 0.05$ genotype × dose interaction] were

increased after treatment with ethanol in mice of both genotypes (Fig.6 c,d). In α3 wild-type and mutant mice, treatment affected the percent of time spent in open arms [$F_{(1,39)} = 13$, $p < 0.001$ main effect of dose; $F_{(1,39)} = 0.3$, $p > 0.05$ main effect of genotype; $F_{(1,39)} = 0.4$, $p > 0.05$ genotype × dose interaction] as well as the percentage of open arm entries [$F_{(1,39)} = 27$, $p < 0.001$ main effect of dose; $F_{(1,39)} = 0.1$, $p > 0.05$ main effect of genotype; $F_{(1,39)} = 0.1$, $p > 0.05$ genotype × dose interaction] (Fig. 6 e,f). No genotype dependent differences were found. Number of entries into the closed arms was reduced after ethanol administration [$F_{(1,39)} = 7.9$, $p < 0.01$ main effect of dose] and this effect was dependent on genotype [$F_{(1,39)} = 4.1$, $p < 0.05$ effect of genotype; $F_{(1,39)} = 0.1$, $p > 0.05$ genotype × dose interaction] (Fig.6 g). In contrast, number of total entries were significantly increased [$F_{(1,39)} = 20$, $p < 0.001$, effect of treatment; $F_{(1,39)} = 5.1$, $p < 0.05$, effect of genotype; $F_{(1,39)} = 0.3$, $p > 0.05$ genotype × dose interaction] after treatment with ethanol in mice of both genotypes (Fig.6 h).

3.6. Ethanol metabolism

There were no differences in metabolism of ethanol (4.0 g/kg) between wild type and any knockout mice (Supplemental Fig.2).

4. DISCUSSION

Taken together, these results show a link between specific $GABA_A$ receptor subunits and specific behavioral actions of ethanol (Table 1) Specifically, CTA induced by ethanol requires the α2 subunit. This is in agreement with data from knock-in mice with mutation of the ethanol-sensitive site in the α2-subunit (Blednov et al., 2011) which also showed a marked reduction in development of ethanol-induced CTA. In contrast, the lack of any of five other subunits of $GABA_AR$ (α1, α3, α4, α5 or δ) did not change ethanol CTA. Although mice lacking the δ subunit showed a difference in saccharin consumption throughout the CTA test, this does not appears to be due to differences in the development of CTA.

Recovery from acute ethanol intoxication (rotarod incoordination) apprears to be controlled by two $GABA_A$ receptor subunits: α2 and α3. Knock-in mice with mutation of the ethanol-sensitive site in the α2-subunit (Blednov et al., 2011) did not show the changes in this behavior, suggesting that it is not due to direct action of ethanol on the receptor, but could be due to increased presynaptic release of GABA (Roberto et al., 2003) or compensatory changes from deletion of the α2 subunit. The absence of any differences in recovery from motor incoordination induced by zolpidem and gaboxadol suggest that the function of α1- or α4/δ- containing receptors is not changed in mice lacking α2- or α3-subunits to an extent that could be detected in our tests. However, it should be noted that the expression of the α4 subunit is significantly upregulated in α2 (-/-) knockout mice (Panzanelli et al., 2011). Further analyses of behavioral effects of low doses of ethanol showed decreased sensitivity to sedative effects of ethanol on pre-habituated motor activity which might lead to faster recovery from ethanol-induced motor incoordination in α2 null mice. Although α3 (-/-) knockout mice did not show any sedation at the doses tested, they showed a small increase of motor stimulation from ethanol and this may cause the longer recovery in rotarod behavior. Thus, it is possible that the behavioral outcome in the rotarod test depends on the balance of signaling from α2- and α3-containing $GABA_A$ receptors. No differences between α2 or α3 (-/-) knockout mice and wild type mice in other types of ataxia-related behaviors such as missteps or grip-strength were found (Table 1). This is consistent with a study of inbred mouse strains which were screened for sensitivity to alcohol-induced intoxication using 11 separate behavioral assays, which found that alcohol sensitivity was influenced by task-specific sets of genes (Crabbe et al., 2005). The pattern of results suggested that there is not a single functional domain that represents ‘balance’ or ‘ataxia’

(Crabbe et al., 2010). $GABA_A$ receptors containing the α3 subunit are found in several brain regions (Möhler, 2007), with high levels of expression in cholinergic and monoaminergic neurons (Fritschy et al., 1992) in several areas of the midbrain and brain stem, including substantia nigra and ventral tegmental area (Fritschy and Möhler, 1995; Pirker et al., 2000; Schwarzer et al., 2001). Thus, the α3 subunits are important for GABAergic inhibition on the dopaminergic, serotoninergic and noradrenergic systems, and therefore may be a pharmacological target for modification of motor activity.

Specific behavioral actions of benzodiazepines have also been linked to GABAA receptor subunits using mutant mice and it is of interest to compare data for ethanol and benzodiazepines. Several conclusions from these studies are that receptors containing the α2 subunit are critical for the antianxiety and myorelaxant actions of benzodiazepines, with α3 subunits having a role in the antianxiety and high dose myorelaxant actions, but neither α2 or α3 subunits have a role in motor sedation (Rudolph et al., 1999; Rudolph and Möhler, 2004; Crestani et al., 2001; Morris et al., 2006; Dixon et al., 2008). There is a remarkable lack of congruence with ethanol studies in GABAA receptor mutant mice where the α2 or α3 subunits are not important for the anxiolytic or myorelaxant actions but contribute to the sedative effects (Table 1).

Human genetic studies associate polymorphisms of the GABRA2 gene encoding the $GABA_A$ α2-subunit with ethanol dependence in variety of populations (Edenberg et al., 2004; Enoch et al., 2006; Covault et al., 2004; Lappalainen et al., 2005). Interestingly, variants in GABRA2 genes have also been associated with addictive behaviours for other drugs such as cocaine (Dixon et al., 2010) and heroin (Enoch et al., 2010). It should be noted, that the same variations are also associated with childhood conduct disorder (Dick et al., 2006; Sakai et al., 2010) and with increased impulsivity (Villafuerte et al., 2012) behavioral traits that may contribute to the development of addictive behaviors. Some data suggest that the influence of GABRA2 haplotypes on the development of addictions is due to an interaction with early life stress (Enoch et al., 2010). Human variation within GABRA2 is associated with attenuated negative responses to alcohol, a known risk factor for vulnerability to alcohol use disorders (Uhart et al., 2012) and twin studies found allelic associations in GABRA2 for alcohol-induced body sway and motor incoordination (Lind et al., 2008). There is little information about the functional consequences of the human polymorphisms, but Haughey et al. (2008) suggested that the risk polymorphisms reduced the levels of GABRA2 mRNA (and perhaps protein) in human prefrontal cortex. This appears consistent with our findings that mice lacking GABRA2 show less aversion to alcohol. Overall, our results suggest that the effects of ethanol at GABAergic synapses containing α2 subunit may be important for behavioral effects of ethanol relevant to the genetic linkage of the α2 subunit with human alcohol phenotypes, particularly aversion and motor incoordination. In addition, the results of this study indicate that the α3 subunit of $GABA_A$ receptor may have an important role in sedative effects of ethanol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We used mutant mice to link specific behavioral actions of ethanol to single subunits of $GABA_A$ receptor.
- Aversion to ethanol induced requires the α2 subunit.
- Recovery from acute ethanol intoxication appears to be controlled by α2 and α3 subunits.
- Our results may be relevant to the genetic linkage of the α2 subunit with human alcohol dependence.

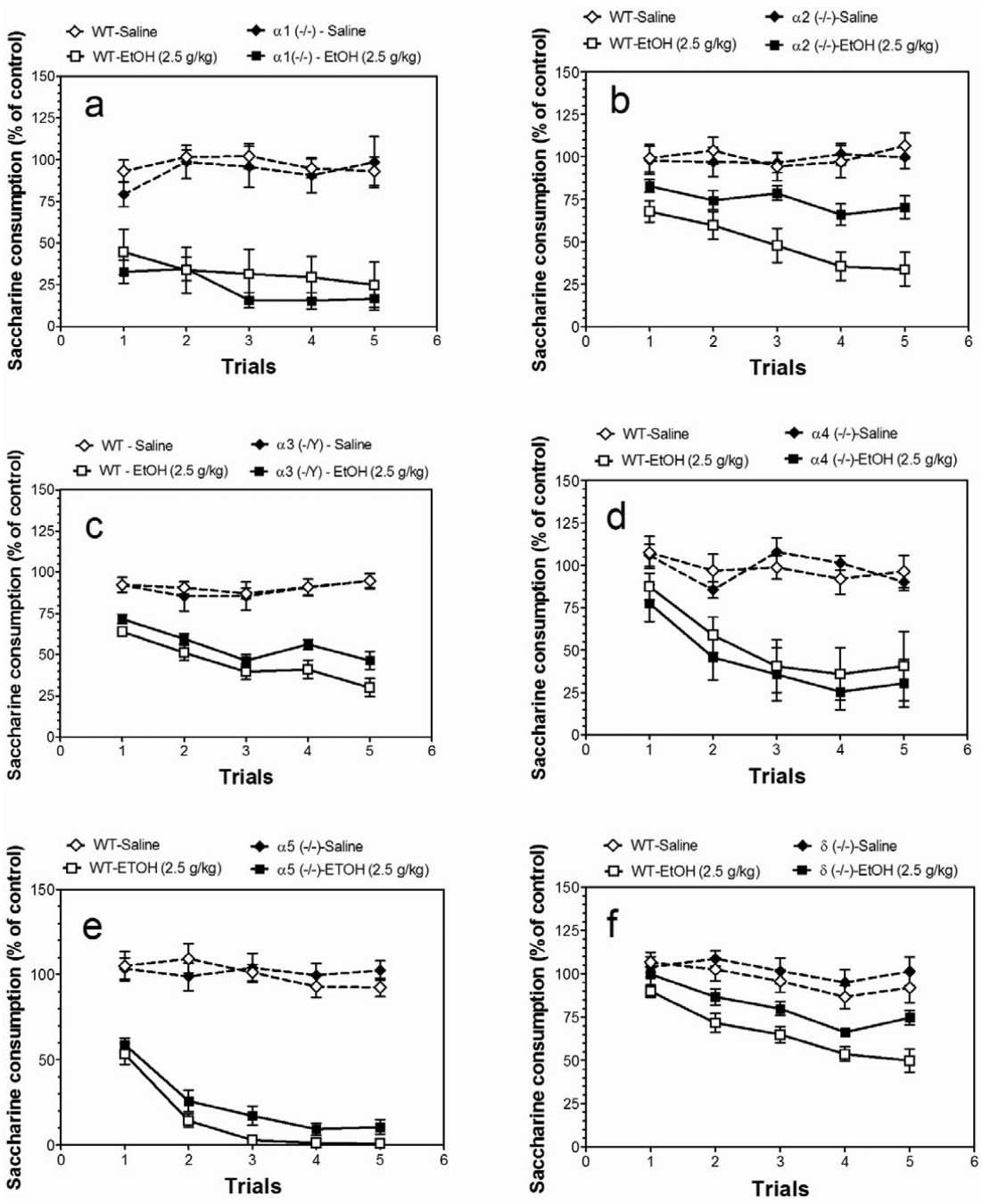

Figure 1. The development of ethanol induced conditioned taste aversion was decreased only in mice lacking of α2 subunit

Changes in saccharin consumption produced by injection of saline or ethanol expressed in percent from control trial (Trial 0). A – Development CTA in α1 (-/-) knockout mice (n = 9 for saline injection for both genotypes; n = 9-14 for groups with ethanol injection). B – Development CTA in α2 (-/-) knockout mice (n = 10 for saline injection for both genotypes; n = 11-14 for groups with ethanol injection). C – Development CTA in α3 (-/Y) knockout mice (n = 7-9 for saline injection for both genotypes; n = 16-19 for groups with ethanol injection). D - Development CTA in α4 (-/-) knockout mice (n = 6 for saline injection for both genotypes; n = 6-7 for groups with ethanol injection). E - Development CTA in α5 (-/-)

knockout mice (n = 10 for saline injection for both genotypes; n = 10-12 for groups with ethanol injection). F - Development CTA in δ (-/-) knockout mice (n = 11 for saline injection for both genotypes; n = 12-13 for groups with ethanol injection). Values represent mean ± SEM.

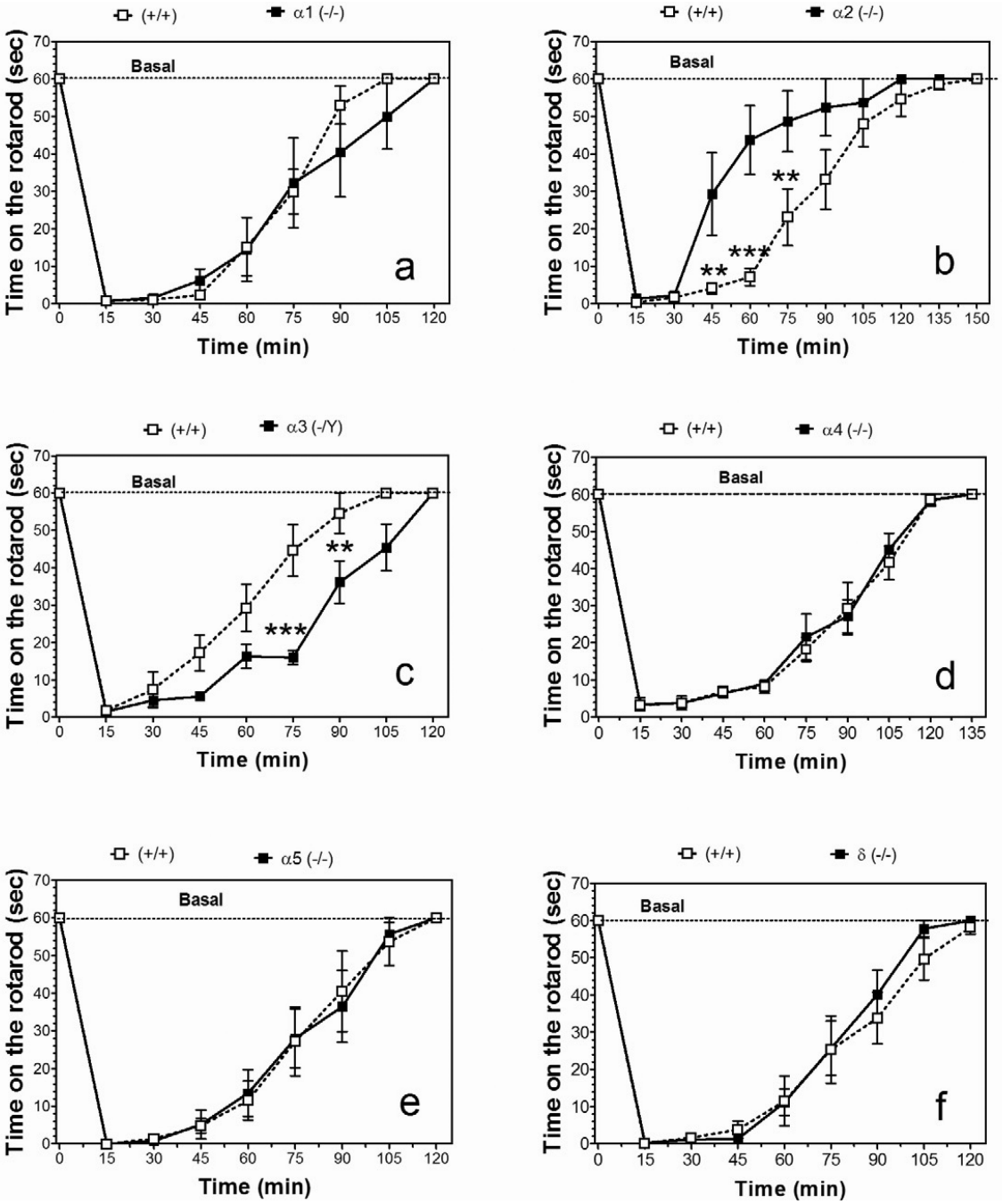

Figure 2. Faster recovery from motor incoordinating effect of ethanol in α2 (-/-) knockout mice and slower recovery in α3 (-/Y) knockout mice

Time on the rotarod (sec) after injection of ethanol (2 g/kg). A – Rotarod recovery in α1 (-/-) knockout mice (n = 5-7 for each genotype). B – Rotarod recovery in α2 (-/-) knockout mice (n = 7 for each genotype). C – Rotarod recovery in α3 (-/Y) knockout mice (n = 5-7 for each genotype). D - Rotarod recovery in α4 (-/-) knockout mice (n = 5 for each genotype). E - Rotarod recovery in α5 (-/-) knockout mice (n = 5-6 for each genotype). F - Rotarod recovery in δ (-/-) knockout mice (n = 6 each genotype). Data are shown as means ± S.E.M. and analyzed by two-way ANOVA with *Bonferroni post hoc* test. ** $P < 0.01$, *** $P < 0.01$ vs wild type group for the time point.

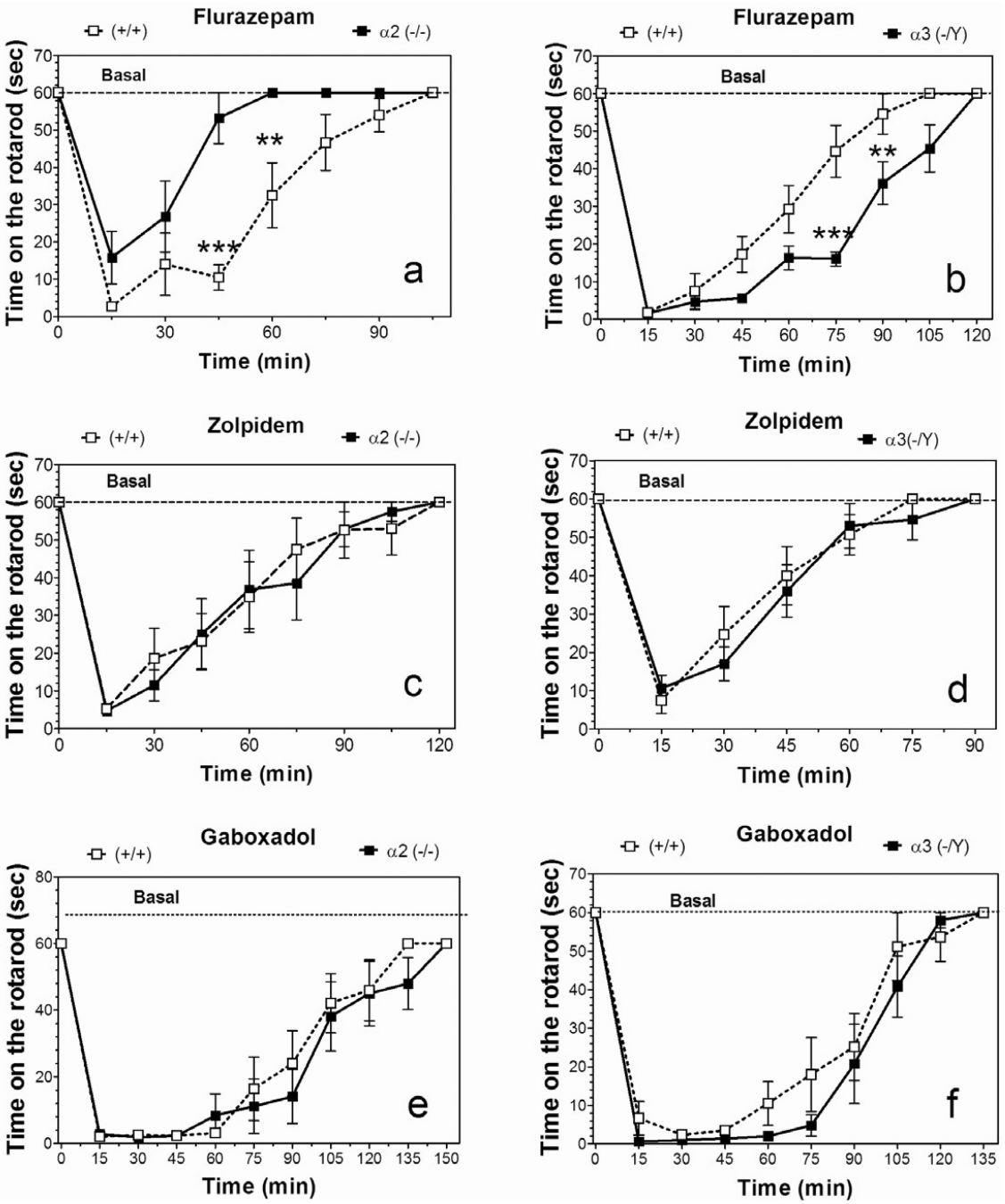

Figure 3. Recovery from motor incoordinating effects of flurazepam, gaboxadol and zolpidem in α2 (-/-) and α3 (-/Y) knockout mice

Time on the rotarod (sec) after injection of flurazepam (35 g/kg), zolpidem (5 mg/kg) and gaboxadol (10 mg/kg). A, C, E – Rotarod recovery in α2 (-/-) knockout mice. B, D, F – Rotarod recovery in α3 (-/Y) knockout mice. A, B – Flurazepam, (n = 5-6 per genotype in α2 colony and n = 6-7 per genotype in α3 (-/Y) colony). C, D – Zolpidem, (n = 6-7 per genotype in α2 colony and n = 6 per genotype in α3 (-/Y) colony). E, F – Gaboxadol, (n = 7-6 per genotype in α2 colony and n = 5-6 per genotype in α3 (-/Y) colony). Data are shown as means ± S.E.M. and analyzed by two-way ANOVA with *Bonferroni post hoc* test. ** $P < 0.01$, *** $P < 0.01$ vs wild type group for the time point.

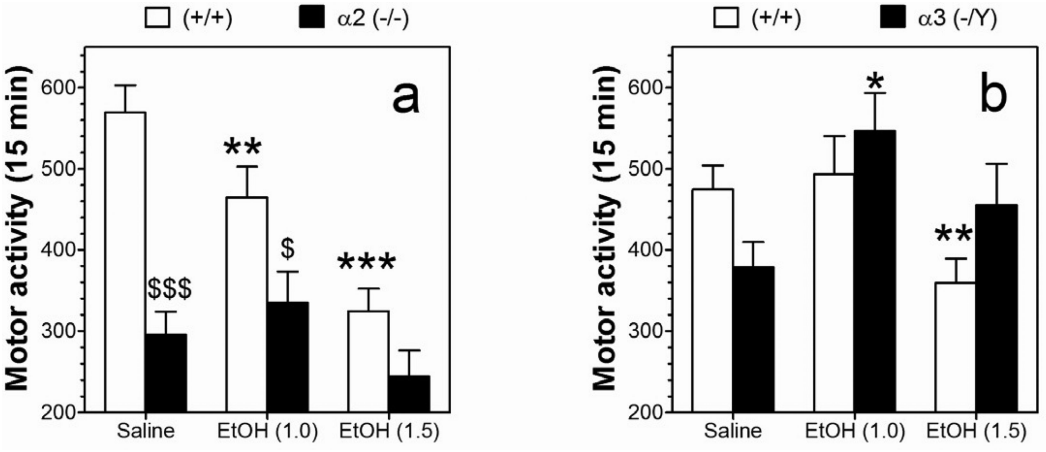

Figure 4. Effect of ethanol on the motor activity of α2 (-/-) and α3 (-/Y) knockout mice after pre-habituation

A – α2 (-/-) knockout mice (n = 20-21 per genotype). B – α3 (-/Y) knockout mice (n = 13-19 per genotype). Data are shown as means ± S.E.M. and analyzed by two-way ANOVA with repeated measures with *Bonferroni post hoc* test ($^{\$}$ $P < 0.05$, $^{\$\$\$}$ $P < 0.001$, vs another genotype for the same condition) and within each genotype by one-way ANOVA with repeated measures with *Dunnett's* test for Multiple comparisons (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs saline group).

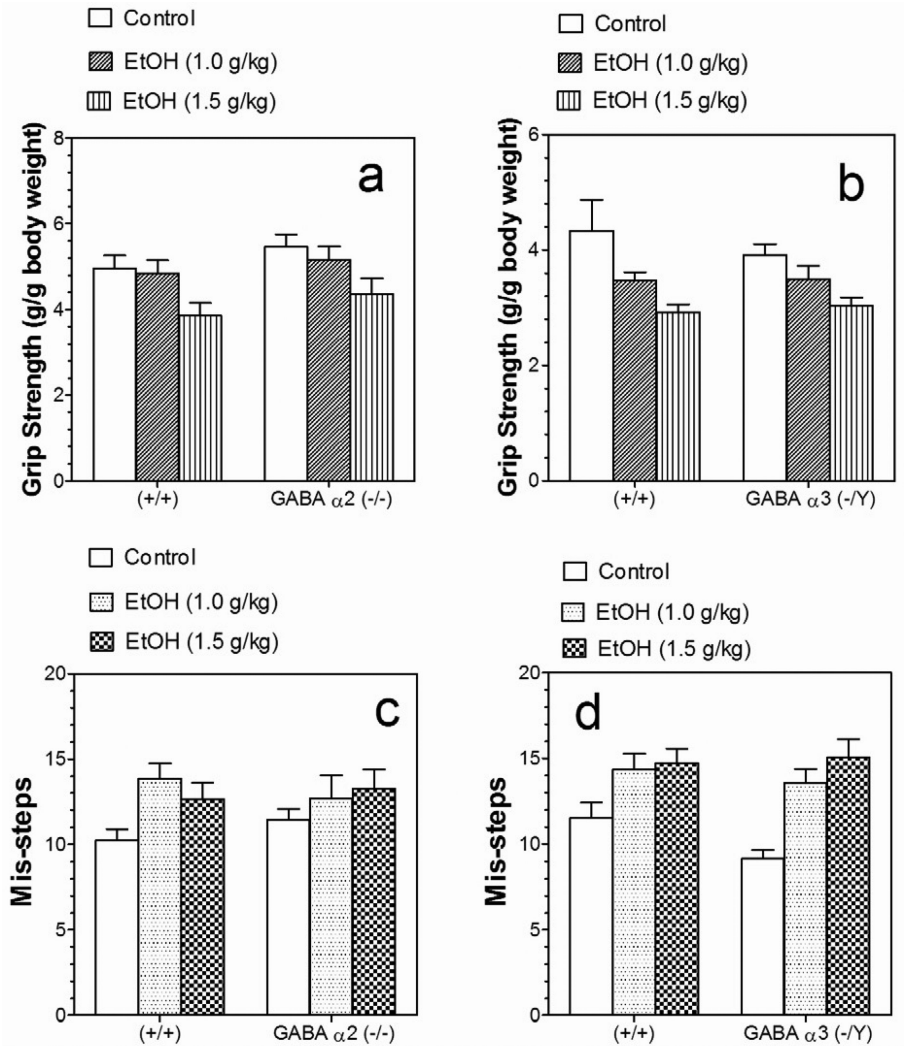

Figure 5. Effect of ethanol on the grip strength and number of missteps in α2 (-/-) and α3 (-/Y) knockout mice

A, C – α2 (-/-) knockout mice. B, D – α3 (-/Y) knockout mice. A, B – grip strength (n = 8-10 per genotype in α2 colony and n = 15-19 per genotype in α3 colony). C, D – number of missteps (n = 8-10 per genotype in α2 colony and n = 15-19 per genotype in α3 colony). Data are shown as means ± S.E.M. and analyzed by two-way ANOVA with repeated measures.

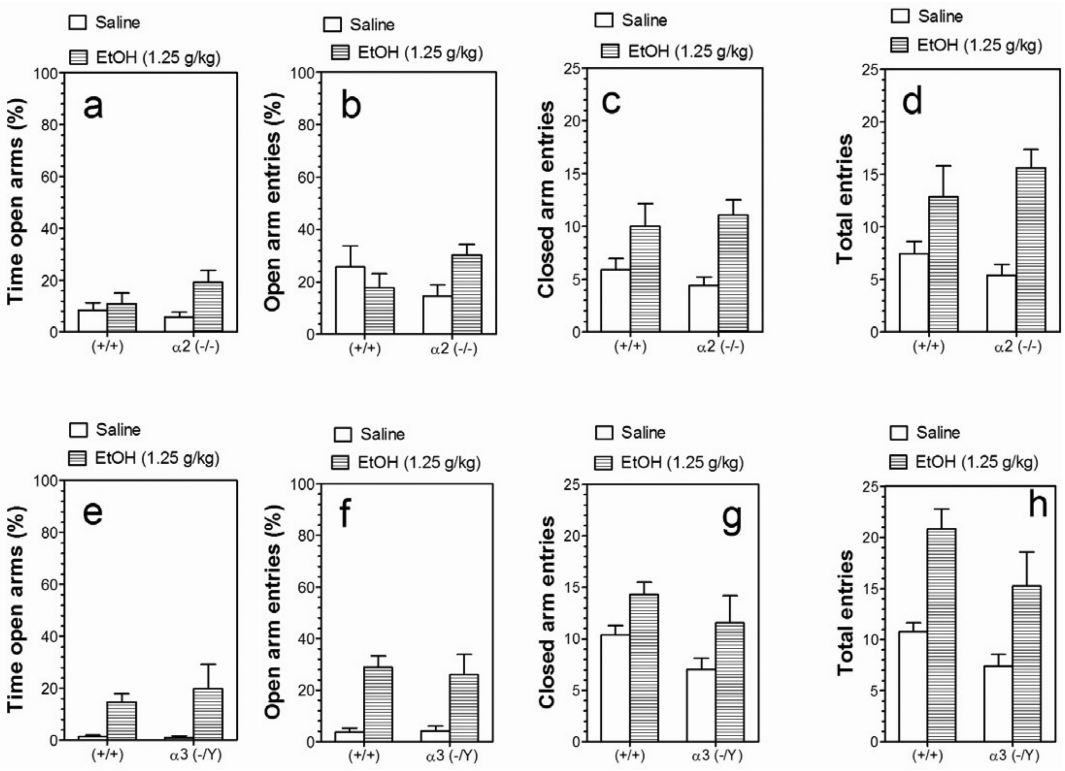

Figure 6. Anxiolytic effect of ethanol in in α2 (-/-) and α3 (-/Y) knockout mice in elevated plus maze test

A, B, C, D - α2 (-/-) knockout mice. E, F, G, H - α3 (-/Y) knockout mice. A, E – percent of time in open arms (n = 7-13 per genotype in α2 colony and n = 15-13 per genotype in α3 (-/Y) colony). B, F – percent of enters into the open arms. C, G – number of enters into the closed arms. D, H – total number of entries. Data from females and males were combined as there were no sex differences. Data are shown as means ± S.E.M. and analyzed by two-way ANOVA

Table 1

Summary of the behavioral effects of ethanol in mice lacking different subunits of GABAA receptors.

Behavior	Drug	α1 KO	α2 KO	α3 KO	α4 KO	α5 KO	δ KO	SHLA α1	SHLA α2
CTA	EtOH (2.5 g/kg)	=	↓	=	=	=	=	↓	↓
Rotarod	EtOH (2.0 g/kg)	=	←	→	=	=	=	←	=
	Flurazepam (35 mg/kg)	→	←	→	←	=	←	→	=
	Gaboxadol (10 mg/kg)	=	=	=	←	=	←	=	=
	Zolpidem (5 mg/kg)		=	=					
Motor activity pre-habituated (decrease)	EtOH (2.0 g/kg)		=	↓					
	EtOH (1.5 g/kg)		↓	↓					
	EtOH (1.0 g/kg)		↓	=					
Motor activity pre-habituated (increase)	EtOH (1.5 g/kg)		=	=					
	EtOH (1.0 g/kg)		=	↑					
Open field (decrease)	EtOH (1.0 g/kg)								↓
Open field (increase)	EtOH (1.5 g/kg)	↑							
	EtOH (1.0 g/kg)	↑			=				
EPM (anxiolysis)	EtOH (0.75 g/kg)	=						↑	
	EtOH (1.0 g/kg)	↑			=			=	=
	EtOH (1.25 g/kg)		↑	=					
	EtOH (1.5 g/kg)	=	=			=	=	=	
Hypothermia	EtOH (2.0 g/kg)		=	=					
Metabolism	EtOH (4.0 g/kg)	=	=	=	=	=	=	=	=

CTA – conditioned taste aversion; EPM - elevated plus maze. ↓ - reduction of behavior in mutant mice compared with correspondent wild type mice; ↑ - increase of behavior in mutant mice compared with correspondent wild type mice; = - no differences compared with correspondent wild type mice; → - right shift and ← - left shift in mutant mice. The results combined in this table were obtained in this study as well as were published previously (Blednov et al., 2003; 2011; Boehm et al., 2004; Chandra et al., 2008; Kralic et al., 2003; Mihalek et al., 2001; Werner et al., 2006). Note, that motor activity under stress conditions of open-field is separated from pre-habituated motor activity.