

Loss of Ethanol Conditioned Taste Aversion and Motor Stimulation in Knockin Mice with Ethanol-Insensitive $\alpha 2$ -Containing GABA_A Receptors

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ABSTRACT

GABA type A receptors (GABA_A-Rs) are potential targets of ethanol. However, there are multiple subtypes of this receptor, and, thus far, individual subunits have not been definitively linked with specific ethanol behavioral actions. Interestingly, though, a chromosomal cluster of four GABA_A-R subunit genes, including $\alpha 2$ (*Gabra2*), was associated with human alcoholism (*Am J Hum Genet* **74**:705–714, 2004; *Pharmacol Biochem Behav* **90**:95–104, 2008; *J Psychiatr Res* **42**:184–191, 2008). The goal of our study was to determine the role of receptors containing this subunit in alcohol action. We designed an $\alpha 2$ subunit with serine 270 to histidine and leucine 277 to alanine mutations that was insensitive to potentiation by ethanol yet retained normal GABA sensitivity in a recombinant

expression system. Knockin mice containing this mutant subunit were tested in a range of ethanol behavioral tests. These mutant mice did not develop the typical conditioned taste aversion in response to ethanol and showed complete loss of the motor stimulant effects of ethanol. Conversely, they also demonstrated changes in ethanol intake and preference in multiple tests. The knockin mice showed increased ethanol-induced hypnosis but no difference in anxiolytic effects or recovery from acute ethanol-induced motor incoordination. Overall, these studies demonstrate that the effects of ethanol at GABAergic synapses containing the $\alpha 2$ subunit are important for specific behavioral effects of ethanol that may be relevant to the genetic linkage of this subunit with human alcoholism.

Introduction

γ -Aminobutyric acid A receptors (GABA_A-Rs) represent the major inhibitory class of neurotransmitter receptors in the mammalian brain. They are pentameric in structure, with five subunits forming an ion pore. Seven classes of GABA_A receptor subunits have been described to date ($\alpha 1$ –6,

$\beta 1$ –3, $\gamma 1$ –3, δ , ϵ , $\theta 1$ –3, π , $\rho 1$ –3), allowing for extensive heterogeneity in receptor subunit composition across neuronal cell types and brain regions. However, most native GABA_A-Rs are thought to consist of two α , two β , and one γ or a δ subunit.

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. There is also considerable evidence that ethanol enhances the function of GABA_A-Rs, although we are only beginning to elucidate the specific roles of each receptor subtype and its component subunits in ethanol-induced behavior modification. (Boehm et al., 2004; Wallner et al., 2006; Harris et al., 2008; Lobo and Harris, 2008).

Subunit composition of GABA_A-Rs have profound effects on receptor pharmacology (Ebert et al., 1997), suggesting

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ABBREVIATIONS: GABA_A-R, GABA type A receptor; LORR, loss of righting reflex; CTA, conditioned taste aversion; ANOVA, analysis of variance; DID, drinking in the dark.

that behavioral sensitivity to ethanol (and other drugs that alter GABA_A-R function) may depend on which subunits are present (or absent) in specific brain circuits. Evidence supporting such pharmacological and behavioral specificity comes from knockin mouse studies. These mice 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 $\alpha 1$ subunit-containing receptors mediate the sedative, amnestic, and anticonvulsant actions of diazepam (Rudolph et al., 1999; McKernan et al., 2000), $\alpha 2$ subunit-containing receptors mediate its anxiolytic actions (Löw et al., 2000; Rudolph and Möhler, 2004). In addition, studies of mice with a mutation in the $\alpha 2$ subunit implicated this subunit in cocaine addiction (Dixon et al., 2010).

A number of studies relating human allelic variation to alcoholism and other alcohol phenotypes have found linkage with GABA_A-R clusters. The Collaborative Studies on Genetics of Alcoholism and other groups have identified a region of chromosome 4p associated with alcoholism, which includes a cluster of four GABA_A-R subunits, wherein the strongest linkage lies with $\alpha 2$ (Edenberg et al., 2004; Enoch et al., 2008; Soyka et al., 2008). Moreover, studies assessing single-nucleotide polymorphisms near the human $\alpha 2$ gene have been shown to modulate the amount of $\alpha 2$ mRNA and protein in human brain and behavioral sensitivity to alcohol (Haughey et al., 2008). In addition, the relative abundance of $\alpha 2$ subunits alters the function and alcohol sensitivity of recombinant GABA_A-Rs (Hurley et al., 2009).

The GABA_A-R $\alpha 2$ subunit accounts for approximately 26% of GABA_A-Rs (McKernan and Whiting, 1996) and is strongly expressed in many brain regions, with the highest levels in hippocampus, amygdala, bed nucleus of the stria terminalis, nucleus accumbens, neocortex, olfactory system, and hypothalamus (Pirker et al., 2000). It coassembles mainly with $\beta 2$ and $\gamma 2$ subunits and is found postsynaptically, although receptors with α , β , and $\gamma 2$ subunits may also occur in extrasynaptic regions (McKernan and Whiting, 1996; Farrant and Nusser, 2005). Given the implication of $\alpha 2$ in alcoholism and its relatively high expression in many brain regions, it is important to determine the role of $\alpha 2$ -containing GABA_A-Rs in the behavioral actions of ethanol.

Traditionally, the approach to investigating the behavioral importance of a gene has been to study global knockout mice. Indeed, this has been done for multiple subunits of GABA_A-R, including $\alpha 2$ (Boehm et al., 2004). Deletion of $\alpha 2$ reduced the duration of the loss of righting reflex (LORR) produced by ethanol but did not markedly affect alcohol consumption or other behavioral effects (Boehm et al., 2004). However, deletion of any GABA_A subunit may lead to the development of compensatory changes in other systems and complicate the interpretation of behavioral data (Ponomarev et al., 2006). Such compensatory changes and other limitations of null mutants can often be avoided by constructing knockin mice in which the wild-type gene is replaced by a mutant sequence possessing a drug-insensitive, but otherwise normally responsive, protein. Indeed, knockin mice with Ser270 to histidine and Leu277 to alanine mutations in the $\alpha 1$ subunit of the GABA_A-R, showed alterations in specific ethanol-induced behavioral effects (Werner et al., 2006). To extend these studies, we generated mice with Ser270 to His

and Leu277 to Ala mutations in the $\alpha 2$ subunit of GABA_A-Rs (Werner et al., 2010).

In the present study, we used these $\alpha 2$ knockin mice to test the hypothesis that $\alpha 2$ -containing GABA_A-Rs mediate specific behavioral responses to ethanol. We demonstrated that mutant $\alpha 2(\text{H270, A277})\beta_{2/3}\gamma_{2S}$ GABA_A-R expressed in *Xenopus* oocytes did not show ethanol enhancement of GABA action. In addition, ethanol-induced behavioral responses were assessed in $\alpha 2$ gene knockin mice harboring the same mutation. In these mice some, but not all, ethanol-induced behavioral responses were reduced or eliminated.

Materials and Methods

Animals. Wild-type mice (homozygous for serine at 270 and leucine at 277; referred to as SL/SL) and knockin mice (homozygous for histidine at 270 and alanine at 277; referred to as HA/HA) used for these experiments were produced from heterozygous (SL/HA) breeding pairs at the University of Pittsburgh (Pittsburgh, PA) or the University of Texas (Austin, TX). All mice were genotyped by Southern blot (University of Pittsburgh) or polymerase chain reaction (University of Texas). Southern blot analysis of tail DNA was performed as described previously (Werner et al., 2010). Mouse genotypes were determined by polymerase chain reactions performed on DNA derived from tail biopsies. Primers 5'-TTG AGC CGA TGA GTA ATG GGT CAC-3' and 5'-GAG GGA ATT TCG AGC ACT GAT GCT-3' amplified a 391-base pair fragment from the wild-type allele. Primers 5'-CAC ATC AGT GCT CGG AAT TCC GC-3' and 5'-CCC TTA AAG GAT CTC AGG CAA GAA-3' amplified a 304-base pair fragment from the knockin allele. To facilitate scoring, wild-type and knockin reactions were performed separately on each sample.

Mice were originally produced on a mixed C57BL/6J \times 129SvJ background and subsequently backcrossed to C57BL/6J for two to four generations as described previously (Werner et al., 2010). After weaning, mice were housed under specific pathogen-free conditions at the University of Pittsburgh and in a conventional facility at the 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 were of the C57BL/6J N4 genetic background and between 8 and 12 weeks of age for behavioral experiments, except as specified below. Both male and female 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 conducted in accordance with National Institutes of Health guidelines with regard to the use of animals in research.

Electrophysiology in *Xenopus* Oocytes. For detailed materials and methods, see Borghese et al. (2006) and Werner et al. (2010). In brief, *Xenopus laevis* oocytes were manually isolated, treated with collagenase, and injected with cDNA encoding GABA_A subunits ($\alpha/\beta/\gamma$ 1:1:3 in ng/oocyte). The cDNAs were human $\alpha 2$ (wild-type and mutated), rat $\beta 2$, human $\beta 3$, and human $\gamma 2$. Recordings were carried out 1 to 3 days after injection. The oocytes were placed in a rectangular chamber, continuously perfused with buffer at room temperature, and voltage-clamped at -70 mV.

All drugs were applied by bath perfusion. All solutions were prepared the day of the experiment. To study the ethanol (1, 10, 100, and 200 mM) modulation of GABA currents, the GABA concentration equivalent to EC₅₋₁₀ was determined after 1 mM GABA gave the maximal current, and then ethanol was coapplied with EC₅₋₁₀ GABA, preceded by 1-min application of ethanol alone. Percentage change was then calculated as the percentage change from the control response to EC₅₋₁₀ GABA in the presence of ethanol. All experiments shown include data obtained from oocytes taken from at least two different frogs.

Pooled data were represented as mean \pm S.E.M. Statistical significance was determined by using either *t* test or two-way ANOVA followed by Bonferroni post tests.

Alcohol Preference Drinking, 24-Hour Access. A two-bottle choice protocol was carried out as described previously (Blednov et al., 2003). In brief, mice were allowed to acclimate for 1 week to individual housing. Two drinking tubes were continuously available to each mouse, and tubes were weighed daily. One tube always contained water. Food was available ad libitum, and mice were weighed every 4 days. After 4 days of water consumption (both tubes), mice were offered 3% ethanol (v/v) versus water for 4 days. Tube positions were changed daily to control for position preferences. Quantity of ethanol consumed (g/kg body weight/24 h) was calculated for each mouse, and these values were averaged for every concentration of ethanol. Immediately after 3% ethanol treatment, a choice between 6% (v/v) ethanol and water was offered for 4 days, then 9% (v/v) ethanol for 4 days, then 12% (v/v) ethanol for 4 days, then 15% (v/v) ethanol for 4 days, and, finally, 18% (v/v) ethanol for 4 days. Throughout the experiment, evaporation/spillage estimates were calculated daily from two bottles placed in an empty cage, one containing water and the other containing the appropriate ethanol solution.

Preference for Nonalcohol Tastants, 24-Hour Access. Wild-type or knockin mice were also tested for saccharin and quinine consumption. One tube always contained water, and the other contained the tastant solution. Mice were serially offered saccharin (2,3-dihydro-3-oxobenzisofurazone sodium salt; Sigma-Aldrich, St. Louis, MO) (0.033 and 0.066%) and quinine (quinine hemisulfate salt monohydrate; Sigma-Aldrich) (0.03 and 0.06 mM), and intakes were calculated. Each concentration was offered for 4 days, with bottle position changed daily. For each tastant, the low concentration was always presented first, followed by the higher concentration. Between tastant testing, mice had access to two bottles with water for 2 weeks.

Ethanol Drinking: Limited Access Drinking in the Dark Phase (One-Bottle DID). Another approach for consumption of ethanol (15% solution) has been described under conditions of limited access, which achieves pharmacologically significant levels of ethanol drinking (Rhodes et al., 2005). In brief, starting at 3 h after lights off, the water bottles were replaced with a bottle containing a 15% ethanol solution. The ethanol bottle remained in place for either 2 h (first 3 days) or 4 h (day 4) and then was replaced with the water bottles. Other than this short period of ethanol drinking, mice had unlimited access to water. The ethanol bottles were weighed before placement and after removal of the bottles from each experimental cage. In a separate experiment, ethanol-naive mice were exposed with ethanol (15%) intake from one bottle for 2 h daily during 9 consecutive days.

Ethanol Drinking: Limited Access in the Dark Phase (Two-Bottle Choice DID). This was similar to the one-bottle DID test described above except that two bottles containing 15% ethanol and water were placed in the cage (Blednov and Harris, 2008). The ethanol and water bottles remained in place for 3 h. After their removal, mice had unlimited access to one bottle of water. The positions of bottles during 3-h access were changed daily to avoid potential side preference. The ethanol and water bottles were weighed before placement and after removal of the bottles from each experimental cage.

Ethanol Drinking: 24-Hour Access Every Other Day (Intermittent Drinking). During the 1970s, several studies showed that intermittent access to ethanol induced high voluntary ethanol consumption (Wayner and Greenberg, 1972; Wise, 1973; Pinel and Huang, 1976). Although the mechanism of this phenomenon is not understood, the behavioral impact is clear, a substantial increase of ethanol intake compared with continuous daily access to ethanol and water. Simms et al. (2008) resurrected this experimental approach and showed that it produces reproducibly high levels of voluntarily ethanol consumption in Long-Evans or Wistar rats. Therefore, we also assessed ethanol consumption by using a paradigm adapted

from Wise (1973) and Simms et al. (2008) using intermittent access to 15% ethanol. Animals were given access to one bottle of ethanol and one bottle of water during 24-h sessions every other day. The placement of the ethanol bottle was alternated each ethanol drinking session (day) to control for side preferences.

Conditioned Taste Aversion. Subjects were adapted to a water-restriction schedule (2 h of water per day) over a 7-day period. At 48-h intervals over the next 10 days (days 1, 3, 5, 7, 9 and 11), all mice received 1-h access to a solution of saccharin [0.15% (w/v) sodium saccharin in tap water]. Immediately after 1-h 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 h after each saccharin access period to prevent dehydration (days 1, 3, 5, 7, and 9). On intervening days, mice had 2-h 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 conditioned taste aversion (CTA).

Loss of Righting Reflex. Sensitivity to ethanol (3.25 or 3.5 g/kg) was determined by using the standard duration of LORR assay in mice of the C57BL/6J N4 (age 9–14 weeks) genetic background. Ethanol was diluted in 0.9% saline (20% v/v) and administered in doses adjusted by injected volumes. Mice were injected with ethanol, and when they became ataxic they were placed in the supine position in V-shaped plastic troughs until they were able to right themselves three times within 30 s. LORR was defined as the time from being placed in the supine position until the mice regained their righting reflex. During all LORR assays normothermia was maintained with the aid of a heat lamp.

Rotarod. Mice were trained on a fixed speed rotarod (Economet; 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. Every 15 min after injection of ethanol (2 g/kg i.p.) 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.

Elevated Plus Maze. Mice (C57BL/6J N3 genetic background; age 8–13 weeks) were evaluated for basal anxiety and ethanol-induced anxiolysis by using the elevated plus maze. Mice were transported to the testing room 1 day before testing. Animals were tested between 9:00 and 11:00 AM under ambient room light. Mice were weighed and injected with 1.0 g/kg ethanol or saline 10 min before testing. Each mouse was placed on the central platform of the maze facing an open arm. Mice were allowed to freely explore the maze for 5 min during which the following measurements were manually recorded: number of open arm entries, number of closed arm entries, total number of entries, time spent in open arms, and time spent in closed arms. A mouse was considered to be on the central platform or any arm when all four paws were within its perimeter.

Motor Activity (Open Field). Wild-type and mutant mice of both sexes were tested for basal locomotor activity and ethanol-induced locomotor stimulation and sedation as described previously (Chandra et al., 2008). In brief, mice (8–12 weeks of age) were injected with saline or 0.75, 1.0, or 1.5 g ethanol/kg body weight 10 min before testing. Locomotor activity was quantified by the number of photobeam breaks in automated activity boxes (MED Associates, St. Albans, VT) during the 10-min test session.

Rationale for the Behavioral Tests. Two-bottle choice is the most widely used test of ethanol preference and intake and allows measurement of voluntary consumption. It seems to be related to other measures of alcohol reward (Green and Grahame, 2008). Other tests for ethanol intake produce high levels of ethanol consumption by limiting access to ethanol. We used one- and two-bottle DID and intermittent access drinking to compare three types of limited access drinking. Because the ethanol produces taste responses (sweet and bitter) it is critical to analyze the sensitivity of the genotypes to bitter (quinine solutions) and sweet (saccharin solutions) tastes to determine whether changes in alcohol consumption are secondary to changes in taste. Conditioned taste aversion is used as the index of

aversive properties to ethanol, and the response in this test is negatively correlated with voluntary ethanol intake (Green and Grahame, 2008). Duration of loss of righting reflex measures the anesthetic or sedative activities of ethanol, and for some mutant mice it is negatively correlated with voluntary ethanol consumption (Crabbe et al., 2006). Acute ethanol withdrawal shows the sensitivity to the development of ethanol physical dependence and also negatively correlates with ethanol intake in the two-bottle choice paradigm (Metten et al., 1998). The rotarod test measures an aspect of motor incoordination and recovery from acute ethanol intoxication. The behavior in the elevated-plus maze and open-field tests serves as an indicator for the level of anxiety and response to the acute stress, behaviors that are regulated by GABAergic systems.

Drug Injection. All injectable ethanol (Aaper Alcohol and Chemical, Shelbyville, KY or Pharmco, Brookfield, CT) solutions were made in 0.9% saline (20% v/v) and injected intraperitoneally with a volume of 0.2 ml/10 g of body weight.

Ethanol Metabolism. Animals were given a single dose of ethanol (3.8 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 values, expressed as milligram of ethanol per milliliter of blood, were determined spectrophotometrically by an enzyme assay (Lundquist, 1959).

Statistical Analysis. Data were reported as the mean \pm S.E.M. The statistics software programs Prism (GraphPad Software Inc., San Diego, CA) or Statview (Abacus Concepts, Berkeley, CA) were used. Statistics for the analysis of data obtained in CTA experiments was performed with Statistica version 6 (StatSoft, Tulsa, OK). Analysis of variance (two-way ANOVA with Fischer's or Bonferroni post hoc tests) and Student's *t* test were carried out to evaluate differences between groups. To evaluate differences within groups, analysis of variance (one-way ANOVA with Fischer's or Bonferroni post-tests) was carried out.

Results

Ethanol Actions on Recombinant Receptors. GABA_A receptors containing either wild-type (S₂₇₀, L₂₇₇, or SL) or mutant (H₂₇₀, A₂₇₇ or HA) $\alpha 2$ subunits along with $\beta 2\gamma 2$ or $\beta 3\gamma 2$ subunits were expressed in *Xenopus* oocytes. Increasing concentrations of ethanol produced potentiation of submaximal GABA responses in $\alpha 2(\text{SL})\beta 3\gamma 2\text{s}$ receptors. In contrast, ethanol did not potentiate GABA responses in $\alpha 2(\text{HA})\beta 3\gamma 2\text{s}$ receptors and even produced a small inhibition (Fig. 1a). A two-way ANOVA of the effect of ethanol on GABA responses in $\alpha 2(\text{SL})\beta 3\gamma 2\text{s}$ and $\alpha 2(\text{HA})\beta 3\gamma 2\text{s}$ receptors showed a significant effect of receptor ($F_{1,39} = 54.99$), ethanol concentration ($F_{1,39} = 10.53$), and interaction ($F_{3,39} = 16.03$); *p* values were in all cases less than 0.0001 ($n = 7-8$).

Similar results were obtained with $\alpha 2\beta 2\gamma 2\text{s}$ receptors: ethanol (200 mM) potentiated $\alpha 2$ wild type-containing receptors ($54 \pm 10\%$), but ethanol had no effect on mutant $\alpha 2$ -containing receptors ($3 \pm 2\%$; $p < 0.01$, Student's *t* test; $n = 4$ each) (Fig. 1b). Recordings from single oocytes are shown in Fig. 1c.

Conditioned Taste Aversion. Consumption of saccharin during the 1-h period varied with sex (male mice: 79 ± 3.4 g/kg and 76 ± 3.6 g/kg body weight for wild-type and mutant mice, respectively; female mice: 117 ± 6.7 g/kg and 116 ± 5.3 g/kg body weight for wild-type and mutant mice, respectively). 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 five subsequent conditioning trials by the amount of saccharin solution consumed on trial 0 (before conditioning). Ethanol-saccharin pairings produced

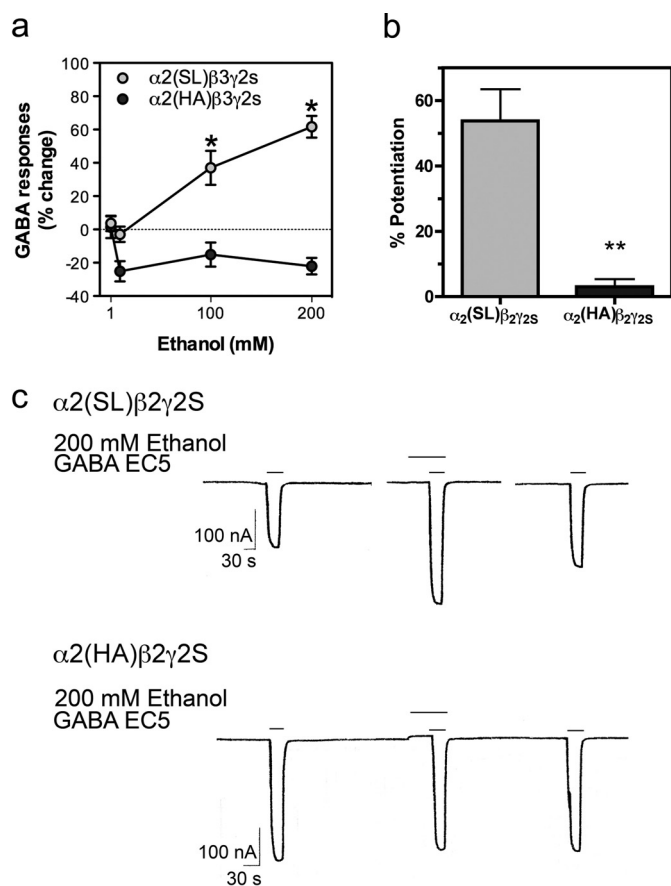


Fig. 1. Ethanol modulation of GABA responses in wild-type (SL) and mutant (HA) $\alpha 2\beta 3\gamma 2\text{s}$ GABA_A receptors expressed in *Xenopus* oocytes. **a**, GABA responses in $\alpha 2\beta 3\gamma 2\text{s}$ GABA_A receptors. Pooled data are represented as mean \pm S.E.M. ($n = 7-8$). Effects of ethanol were tested with EC₅₋₁₀ GABA. *, $p < 0.01$, significant differences from wild type for the same concentration of ethanol (two-way ANOVA followed by Bonferroni post test). **b**, GABA responses in $\alpha 2\beta 2\gamma 2\text{s}$ GABA_A receptors. Pooled data are represented as mean \pm S.E.M. ($n = 4$ each). **, $p < 0.01$, Student's *t* test; difference between responses in wild-type and mutant GABA receptors. **c**, the actual tracing from oocyte recording with $\alpha 2\beta 2\gamma 2\text{s}$ GABA_A receptors.

reduction in saccharin intake across trials compared with saline-saccharin pairings (Fig. 2), indicating the development of CTA in wild-type mice of both sexes (males: $F_{1,45} = 139$, $p < 0.001$, effect of treatment; $F_{4,45} = 9.7$, $p < 0.001$, effect of trial; $F_{4,45} = 5.2$, $p < 0.01$, treatment \times trial interaction and females: $F_{1,65} = 63$; $p < 0.001$, effect of treatment) as well as for knockin female mice: $F_{1,60} = 38$, $p < 0.001$, effect of treatment. However, the mutant male mice did not develop CTA; there were no differences between saline- and ethanol-treated groups of male mutant mice (Fig. 2a). Comparison of saline-treated groups of wild-type and mutant mice of corresponding sex also did not reveal significant differences over time. Dependence on trial was present only for the saline-saline comparison of male mice ($F_{4,40} = 3.9$, $p < 0.001$, main effect of trial). However, wild-type mice of both sexes developed significantly stronger CTA than knockin mice of both sexes (comparison of ethanol-treated groups of wild-type and mutant mice of corresponding sex) (males: $F_{1,45} = 149$, $p < 0.001$, effect of treatment; $F_{4,45} = 8.5$, $p < 0.001$, effect of trial; $F_{4,45} = 6$, $p < 0.001$, treatment \times trial interaction and females: $F_{1,85} = 16$, $p < 0.001$, effect of treatment; $F_{4,85} = 3.3$, $p < 0.05$, effect of trial).

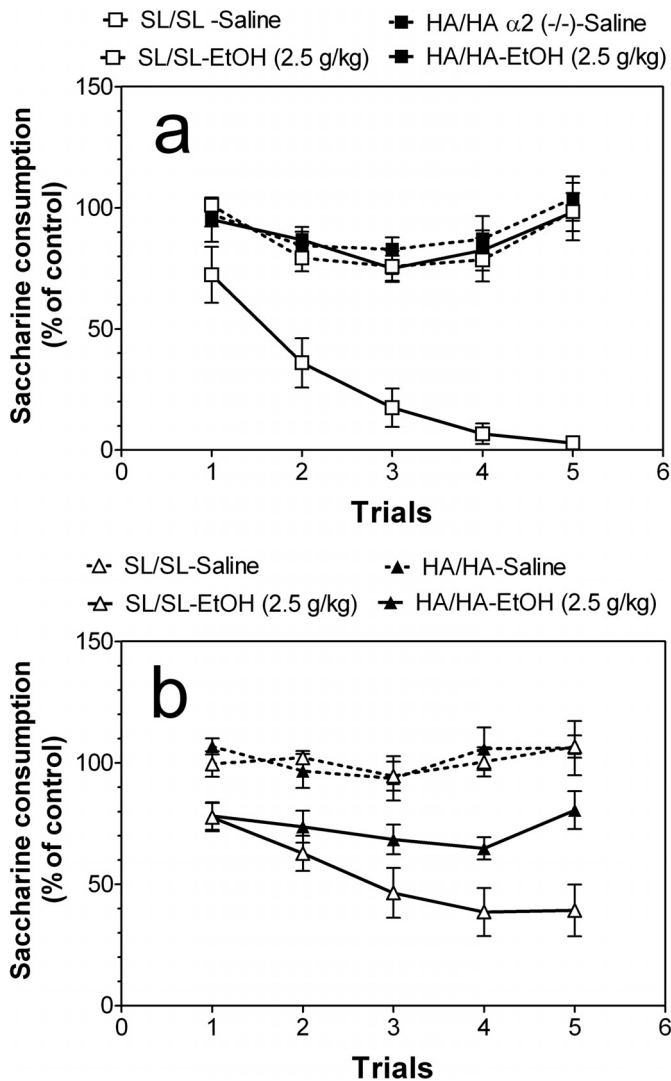


Fig. 2. $\alpha 2$ Knockin mice develop weaker conditioned taste aversion for ethanol. a, male mice ($n = 5$ for saline injection for both genotypes; $n = 5-6$ for groups with ethanol injection). b, female mice ($n = 5$ for saline injection for both genotypes; $n = 9-10$ for groups with ethanol injection). No differences between saline-treated groups of wild-type and mutant mice of corresponding sex were found (two-way ANOVA). Wild-type mice of both sexes developed significantly stronger CTA than knockin mice of both sexes (comparison of ethanol-treated groups of wild-type and mutant mice of corresponding sex) (males: $p < 0.001$; females: $p < 0.001$; two-way ANOVA). Values represent mean \pm S.E.M.

Ethanol Consumption. In limited access to 15% ethanol without free choice (one-bottle DID model), mutant and wild-type male mice consumed similar amounts of ethanol both during the first 3 days with 2-h access (a trend toward significance: $F_{1,51} = 2.9, p = 0.09$, main effect of genotype) and at day 4 with 4-h access to ethanol (Fig. 3a). On the contrary, female mutant mice (Fig. 3b) consumed slightly larger amounts of ethanol during the first 3 days with 2-h access ($F_{1,42} = 7.5, p < 0.01$, main effect of genotype). No differences in the amount of consumed ethanol were found at day 4 with 4-h access to ethanol in either sex (Fig. 3, a and b). However, during 9 days with 2-h daily access mutant mice of both sexes consumed larger amounts of ethanol than their wild-type littermates ($F_{1,153} = 6.9, p < 0.01$, main effect of genotype for male mice; $F_{1,126} = 23, p < 0.001$, main effect of genotype for female mice) (Fig. 3, c and d).

Over 18 days of limited daily access to 15% ethanol but with free choice between ethanol and water (two-bottle DID model: 15%) male mutant mice consumed slightly larger amounts of ethanol ($F_{1,126} = 6.3, p < 0.05$, main effect of genotype; $F_{8,126} = 2.3, p < 0.05$, main effect of days) (Fig. 4a) and showed slightly higher preference for ethanol ($F_{1,126} = 6.6, p < 0.05$, main effect of genotype; $F_{8,126} = 3.7, p < 0.001$, main effect of days) (Fig. 4b). No differences between genotypes for male mice in the amount of consumed fluid were found (Fig. 4c). Female mutant mice also consumed larger amounts of ethanol ($F_{1,108} = 9.9, p < 0.01$, main effect of genotype; $F_{8,108} = 2.2, p < 0.05$, main effect of days) (Fig. 4d) and larger amount of fluid ($F_{1,108} = 31, p < 0.001$, main effect of genotype; $F_{8,108} = 2, p < 0.05$, main effect of days) (Fig. 4f). However, no differences were observed between genotypes for female mice in preference for ethanol (Fig. 4e).

Mutant male mice consumed ethanol with a slightly higher preference than wild-type male mice over 1 month of intermittent drinking (every other day drinking) ($F_{1,180} = 4.7, p < 0.05$, main effect of genotype) (Fig. 5b). No significant differences in the amount of ethanol consumed and the total amount of fluid consumed were found for male mice (Fig. 5, a and c). However, mutant female mice consumed larger amounts of ethanol ($F_{1,180} = 40, p < 0.001$, main effect of genotype) (Fig. 5d) with higher preference for ethanol ($F_{1,180} = 24, p < 0.001$, main effect of genotype) (Fig. 5e) than their wild-type littermates. Total fluid intake was also slightly elevated in mutant female mice ($F_{1,180} = 5.3, p < 0.05$, main effect of genotype) (Fig. 5f).

In the two-bottle free-choice paradigm in which mice could drink either water or an increasing series of ethanol concentrations the amount of ethanol consumed by mutant HA/HA male mice was significantly reduced compared with wild type (SL/SL) ($F_{1,108} = 26, p < 0.001$, main effect of genotype; $F_{5,108} = 5.3, p < 0.001$; no genotype \times concentration interaction was found) (Fig. 6a). Mutant male mice also demonstrated significantly reduced preference for ethanol ($F_{1,108} = 40, p < 0.001$, main effect of genotype; $F_{5,108} = 6.6, p < 0.001$; no genotype \times concentration interaction was found) (Fig. 6b) and increased total fluid intake ($F_{1,126} = 23, p < 0.001$, main effect of genotype) (Fig. 6c). No main effect of concentration and genotype \times concentration interaction was found. In contrast, ethanol intake in female mice depended only on concentration of ethanol ($F_{1,102} = 18, p < 0.001$) (Fig. 6d). No differences between mutant and wild-type female mice in preference for ethanol or in the total amount of fluid consumed were found (Fig. 6, e and f).

Given that ethanol intake in the continuous two-bottle choice paradigm depends strongly on taste (Blednov et al., 2008), the preferences for nonalcohol tastants such as saccharine and quinine were measured. No differences in preference for saccharin between mutant mice and wild-type mice of both sexes were found (Fig. 7, a and b). In male mice preference for saccharin depended on concentration of tastant ($F_{1,36} = 5.6, p < 0.05$, main effect of concentration). However, only mutant male mice demonstrated stronger avoidance for the bitter quinine solution ($F_{1,36} = 14, p < 0.001$, main effect of genotype) (Fig. 7c). In female mice the quinine intake depended only on concentration of tastant ($F_{1,34} = 9.2, p < 0.01$, main effect of concentration) (Fig. 7d).

Elevated Plus Maze. Locomotor activity was assessed by the total number of entries, whereas anxiety was measured

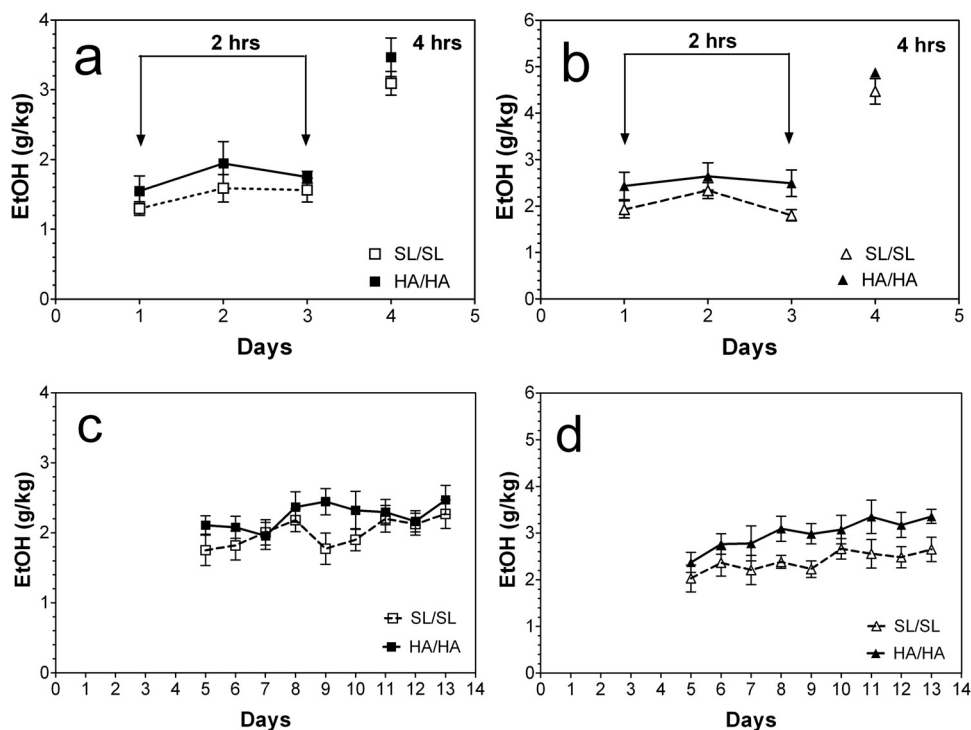


Fig. 3. Ethanol intake in a limited access (one-bottle DID) model. a, male mice ($n = 8-11$ per genotype). The amount of ethanol consumed (g/kg) with 2- or 4-h access periods is shown. No differences between wild-type and mutant male mice were found (two-way ANOVA). b, female mice ($n = 7-9$ per genotype). Female mutant mice consumed larger amounts of ethanol during first 3 days with 2-h access ($p < 0.01$; two-way ANOVA). c, male mice ($n = 8-11$ per genotype). Male mutant mice consumed larger amounts of ethanol during 9 days with 2-h access ($p < 0.01$; two-way ANOVA). d, female mice ($n = 7-9$ per genotype). Female mutant mice consumed larger amounts of ethanol during 9 days with 2-h access ($p < 0.001$; two-way ANOVA). Values represent mean \pm S.E.M.

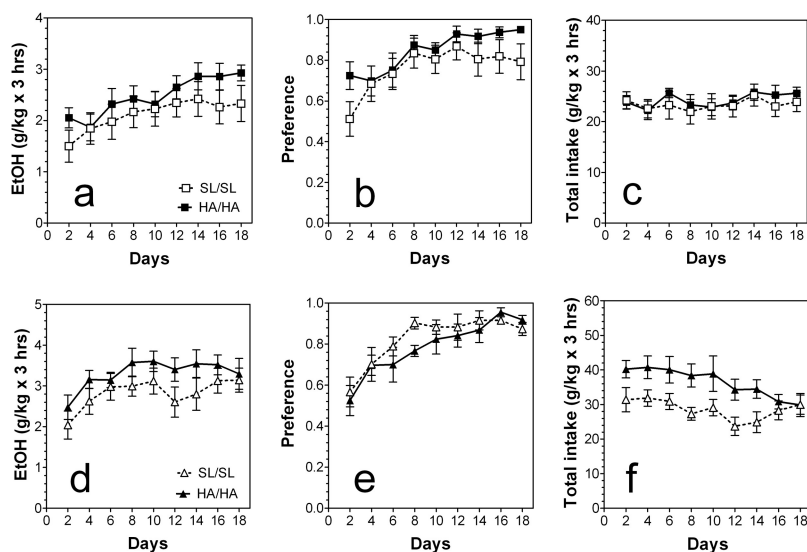


Fig. 4. Ethanol intake in limited access (two-bottle DID) model. a-c, male mice. a, amount of ethanol consumed given as g/kg/3 h. b, preference for ethanol as a percentage of fluid intake. c, total fluid intake (alcohol solution + water) given as g/kg/3 h. Male mutant mice consumed slightly larger amounts of ethanol ($p < 0.05$; two-way ANOVA) and showed slightly higher preference for ethanol ($p < 0.05$; two-way ANOVA) than wild-type littermates. No differences between genotypes for male mice in the amount of consumed fluid were found (two-way ANOVA) ($n = 7-9$ per genotype). d-f, female mice. d, amount of ethanol consumed given as g/kg/3 h. e, preference for ethanol as a percentage of fluid intake. f, total fluid intake given as g/kg/3 h. Female mutant mice consumed larger amounts of ethanol ($p < 0.01$; two-way ANOVA) and larger amounts of fluid ($p < 0.001$; two-way ANOVA) than wild-type littermates. No differences were observed between genotypes for female mice in preference for ethanol (two-way ANOVA) ($n = 6-8$ per genotype). Values represent mean \pm S.E.M.

by percentage of time spent in open arm entries and percentage of open arm entries after injection of saline or ethanol. Because no gender-dependent differences were found, the data obtained from male and female mice were combined for the final analysis. Treatment affected only the percentage of time spent in open arms ($F_{1,37} = 10$, $p < 0.01$) (Fig. 8a). Percentage of open arm entries demonstrated slight dependence on genotype ($F_{1,37} = 4$, $p < 0.05$) and treatment ($F_{1,37} = 8$, $p < 0.01$) (Fig. 8b). The total number of entries also depended on genotype ($F_{1,37} = 8$, $p < 0.01$) and treatment ($F_{1,37} = 7$, $p < 0.05$) (Fig. 8c). Post hoc analysis showed that ethanol significantly increased the activity in wild-type mice ($p < 0.05$) but not knockin mice, and elevation of activity after ethanol injection was significantly higher in wild-type mice compared with mutant mice ($p < 0.05$).

Motor Activity (Open Field). Because no gender-dependent differences were found, data from male and female mice were combined for the final analysis. Analysis of variance (two-way ANOVA) showed that the effect of ethanol on motor activity in the open field depended on genotype ($F_{1,118} = 51$, $p < 0.001$) and dose of ethanol ($F_{3,118} = 4.3$, $p < 0.01$) and showed significant genotype \times dose interaction ($F_{3,118} = 4.3$, $p < 0.01$) (Fig. 9). Post hoc analysis showed that at 0.75 and 1.0 g/kg doses of ethanol, motor activity was higher in wild-type (SL/SL) mice compared with mutant (HA/HA) mice. Additional within-groups analyses of variance (one-way ANOVA) showed a strong effect of ethanol in wild-type mice ($F_{3,60} = 7$, $p < 0.001$) and no effect of ethanol in knockin mice ($F_{3,58} = 0.4$, $p > 0.05$). At a dose of 1.0 g/kg, ethanol significantly

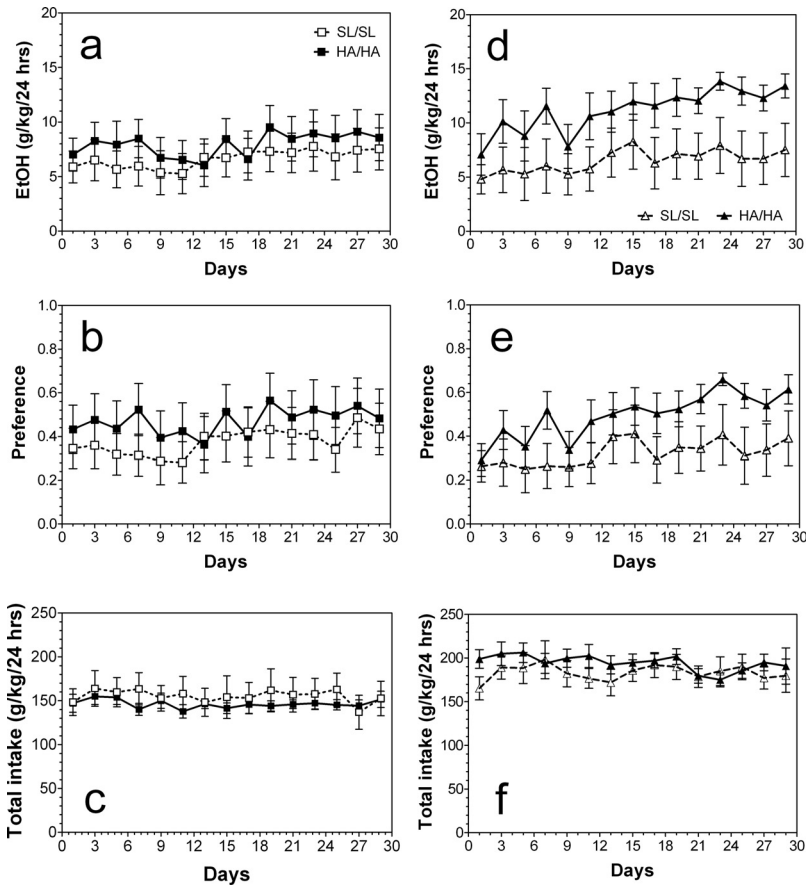


Fig. 5. Ethanol intake in a two-bottle choice test with intermittent access to ethanol (every other day drinking). a–c, male mice. a, amount of ethanol consumed given as g/kg/24 h. b, preference for ethanol as a percentage of fluid intake. c, total fluid intake given as g/kg/24 h. Mutant male mice consumed ethanol with a slightly higher preference than wild-type male mice ($p < 0.05$; two-way ANOVA). No significant differences in amount of ethanol consumed and total amount of fluid consumed were found (two-way ANOVA) ($n = 7$ per genotype). d–f, female mice. d, amount of ethanol consumed given as g/kg/24 h. e, preference for ethanol as a percentage of fluid intake. f, total fluid intake given as g/kg/24 h. Mutant female mice consumed larger amounts of ethanol ($p < 0.001$; two-way ANOVA) with higher preference for ethanol ($p < 0.001$; two-way ANOVA) than their wild-type littermates. Total fluid intake was also slightly elevated in mutant female mice ($p < 0.05$; two-way ANOVA) ($n = 7$ per genotype). Values represent mean \pm S.E.M.

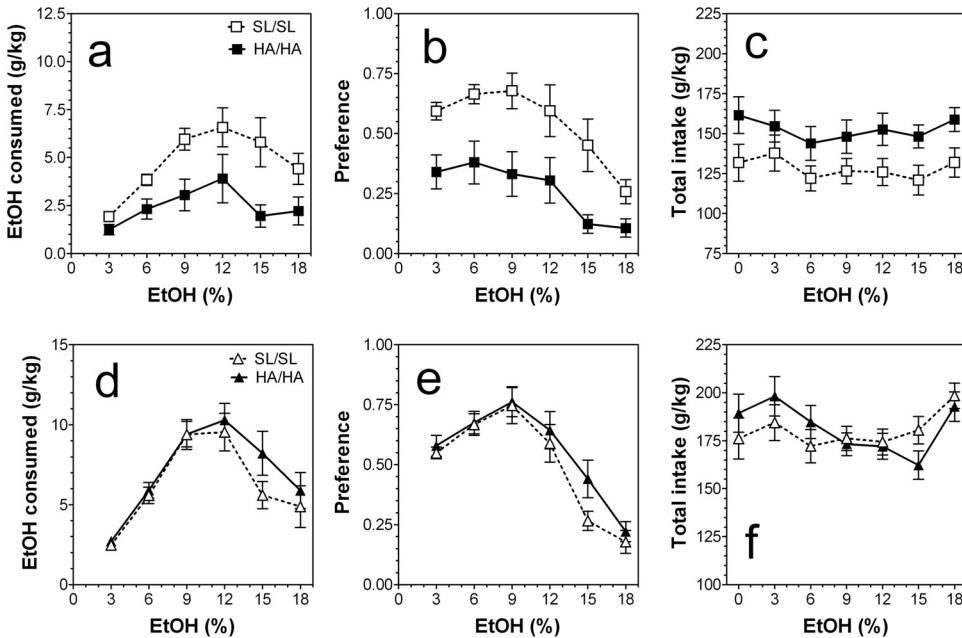


Fig. 6. Ethanol intake in a two-bottle choice test with 24-h continuous access to ethanol. a–c, male mice. a, amount of ethanol consumed given as g/kg/24 h. b, preference for ethanol as a percentage of fluid intake. c, total fluid intake given as g/kg/24 h. Mutant male mice consumed smaller amounts of ethanol ($p < 0.001$; two-way ANOVA) with reduced preference for ethanol ($p < 0.001$; two-way ANOVA) than their wild-type littermates. Total fluid intake was elevated in mutant male mice ($p < 0.001$; two-way ANOVA) ($n = 10$ per genotype). d–f, female mice. d, amount of ethanol consumed given as g/kg/24 h. e, preference for ethanol as a percentage of fluid intake. f, total fluid intake given as g/kg/24 h. No differences between mutant and wild-type female mice in ethanol intake, preference for ethanol, or total amount of fluid consumed were found (two-way ANOVA) ($n = 9$ – 10 per genotype). Values represent mean \pm S.E.M.

increased the motor activity in wild-type mice ($p < 0.001$, Fischer's post hoc test).

Sedative/Hypnotic and Motor Ataxic Effects of Ethanol. Because no gender-dependent differences were found, data from male and female mice were combined for the final analysis. Acute administration of ethanol (2 g/kg) produced motor ataxia as measured by the rotarod test in wild-type

and mutant mice (Fig. 10a). There were no differences between wild-type and mutant mice in recovery from this motor incoordination ($F_{1,180} = 0.4$, $p > 0.05$, dependence on genotype; $F_{9,180} = 297$, $p < 0.001$, dependence on time; $F_{9,180} = 1.9$, $p > 0.05$, genotype \times time interaction).

In animals from the N4 generation of backcrossing, ethanol produced a significantly longer duration of LORR in mutant

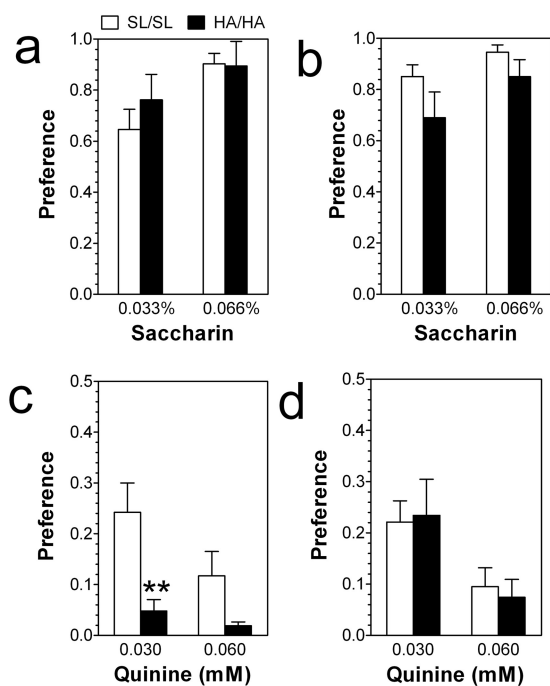


Fig. 7. Saccharin and quinine intake in a two-bottle choice test with 24-h continuous access to tastants. a and c, male mice ($n = 10$ per genotype). a, preference for saccharin. c, preference for quinine. b and d, female mice ($n = 10$ per genotype). b, preference for saccharin. d, preference for quinine. No differences in preference for saccharin between mutant mice and wild-type mice of both sexes were found (two-way ANOVA). Only mutant male mice demonstrated stronger avoidance for the bitter quinine solution ($p < 0.001$, main effect of genotype; two-way ANOVA). **, $p < 0.01$, significant differences relative to wild-type mice for the same dose of quinine (Bonferroni post hoc test). Values represent mean \pm S.E.M.

mice than in wild-type mice ($p < 0.001$, Student's t test) (Fig. 10b).

Ethanol Metabolism. There were no differences in metabolism of ethanol (after 3.8 g/kg dose) between wild-type and knockin mice (data not shown). The slopes of the regression lines were -0.47 ± 0.02 and -0.52 ± 0.02 for wild-type mice ($n = 4$) and knockout mice ($n = 4$), respectively.

Discussion

Engineering a mutant $\alpha 2$ subunit with normal sensitivity to GABA (Werner et al., 2010) but complete resistance to modulation by ethanol allows us to ask which, if any, behavioral effects of ethanol might be caused by the direct action of ethanol on GABA_A-R-containing $\alpha 2$ subunits. Any behavior resulting from ethanol action on such receptors should be

reduced or eliminated in the knockin mice. There are two ethanol-induced behaviors that meet this criterion as summarized in Table 1: conditioned taste aversion and stimulation of motor activity. These mutations also tended to increase ethanol consumption, and this may be related to decreased aversive (CTA) properties of the drug in the mutant mice consistent with the relationship between CTA and alcohol consumption shown in many other studies (Green and Grahame, 2008). It is noteworthy that these effects on consumption depend on the specific test used as well as the sex of the mice, and this complexity has been noted in other studies of alcohol consumption by mutant mice (Blednov and Harris, 2008). The male $\alpha 2$ knockin mice also showed increased avoidance of a bitter tastant (quinine), and this may also influence alcohol consumption in this sex. In particular, the two-bottle choice test provides increasing concentrations of ethanol, whereas the other tests used a fixed (15%) concentration of ethanol. Bitter taste may play a different role in these two types of tests because males decreased their consumption in the two-bottle choice test, but males and females increased consumption in the one-bottle and two-bottle DID tests. These findings raise the question of what regulates, or limits, alcohol consumption in each of these tests. Our studies of mutant mice with taste deficiencies suggest that sweet taste is very important for alcohol consumption in the two-bottle choice test (Blednov et al., 2008) but not as important for limited-access drinking (unpublished data). The DID models provide a model where voluntary consumption produces appreciable levels of blood alcohol and, in view of the linkage between $\alpha 2$ and human alcoholism, it is intriguing that mutation of $\alpha 2$ increased consumption in these tests. Apart from ethanol drinking behavior, it is somewhat surprising that the knockin mutations enhanced one behavioral action of ethanol, duration of LORR. This effect was robust and evident on two different genetic backgrounds. This contrasts with the enhanced duration of the LORR in response to isoflurane that was observed in these mice only on the N2 background (Werner et al., 2010). The mechanism for this paradoxical increase in ethanol sensitivity is unknown at present. It is possible that because the $\alpha 2$ mutation ablated the locomotor stimulatory action of ethanol it changed the balance between inhibitory and stimulatory effects and enhanced the depressant actions thus exacerbating LORR. It is also interesting that these same mutations in the $\alpha 1$ subunit of the GABA_A-R also paradoxically increased sensitivity to ethanol, albeit on a different behavioral response, the anxiolytic effect (Werner et al., 2006).

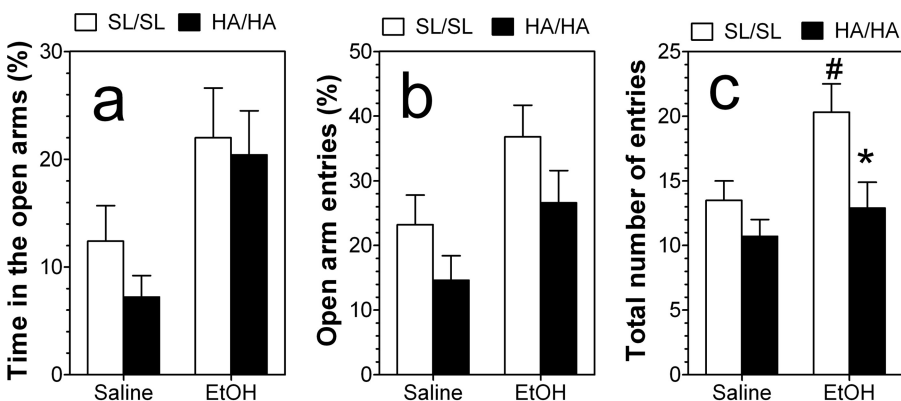


Fig. 8. Evaluation of anxiety and activity using the elevated plus maze. a, percentage of total time spent in open arms. There was a dependence only on treatment ($p < 0.01$; two-way ANOVA). b, percentage of open arm entries. There was a dependence on genotype ($p < 0.05$; two-way ANOVA) and treatment ($p < 0.01$; two-way ANOVA). c, total arm entries. There was a dependence on genotype ($p < 0.01$; two-way ANOVA) and treatment ($p < 0.05$; two-way ANOVA) ($n = 9-11$ per genotype). *, $p < 0.05$, significant differences relative to wild-type mice for the same concentration of ethanol (two-way ANOVA Fischer's post hoc test). #, $p < 0.05$, significant differences between ethanol-injected and control groups of wild-type mice (two-way ANOVA Fischer's post hoc test). Values represent mean \pm S.E.M.

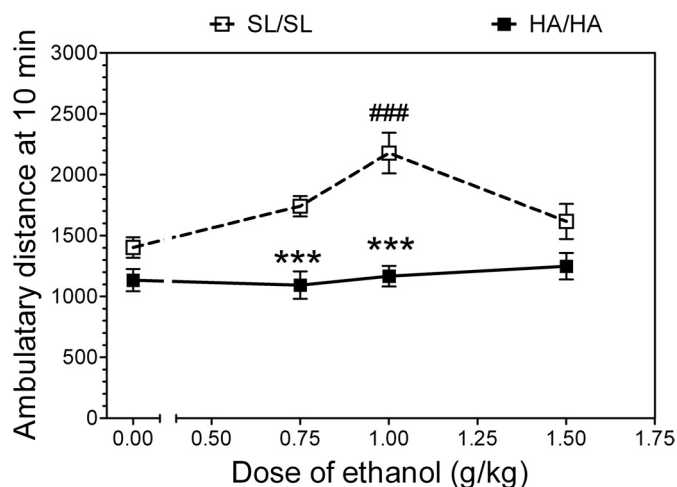


Fig. 9. $\alpha 2$ Knockin mice are less sensitive to ethanol-induced motor stimulation in the open field. There was a dependence on genotype ($p < 0.001$; two-way ANOVA) and dose of ethanol ($p < 0.01$; two-way ANOVA). Within-groups analyses of variance showed strong effect of ethanol in wild-type mice ($p < 0.001$; one-way ANOVA) and no effect of ethanol in knockin mice. ###, $P < 0.001$, significant difference from saline control for the same genotype (one-way ANOVA, Fischer's post hoc test). ***, $P < 0.001$, significant difference from the same dose of ethanol between two different genotypes (two-way ANOVA, Fischer's post hoc test). Each point represents an independent group of animals ($n = 15$ – 16 per each group of each genotype).

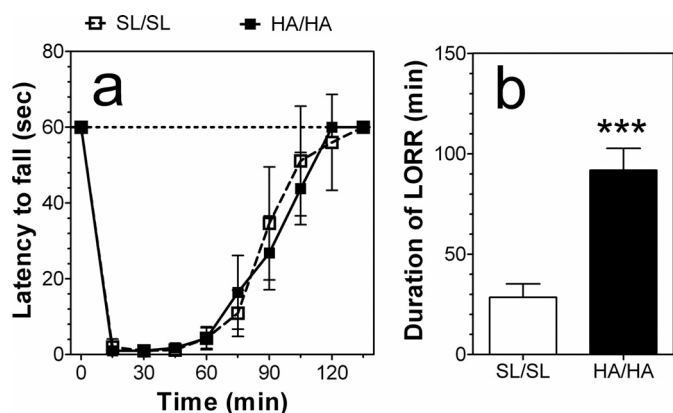


Fig. 10. Depressant effects of ethanol in $\alpha 2$ knockin mice. a, time on the rotarod in seconds before and after motor incoordination induced by ethanol (2 g/kg) ($n = 6$ per genotype). No differences between wild-type and mutant mice in recovery from ethanol-induced motor incoordination were found (two-way ANOVA). b, duration of LORR in minutes after injection of ethanol (3.25 g/kg) ($n = 6$ per genotype). ***, $P < 0.05$, significant difference between genotypes (Student's t test). Values represent mean \pm S.E.M.

Also surprising is the lack of importance of $\alpha 2$ for the anxiolytic actions of ethanol found in the present work and in an earlier study of $\alpha 2$ null mutant mice (Boehm et al., 2004). The anxiolytic actions of benzodiazepines seem to be solely caused by actions on GABA_A-R-containing $\alpha 2$ subunits (Löw et al., 2000), and one might expect that the mutants would display reduced anxiolytic effects of ethanol. However, the data from $\alpha 2$ mutant mice indicate clear differences in the anxiolytic actions of ethanol and benzodiazepines.

As noted earlier, our interest in the GABA_A-R $\alpha 2$ subunit was stimulated by the many human genetic studies showing linkage of polymorphisms near this gene with alcoholism (Edenberg et al., 2004; Enoch et al., 2008; Soyka et al., 2008). In a human behavioral study, Haughey et al. (2008)

TABLE 1

Summary of the behavioral effects of ethanol in HA/HA knockin mice

Test	Behavior	Males	Females
2-BC (ethanol)	EtOH (g/kg/24 h)	↓	=
	Preference	↓	=
	Fluid intake (g/kg/24 h)	↑	=
2-BC (saccharin)	Preference	=	=
	Preference	↓	=
2-BC (quinine)	Preference	↓	=
1B-DID (short)	EtOH (g/kg/2 h)	=	↑
1B-DID (long)	EtOH (g/kg/2 h)	↑	↑
2B-DID	EtOH (g/kg/3 h)	↑	↑
	Preference	↑	=
	Fluid intake (g/kg/3 h)	=	↑
2-BCI	EtOH (g/kg/24 h)	=	↑
	Preference	↑	↑
	Fluid intake (g/kg/24 h)	=	↑
CTA	Taste aversion	↓	↓
LORR	Duration (min)	↑	↑
EPM	Anxiety	=	=
	Activity	↓	↓
OF	Motor stimulation	↓	↓
Rotarod	Motor incoordination	=	=

2-BC, two-bottle choice with 24-h access; 2-BCI, two-bottle choice with intermittent 24-h access; 1B-DID, limited access with one bottle; 2B-DID, limited access with two bottles; EPM, elevated plus maze; OF, open field. ↓, reduction of behavior in mutant mice; ↑, increase of behavior in mutant mice; =, no differences.

showed that two GABA_A-R $\alpha 2$ single-nucleotide polymorphisms were associated with sensitivity to the acute effects of alcohol. Specifically, several measures of subjective responses to alcohol, including the hedonic value, were linked with *Gabra2* polymorphisms. Based on a haplotypic association of alcohol dependence with *Gabra2*, Pierucci-Lagha et al. (2005) also provided evidence that the risk of alcoholism associated with *Gabra2* alleles may be related to differences in the subjective response to alcohol. It is difficult to directly link these human responses to alcohol with mouse behavior, but it should be noted that the most dramatic effects of mutation were in development of CTA, a test that probably reflects aversive properties of ethanol (Green and Grahame, 2008). In addition, we found a marked reduction of CTA in mice with global deletion of the GABA_A-R $\alpha 2$ subunit (Y. A. Blednov, unpublished data).

The $\alpha 2$ subunit coassembles with $\beta 2$ or $\beta 3$ and $\gamma 2$ to form functional GABA_A-R. However, these receptors, at least when tested in vitro in recombinant systems, require high concentrations (50–100 mM) of ethanol for enhancement of channel function. It should be noted that these concentrations are not so far removed from those encountered in vivo. Most of our behavioral tests used intraperitoneal doses of 2.5 to 3.5 g/kg, which will result in peak brain ethanol concentrations of approximately 50 to 100 mM (Deitrich and Harris, 1996). Thus, it might be expected that they could be important only for the behavioral effects produced by large doses of ethanol, such as loss of righting reflex or motor incoordination. However, the two behaviors that are among the most sensitive to ethanol, stimulation of motor activity and CTA, were remarkably sensitive to the mutation, whereas the behaviors requiring higher doses of ethanol were not reduced in the knockin mice. The results clearly indicate that direct action of ethanol on GABA_A-Rs with $\alpha 2$ subunits is important for several low-dose behaviors, although the basis of this unexpected sensitivity is not apparent. A caveat of any study of mutant mice is that the mutation may produce changes in other neuronal functions, and this has been found with null mutant mice lacking GABA_A-R subunits (Ponomarev et al.,

2006). Although we expect such changes to be less pronounced in knockin mice than in null mutants, Borghese et al. (2006) found changes in the protein levels of several GABA_A-R subunits in the cortex of wild-type and knockin mice with a mutation in the $\alpha 1$ subunit. We have examined global gene expression in brain of $\alpha 2$ knockin mice and found very few changes in contrast to $\alpha 1$ knockin or knockout mice (Harris et al., 2010), suggesting that this knockin mutation may be relatively “silent.”

In summary, we have demonstrated that mutation of two amino acids in the $\alpha 2$ subunit prevents ethanol modulation of GABA_A-R in vitro. Moreover, knockin mice containing these mutant receptors are resistant to ethanol-induced conditioned place aversion and motor stimulation. Thus, $\alpha 2$ -containing GABA_A-Rs may play a role in several behaviors, including ethanol intake, that are relevant for human alcoholism and may explain the association of polymorphisms in *Gabra2* with alcoholism.

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Authorship Contributions

Participated in research design: Blednov, Harrison, Homanics, and Harris.

Conducted experiments: Blednov, Borghese, McCracken, Benavidez, Geil, Osterndorff-Kahanek, Iyer, Werner, and Swihart.

Performed data analysis: Blednov, Borghese, McCracken, Iyer, Werner, Homanics, and Harris.

Wrote or contributed to the writing of the manuscript: Blednov, Borghese, Werner, Harrison, Homanics, and Harris.

Other: Blednov, Homanics, and Harris acquired funding for the research.

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