

# Knockin Mice with Ethanol-Insensitive $\alpha 1$ -Containing $\gamma$ -Aminobutyric Acid Type A Receptors Display Selective Alterations in Behavioral Responses to Ethanol

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## ABSTRACT

Despite the pervasiveness of alcohol (ethanol) use, it is unclear how the multiple molecular targets for ethanol contribute to its many behavioral effects. The function of GABA type A receptors (GABA<sub>A</sub>-Rs) is altered by ethanol, but there are multiple subtypes of these receptors, and thus far, individual subunits have not been definitively linked with specific behavioral actions. The  $\alpha 1$  subunit of the GABA<sub>A</sub>-R is the most abundant  $\alpha$  subunit in the brain, and the goal of this study was to determine the role of receptors containing this subunit in alcohol action. We designed an  $\alpha 1$  subunit with serine 270 to histidine and leucine 277 to alanine mutations that was insensitive to potentiation by ethanol yet retained normal GABA sensitivity and

constructed knockin mice containing this mutant subunit. Hippocampal slice recordings from these mice indicated that the mutant receptors were less sensitive to ethanol's potentiating effects. Behaviorally, we observed that mutant mice recovered more quickly from the motor-impairing effects of ethanol and etomidate, but not pentobarbital, and showed increased anxiolytic effects of ethanol. No differences were observed in ethanol-induced hypnosis, locomotor stimulation, cognitive impairment, or in ethanol preference and consumption. Overall, these studies demonstrate that the postsynaptic effects of ethanol at GABAergic synapses containing the  $\alpha 1$  subunit are important for specific ethanol-induced behavioral effects.

Alcohol (ethanol) has a prominent role in society and is one of the most frequently used and abused drugs. Despite the prevalence of alcohol use, the molecular mechanisms underlying its behavioral effects remain unclear. Ethanol intoxication elicits a diverse array of behavioral effects including cognitive impairment and motor incoordination, and these behavioral effects are likely due to actions of ethanol on multiple brain proteins (Harris, 1999). GABA type A receptors (GABA<sub>A</sub>-Rs) are ligand-gated ion channels that mediate

the majority of rapid inhibitory neurotransmission in the brain, and there is evidence that ethanol enhances the function of these receptors (Grobin et al., 1998).

GABA<sub>A</sub>-Rs are pentameric chloride channels composed of multiple subunits. Numerous subunits have been cloned and are categorized into different classes, with some containing several isoforms: six  $\alpha$ , three  $\beta$ , three  $\gamma$ , one  $\delta$ , one  $\epsilon$ , one  $\pi$ , one  $\theta$ , and three  $\rho$  (Sieghart and Sperk, 2002). However, the majority of GABA<sub>A</sub>-Rs are thought to consist of two  $\alpha$ , two  $\beta$ , and a  $\gamma$  or  $\delta$  subunit (Tretter et al., 1997). Because of the numerous subunits, it is quite possible that receptors with a specific subunit composition can mediate specific ethanol-induced behavior as is the case for benzodiazepines (Atack, 2005) and i.v. anesthetics (Grasshoff et al., 2005).

We hypothesized that  $\alpha 1$  subunit-containing GABA<sub>A</sub>-Rs

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**ABBREVIATIONS:** GABA<sub>A</sub>-R, GABA type A receptor; QX-314, *N*-(2,6-dimethyl-phenylcarbonylmethyl)-triethylammonium bromide; IPSC, inhibitory postsynaptic current; HIC, handling-induced convulsion; ANOVA, analysis of variance; LORR, loss of righting reflex; BEC, blood ethanol concentration; FLU, flunitrazepam; PB, pentobarbital.

are important for ethanol action based on several lines of evidence. The  $\alpha 1$  subunit is the most abundant  $\alpha$  subunit in adult brain and is present in ~50% of all GABA<sub>A</sub>-Rs (McKernan and Whiting, 1996; Sieghart and Sperk, 2002).  $\alpha 1$ -Containing GABA<sub>A</sub>-Rs mediate benzodiazepine-induced sedation and memory impairment (Rudolph et al., 1999; McKernan et al., 2000) and pharmacologic studies using the  $\alpha 1$ -selective ligand zolpidem have suggested a role for this subunit in ethanol sensitivity (Criswell et al., 1995). In addition, previous work with  $\alpha 1$  global knockout mice suggests this subunit is important for certain ethanol-related effects. Specifically, abolition of  $\alpha 1$  increases ethanol-induced locomotor activity (Blednov et al., 2003b; Kralic et al., 2003), decreases ethanol-induced hypnosis (Blednov et al., 2003a), and non-sedative doses of ethanol eliminate essential tremor observed in knockouts (Kralic et al., 2005).

The 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. This has proven very useful for defining the role of specific GABA<sub>A</sub>-R subunits in behavioral actions of i.v. anesthetics and benzodiazepines. For example, construction of mice containing mutant GABA<sub>A</sub>-Rs with normal responses to GABA, but lacking modulation by benzodiazepines, have shown that the  $\alpha 1$ , 2, and 3 subunits are each responsible for distinct actions (e.g., sedative, antianxiety, myorelaxation, respectively) of these drugs (e.g., Rudolph et al., 1999). Two previous attempts to construct knockin mice with glycine or GABA<sub>A</sub>-Rs resistant to ethanol were not completely successful because the normal function of these receptors was impaired by the mutations (Findlay et al., 2003; Homanics et al., 2005).

Numerous studies have shown that mutation of amino acids in the transmembrane domains of the GABA<sub>A</sub>-R  $\alpha$  subunits can eliminate alcohol modulation, suggesting that construction of a knockin mouse with alcohol-resistant GABA<sub>A</sub>-Rs is feasible. Because the serine 270 to histidine (S270H) mutation in the TM2 region of the  $\alpha 1$  subunit eliminated GABA<sub>A</sub>-R enhancement by ethanol (Ueno et al., 2000), we initially used this mutation for knockin mice. However, this mutation by itself resulted in receptors that were hypersensitive to GABA (Nishikawa et al., 2002), and mice bearing this mutation were behaviorally impaired (Homanics et al., 2005). However, incorporating a second mutation, leucine 277 to alanine (L277A), resulted in receptors that displayed normal GABA sensitivity but no modulation by ethanol and a reduced potentiation by etomidate and pentobarbital. Importantly, these receptors showed normal modulation by flunitrazepam, and mice harboring this mutation appeared normal (Borghese et al., 2006b).

In the present study, we used these knockin mice to test the hypothesis that  $\alpha 1$ -containing GABA<sub>A</sub>-Rs mediate specific behavioral responses to ethanol. We demonstrate that some, but not all, ethanol-induced behavioral responses are altered in these knockin mice.

## Materials and Methods

### Mouse Production

Wild-type (homozygous for serine at 270 and leucine at 277; genotype referred to as SL/SL) and knockin (homozygous for histidine

at 270 and alanine at 277; genotype referred to as HA/HA) mice used for these experiments were produced from heterozygous (SL/HA) breeding pairs at the University of Pittsburgh (Pittsburgh, PA), the University of Texas (Austin, TX), or the University of Memphis (Memphis, TN). All mice were genotyped by Southern blot or polymerase chain reaction analysis of tail DNA as described previously (Borghese et al., 2006b). All mice were of a mixed C57BL/6Jx129SvJ background of the F<sub>3</sub> to F<sub>6</sub> generations. After weaning, mice were housed under specific pathogen-free conditions with ad libitum access to rodent chow and water with 12-h light/dark cycles (lights on at 7:00 AM). All mice were between 8 and 12 weeks of age for behavioral studies and 3 to 5 months for electrophysiological experiments. For most experiments, only male mice were used; however, in cases where sufficient numbers of male mice were not available, both male and female mice were used, and this is noted in the figure legends. 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.

### Electrophysiology in Hippocampal Slices

Brain slice electrophysiological experiments were carried out on 3- to 5-month old male and female mice. Mice were anesthetized with halothane and euthanized by decapitation. Experiments on SL/SL and HA/HA mice were performed on an alternating day schedule, and experimenters were blinded to genotype. Brain slice preparation and electrophysiological recordings were carried out as described previously (Ariwodola et al., 2003; Ariwodola and Weiner, 2004). In brief, transverse hippocampal slices (400  $\mu$ m) were prepared using a vibrating tissue slicer (Leica VT 1000S; Leica Microsystems, Bannockburn, IL), and slices were maintained at ambient temperature for at least 2 h in oxygenated artificial cerebrospinal fluid containing: 124 mM NaCl, 3.3 mM KCl, 2.4 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM D-glucose, and 25 mM NaHCO<sub>3</sub>, saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Whole-cell patch-clamp recordings of hippocampal CA1 pyramidal neurons were performed at ambient temperature using a patch pipette filling solution containing: 130 mM potassium gluconate, 10 mM KCl, 1 mM EGTA, 100  $\mu$ M CaCl<sub>2</sub>, 2 mM Mg-ATP, 200  $\mu$ M Tris-guanosine 5'-triphosphate, 5 mM QX-314, and 10 mM HEPES (pH adjusted with KOH; 275–280 mOsm). Neurons were voltage-clamped at  $-45$  to  $-60$  mV, and only cells with a stable access resistance of 5 to 20 M $\Omega$  were included in the data analysis. GABA<sub>A</sub> IPSCs were evoked every 20 s by electrical stimulation (0.2-ms duration, constant current) using a concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) placed near the CA1 somatic layer ("proximal" stimulation) (Weiner et al., 1997). Stimulation intensity was adjusted to evoke responses that were 10 to 20% of maximal currents (typically 50–200 pA). GABA<sub>A</sub> IPSCs were pharmacologically isolated using a cocktail of 50  $\mu$ M 2-amino-5-phosphonovalerate and 20  $\mu$ M 2,3-dihydroxy-6,7-dinitroquinoxaline to block *N*-methyl-D-aspartate and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptors, respectively. Unless otherwise stated, all drugs used were purchased from Sigma-Aldrich (St. Louis, MO). Drugs were made up as 100- to 400-fold concentrates and applied to slices via calibrated syringe pumps (Razel Scientific Instruments, Stamford, CT). A 4 M ethanol solution was prepared immediately before each experiment from a 95% stock solution (Aaper Alcohol and Chemical, Shelbyville, KY) kept in a glass storage bottle. Drug effects were quantified as the percentage change in the area under the curve of synaptic currents relative to the mean of control and washout values. Statistical analyses of drug effects were carried out using the two-tailed Student's paired or unpaired *t* tests or analysis of variance followed by the Newman-Keuls post hoc test.

### Behavioral and Pharmacokinetic Analyses

**Acute Withdrawal.** Mice were scored for handling-induced convulsion (HIC) severity 30 min and immediately before i.p. ethanol

administration. These two predrug baseline scores were averaged. A dose of 4.0 g/kg ethanol in saline was injected i.p., and the mice were tested for HIC every hour until the HIC level reached baseline. Acute withdrawal was quantified as the area under the curve but above basal response (Crabbe et al., 1991). In brief, each mouse was picked up gently by the tail and, if necessary, gently rotated 180°, and the HIC was scored as follows: 5, tonic-clonic convulsion when lifted; 4, tonic convulsion when lifted; 3, tonic-clonic convulsion after a gentle spin; 2, no convulsion when lifted, but tonic convulsion elicited by a gentle spin; 1, facial grimace only after a gentle spin; and 0, no convulsion. HIC response curves were integrated to determine the area both under the curve and above basal response. Data were analyzed by Student's *t* test.

**Rotorod.** Mice were trained on a fixed speed (5.0 rpm) rotorod [Ecomex; Columbus Instruments (Columbus, OH) for ethanol and etomidate studies; Ugo Basile Rota-Rod, model 7650; Stoelting Co. (Wood Dale, IL) for pentobarbital studies], and training was considered complete when mice were able to remain on the rotorod for 60 s. After drug administration, each mouse was placed back on the rotorod, and time spent on the rotorod was measured for up to 60 s. Behavior of mice was measured at 30-, 5-, or 10-min intervals for ethanol (2.0 g/kg i.p.), etomidate (20 mg/kg i.p.), or pentobarbital (35 mg/kg i.p.), respectively. Data were analyzed using two-way repeated measures ANOVA. Time was the repeated measure factor and genotype was the between-group factor.

**Loss of Righting Reflex.** Mice were tested for the sedative/hypnotic effects of ethanol (3.8 g/kg i.p.) and etomidate (20 or 30 mg/kg i.p.). Mice were injected with drug and observed for loss of righting reflex (LORR). Once this occurred, mice were placed on their backs in v-shaped troughs and monitored until they were able to right themselves three times in a 30-s period. LORR was determined as the length of time from when the mouse was placed in a supine position until it was able to right itself. Data were analyzed using unpaired Student's *t* test.

**Two-Bottle Choice.** The two-bottle choice protocol was carried out as described previously (Blednov et al., 2003b). In brief, mice were allowed to acclimate for 1 week to individual housing. Experiments were carried out in standard 7.5- × 12.5-inch cages in sliding racks. 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 available on both tubes, mice were offered 3% (v/v) ethanol versus water for 4 days. Tube positions were changed every day to control for position preferences. Quantity of ethanol consumed (grams per kilogram of body weight per 24 h) was calculated for each mouse, and these values were averaged for every concentration of ethanol. Immediately following 3% ethanol, a choice between 6% (v/v) ethanol and water was offered for 4 days, then 9% (v/v) ethanol and water for 4 days, and finally 12% (v/v) ethanol and water for 4 days. Throughout the experiment, evaporation/spillage estimates were calculated every day from two bottles placed on an empty cage; one contained water, and the other contained the appropriate ethanol solution. Wild-type and 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 (0.033 and 0.066%) and quinine hemisulfate (0.03 and 0.06 mM), and intakes were calculated. Each concentration was offered for 4 days, with bottle position changed every day. Within each tastant, the low concentration was always presented first, followed by the higher concentrations. Between tastants, mice had two bottles with water for 2 weeks. Preference was defined as the ratio between the amount of consumed ethanol or tastant solution and total liquid intake. If the ratio was less than 0.5, the behavior was considered avoidance, and if it was more than 0.5, the behavior was considered preference. Data were analyzed using two-way repeated measures ANOVA with Bonferroni post hoc test when necessary. Ethanol, saccharin, or quinine concentration was

the repeated measure factor, and genotype was the between-group factor.

**Hypothermia.** Ethanol-induced hypothermia was determined by measuring rectal body temperature via a digital thermometer (Thermalert Model TH-8 with probe RET-3; Physiotemp Instruments, Clifton, NJ). Mice were injected with either 2.0 or 3.0 g/kg ethanol. Body temperatures were measured at times of 15, 30, 45, 60, 90, and 120 min postinjection. Data were assessed using repeated measures ANOVA with time as the repeated measure factor and genotype or dose as the between-group factor.

**Morris Water Maze.** The water maze consisted of a galvanized steel, circular tub 3 feet in diameter and 24 inches high. Four points labeled along the rim corresponded to compass points and served as start points during training day sessions. Water level was raised 0.25 inches above the level of the clear, Plexiglas escape platform and made invisible by clouding the water with water-based paint. The water maze was located in a room that was rich with distal cues in fixed locales. Recording measurements were made using a video camera (video tracking system; HVS Image, Co., Buckingham, UK) mounted above the pool. Animals were trained using the standard spatial version of the Morris water maze task (Morris, 1981). The procedure occurred over a successive 10-day period. Training took place on days 1 through 9 with testing occurring on day 10. Escape platform location was held constant throughout the procedure. Mice were given four trials a day, one from each starting position, with each trial lasting 45 s. A Latin square design allowed for a unique starting position order each day. For each trial, the investigator lowered mice into the pool facing the wall while simultaneously activating the tracking system. Latency to reach the platform was assessed for each trial. On test day, mice received 2.25 g/kg ethanol i.p. 30 min prior to testing. Dose was based on previous work demonstrating reliably impaired spatial memory (Berry and Matthews, 2004). Animals were tested in the same manner as employed during training. Data were analyzed first by multivariate ANOVA [genotype by day (last day training, testing under ethanol)] followed by a direct comparison between conditions using a Student's *t* test.

**Elevated Plus Maze.** Mice were evaluated for basal anxiety as well as ethanol-induced anxiolysis using the elevated plus maze. Mice were transported to the testing room 1 day prior to testing. Animals were tested between 9:00 and 11:00 AM under ambient room light. Mice were weighed and injected with 0.75, 1.0, or 1.5 g/kg ethanol or saline 10 min prior to 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. Data were analyzed using ANOVA with genotype and dose as the between-subject factors. Data were further analyzed with Fischer's post hoc test, or pair-wise comparisons were made with Student's *t* test where appropriate.

**Ethanol Metabolism and Clearance.** Following injection of ethanol (3.5 g/kg i.p.), blood was collected from the retro-orbital sinus at 30, 60, 90, and 120 min postinjection. Blood ethanol concentrations (BECs) were determined as described previously (Harris et al., 1995). In brief, blood samples were collected in heparinized capillary tubes, mixed with 3% perchloric acid, and centrifuged for 10 min at 1500g at 4°C. Supernatants were subsequently used to assess ethanol concentration via an alcohol dehydrogenase enzymatic spectrophotometric method. The rate of clearance was determined by linear regression analysis. Data were analyzed by Student's *t* test.

## Results

**Hippocampal Electrophysiology.** Whole-cell recordings were made from CA1 pyramidal neurons, and pharmacologically isolated GABA<sub>A</sub> IPSCs were evoked as described in the

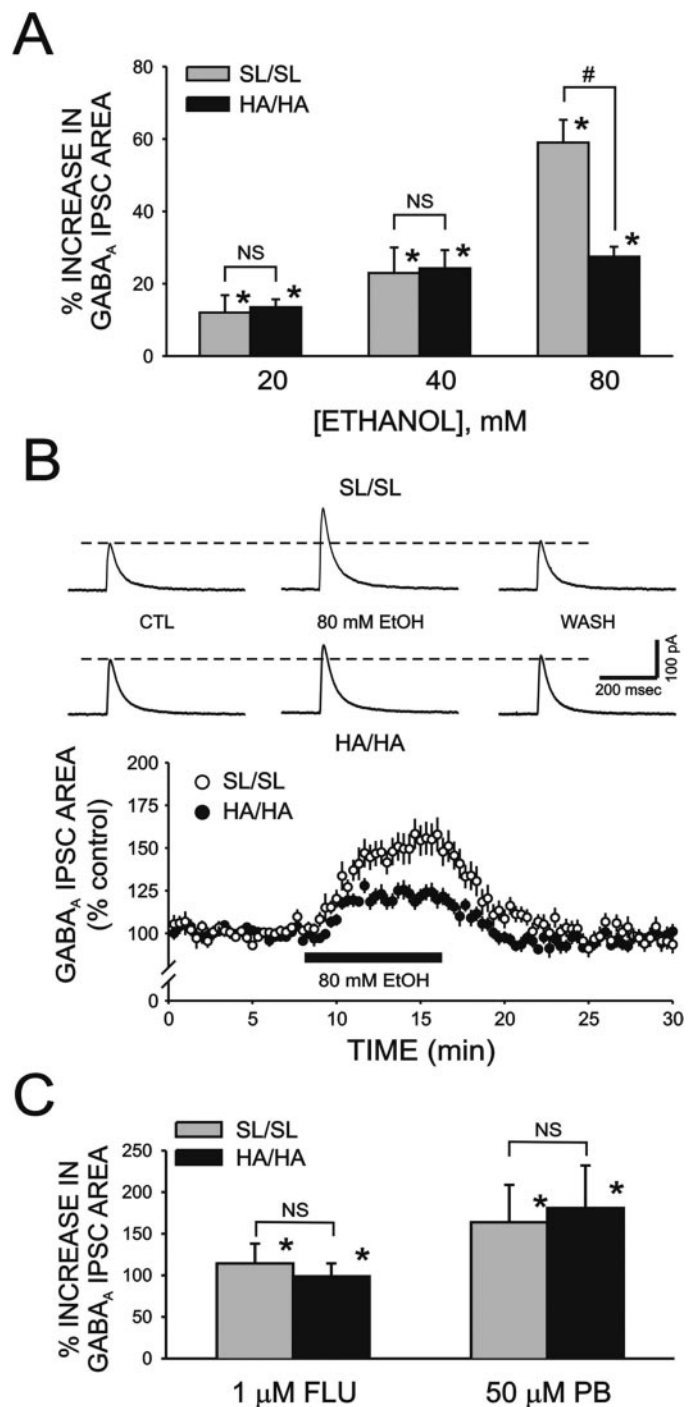
methods. Experiments were carried out on male and female mice, and since no sex differences in the acute effects of ethanol on IPSCs were noted, data from males and females were pooled for the genotype comparisons. Bath application of ethanol significantly potentiated the area of GABA<sub>A</sub> IPSCs in both groups of mice in a concentration-dependent manner (Fig. 1A). The potentiation was significant at all three concentrations tested, and there were no genotype differences in the magnitude of the enhancement at 20 and 40 mM ethanol (e.g., 40 mM: SL/SL = 23.0 ± 7.6%; HA/HA = 24.7 ± 4.6%;  $p > 0.05$ ). In contrast, at 80 mM ethanol, IPSCs recorded from HA/HA slices were markedly less sensitive to ethanol (SL/SL = 59.1 ± 6.3%; HA/HA = 27.7 ± 3.5%,  $p < 0.01$ ). As illustrated in the summary time course graphs (Fig. 1B), the ethanol enhancement was stable throughout the duration of an 8-min application in both groups and reversed completely upon washout.

Additional experiments were carried out to examine the effects of two well characterized positive allosteric modulators of GABA<sub>A</sub> receptor function on IPSCs in SL/SL and HA/HA hippocampal slices. Bath application of 1 μM flunitrazepam (FLU) and 50 μM pentobarbital (PB) significantly enhanced GABA<sub>A</sub> IPSC area in all cells tested, and no group differences in the sensitivity of IPSCs to these two drugs were noted (FLU: SL/SL = 114.0 ± 23.8%, HA/HA = 98.7 ± 15.7%,  $p > 0.05$ ; PB: SL/SL = 163.8 ± 45.0%, HA/HA = 181.1 ± 51.1%,  $p > 0.05$ ) (Fig. 1C).

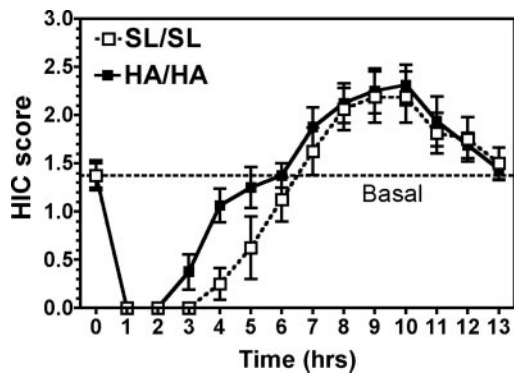
**Acute Withdrawal.** HICs were scored before and after injection of ethanol. The initial effect of ethanol is to eliminate the HIC, which returns to preinjection baseline after approximately 6 h, and then increases above baseline during h 6 to 10. The increase above baseline is a measure of acute withdrawal and was not different for the SL/SL and HA/HA mice (areas under the curve but above basal response 3.5 ± 0.8 and 4.3 ± 1.2, respectively) (Fig. 2). However, the recovery of HIC appeared to be somewhat more rapid for the HA/HA mice (Fig. 2), although the area below baseline was not significantly different for the two genotypes (6.3 ± 0.6 and 4.5 ± 0.8, respectively,  $p = 0.08$ ).

**Motor Coordination on the Rotorod.** Because of the suggestion from the HIC scores that the HA/HA mice might recover more quickly from the sedative/anticonvulsant actions of alcohol, motor coordination was measured at different times after injection of 2.0 g/kg ethanol using the fixed-speed rotorod. There were significant main effects of genotype ( $F_{1,95} = 28.35$ ,  $p < 0.0001$ ), time ( $F_{5,95} = 128.12$ ,  $p < 0.0001$ ), and interaction of genotype × time ( $F_{5,95} = 11.71$ ,  $p < 0.0001$ ). This indicated HA/HA mice recovered more quickly from ethanol's motor-impairing effects compared with SL/SL mice (Fig. 3A).

To determine whether this rapid recovery also occurred with other sedative drugs, etomidate (20 mg/kg) and pentobarbital (35 mg/kg) were also tested. After etomidate administration, there were significant main effects of genotype ( $F_{1,300} = 10.96$ ,  $p < 0.01$ ), time ( $F_{25,300} = 31.65$ ,  $p < 0.0001$ ), and interaction of genotype × time ( $F_{25,300} = 3.93$ ,  $p < 0.0001$ ). This analysis indicated that the knockin mutations also markedly increased the recovery of motor coordination after etomidate (Fig. 3B). Following pentobarbital administration, there was a significant effect of time ( $F_{6,108} = 167$ ,  $p < 0.0001$ ), but genotype and the interaction of genotype ×



**Fig. 1.** Pharmacological characterization of GABA<sub>A</sub> mIPSCs recorded from SL/SL and HA/HA CA1 pyramidal neurons. **A**, bar graph summarizing the effect of 20, 40, and 80 mM ethanol on the area of pharmacologically isolated GABA<sub>A</sub> IPSCs recorded from SL/SL and HA/HA CA1 pyramidal neurons.  $n = 12$ –38 neurons/group. \*,  $p < 0.05$ , significant difference relative to control; #,  $p < 0.01$ , significant difference between SL/SL and HA/HA; NS, no significant difference. **B**, summary time courses of the effect of an 8-min bath application of 80 mM ethanol on the area of evoked GABA<sub>A</sub> IPSCs recorded from SL/SL ( $n = 38$ ) and HA/HA ( $n = 34$ ) CA1 pyramidal neurons. Traces above the graph are averages of 8 to 10 IPSCs recorded from a representative SL/SL and HA/HA neuron prior to, during, and 10 min after 80 mM ethanol treatment. **C**, bar graph summarizing the effect of 1 μM FLU and 50 μM PB on the area of GABA<sub>A</sub> IPSCs recorded from SL/SL and HA/HA CA1 pyramidal neurons.  $n = 5$ –7 neurons/group. \*,  $p < 0.05$ , significant difference relative to control; NS, no significant difference between SL/SL and HA/HA.



**Fig. 2.** Severity of acute ethanol withdrawal using HICs. Mice were injected with 4 g/kg ethanol at time 0, and handling-induced convulsions were scored hourly for 13 h. The increase above basal levels is a measure of acute alcohol withdrawal and showed a similar withdrawal severity for SL/SL and HA/HA mice. Values are mean  $\pm$  S.E.M.,  $n = 8$  per genotype.

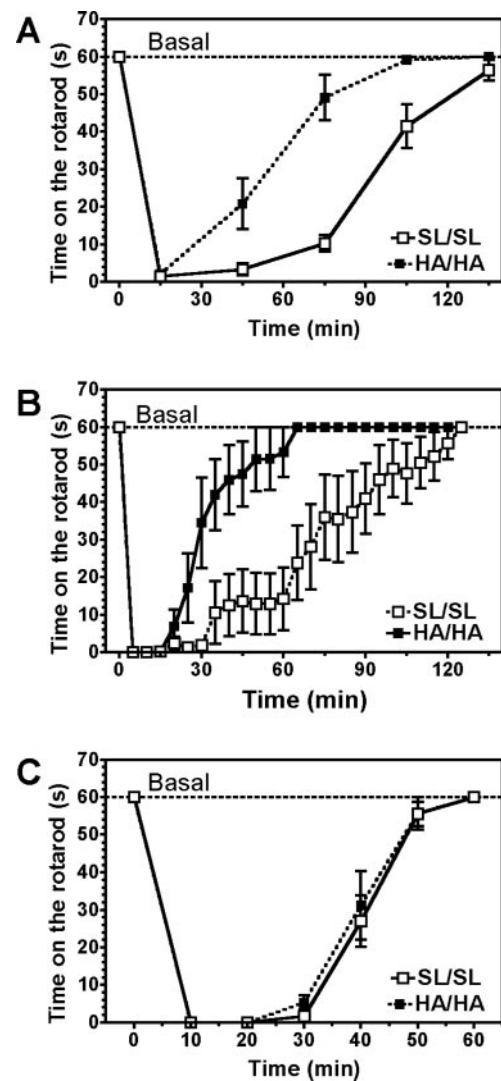
time were not significant. Thus, the knockin mutations did not affect recovery from pentobarbital-induced motor ataxia.

**Loss of Righting Reflex.** The LORR assay was used to examine the hypnotic effects of ethanol and etomidate. For ethanol, there were no significant differences between genotypes (Fig. 4A), but for etomidate, the knockin mice displayed a shorter duration of LORR than the wild type (Fig. 4, B and C). It should be noted that etomidate gave somewhat variable LORR values, and these experiments were independently conducted in two different laboratories using a large number of mice ( $n = 17$ – $31$ ) to assure the validity of this conclusion.

**Voluntary Consumption of Ethanol, Saccharin, and Quinine.** The consumption of ethanol, saccharin, or quinine solutions, as well as total fluid intake, was measured using the two-bottle choice technique with several different concentrations of ethanol, saccharin, or quinine.

For ethanol consumption, there was a significant main effect of concentration ( $F_{3,51} = 15.96$ ,  $p < 0.0001$ ) but no effect of genotype or the interaction of genotype  $\times$  concentration (Fig. 5A). Analysis of total fluid intake (ethanol + water) indicated a main effect of concentration ( $F_{3,51} = 4.55$ ,  $p < 0.01$ ) and genotype ( $F_{1,51} = 18.47$ ,  $p < 0.001$ ) but no interaction of genotype  $\times$  concentration (Fig. 5B). Subsequent pairwise comparisons indicate HA/HA mice had an increased total fluid intake at 6% (v/v) ethanol ( $p < 0.05$ ). Analysis of ethanol preference revealed no effect of concentration, genotype, or interaction of concentration  $\times$  genotype (Fig. 5C). Overall, these results indicate that although genotypes do not differ in preference for ethanol, there is an increase in total fluid intake in HA/HA mice.

Analysis of preference for saccharin indicated a significant main effect of concentration ( $F_{1,17} = 35.20$ ,  $p < 0.0001$ ) but no effect of genotype or interaction of genotype  $\times$  concentration (Fig. 6A). Analysis of total fluid intake for this experiment revealed significant main effects of genotype ( $F_{1,17} = 5.34$ ,  $p < 0.05$ ), concentration ( $F_{1,17} = 18.88$ ,  $p < 0.005$ ), but no interaction of genotype  $\times$  concentration (Fig. 6B). Subsequent pairwise comparisons revealed no differences with respect to genotype. Analysis of avoidance of quinine solutions revealed no effect of concentration, genotype, or interaction of concentration  $\times$  genotype (Fig. 6C). Analysis of total fluid intake for this experiment indicated a significant main effect of genotype ( $F_{1,17} = 12.38$ ,  $p < 0.005$ ) but no effect of concentration or interaction of genotype  $\times$  concentration

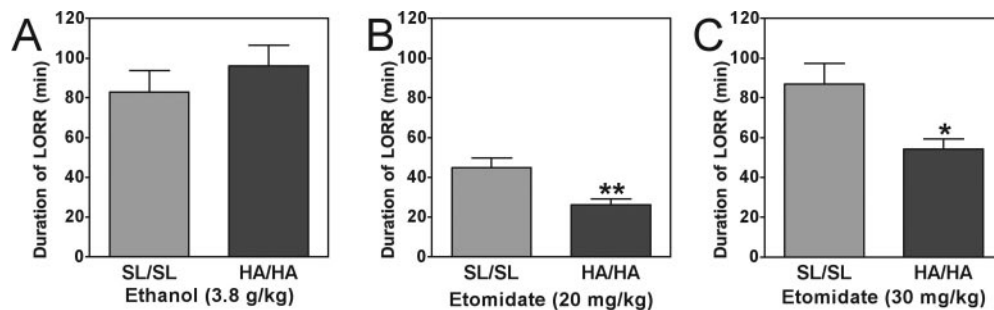


**Fig. 3.** Effects of ethanol, etomidate, and pentobarbital on motor coordination using rotarod. Mice were injected with 2 g/kg ethanol (A), 20 mg/kg etomidate (B), or 35 mg/kg sodium pentobarbital (C), and the ability to balance on a rotarod was measured at 30-, 5-, and 10-min intervals, respectively. HA/HA mice recovered motor coordination more rapidly than SL/SL mice after ethanol ( $p < 0.0001$ ) and etomidate ( $p < 0.01$ ) administration, but the lines did not differ for pentobarbital. Values are mean  $\pm$  S.E.M.,  $n = 7$ – $11$  per genotype. Male and female mice were used for pentobarbital experiments, but no gender differences were observed.

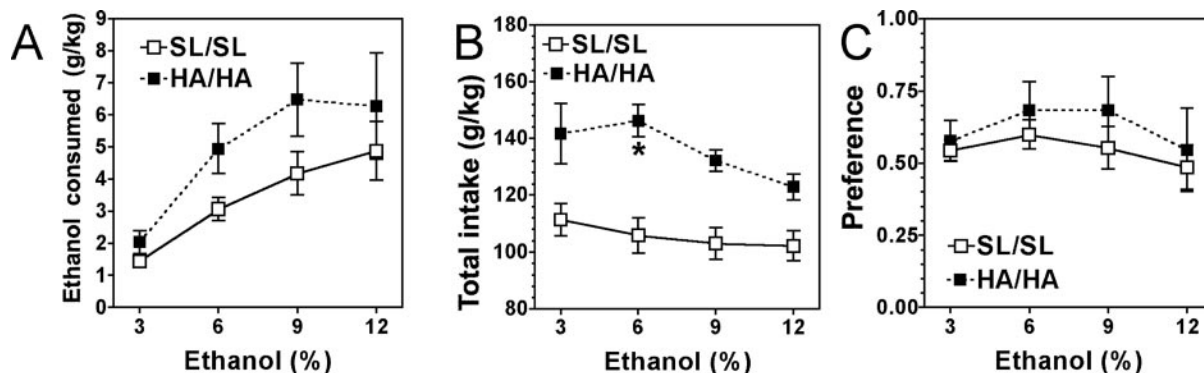
(Fig. 6D). Subsequent pairwise comparisons indicate HA/HA mice had an increased total fluid intake at 0.06 mM quinine ( $p < 0.05$ ). Overall, the differences with respect to genotype for total fluid intake for saccharin and quinine also indicate knockin mice have increased fluid consumption.

**Hypothermia.** We next asked whether genotypes differed in ethanol-induced decreases in body temperature. Treatment with ethanol (2 or 3 g/kg) reduced body temperature (Fig. 7) at all of the time points measured compared with preinjection baseline. Analysis indicated a significant main effect of dose ( $F_{1,40} = 29$ ,  $p < 0.0001$ ), time ( $F_{6,40} = 211$ ,  $p < 0.0001$ ), and interaction of dose  $\times$  time ( $F_{6,40} = 9$ ,  $p < 0.0001$ ). No difference was observed with respect to genotype.

**Morris Water Maze.** Mice were tested for ethanol-induced impairment of cognition using a spatial memory task, the Morris water maze. Analysis indicated a significant main effect of genotype ( $F_{1,36} = 7.46$ ,  $p < 0.025$ ) and trial (last day



**Fig. 4.** Loss of righting reflex produced by ethanol or etomidate. Mice were injected i.p. with 3.8 g/kg ethanol (A) or with 20 (B) or 30 (C) mg/kg etomidate, and the duration of LORR was measured. The HA/HA mice did not differ from SL/SL mice for ethanol-induced LORR ( $n = 6-7$ ), but the etomidate LORR was significantly shorter for HA/HA than for SL/SL mice ( $p < 0.005$ ,  $n = 24-31$  for 20 mg/kg;  $p < 0.01$ ,  $n = 17-18$  for 30 mg/kg). Values are mean  $\pm$  S.E.M. Male and female mice were used for etomidate experiments, but no gender differences were observed.



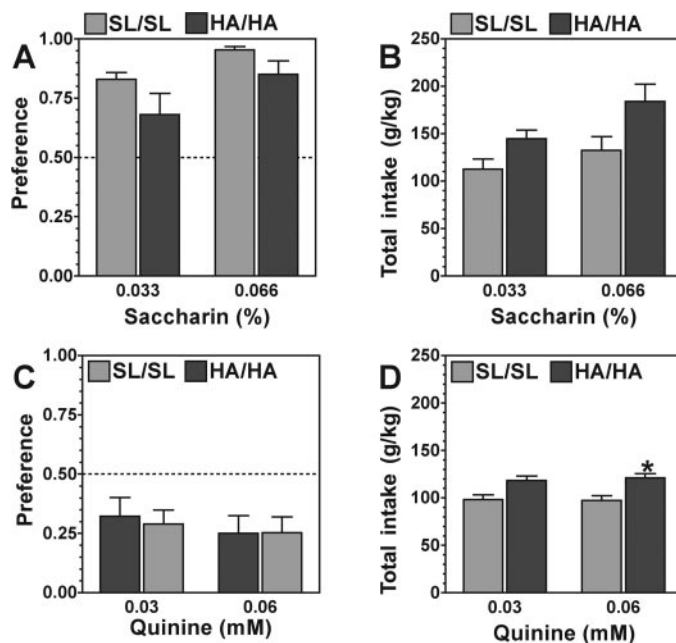
**Fig. 5.** Voluntary consumption of ethanol and water. The two-bottle choice test was used to measure voluntary consumption of ethanol solutions and water. HA/HA mice showed increased total fluid (alcohol + water) consumption at 6% (v/v) ethanol (B) compared with SL/SL mice, but alcohol consumption (A) and preference (C) were not different between HA/HA and SL/SL mice. Values are mean  $\pm$  S.E.M.,  $n = 9-10$ . \*,  $p < 0.05$ .

of training versus testing under ethanol,  $F_{1,36} = 36.98$ ,  $p < 0.0001$ ) but no effect of the interaction of genotype  $\times$  trial. Subsequent pair-wise comparisons indicated acute ethanol administration impaired spatial memory (Fig. 8,  $p < 0.001$ ), but the impairment was not dependent upon genotype.

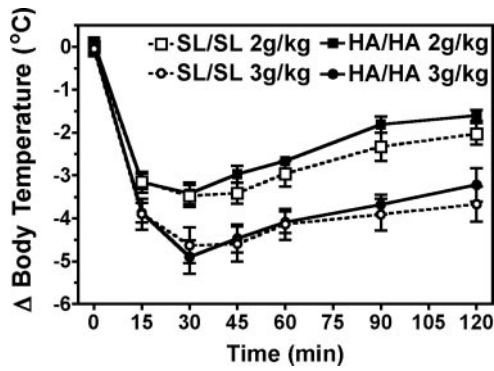
**Elevated Plus Maze.** Locomotor activity was assessed by total number of entries, whereas anxiety was measured by percentage of time spent in open arm entries and percentage of open arm entries. Basal performance on the elevated plus maze (following saline injection) did not differ with respect to genotype for these measures (Fig. 9, A–C). Thus, locomotor activity and indicators of anxiety-like behavior were not altered by the mutations in the GABA<sub>A</sub>-R  $\alpha 1$  subunit.

Using total number of arm entries to assess the effect of ethanol on locomotor activity, we observed significant main effects of dose and genotype (Fig. 9A), as assessed by ANOVA (dose  $F_{3,77} = 6.7$ ,  $p < 0.001$ ; genotype  $F_{1,79} = 4.3$ ,  $p < 0.05$ ), after initial analysis using two-way ANOVA indicated no interaction. Subsequent pair-wise comparisons revealed a stimulating effect of ethanol in both genotypes at the lowest dose of ethanol tested (0.75 g/kg). Interestingly, HA/HA mice were more sensitive to the locomotor stimulant effect of ethanol at 1 g/kg compared with wild-type mice ( $p < 0.01$ ). Ethanol also had a stimulant effect at 1.5 g/kg in HA/HA mice compared with saline, whereas no effect was observed in wild-type mice compared with saline controls.

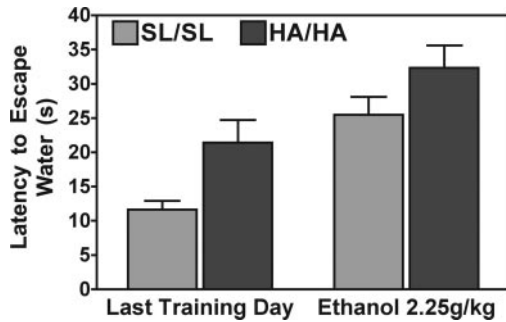
With respect to the anxiolytic effects of ethanol, ethanol increased the percentage of open arm entries relative to total number of entries (Fig. 9B) and percentage of time spent in open arms relative to the total time (Fig. 9C). Two-way



**Fig. 6.** Voluntary consumption of saccharin and quinine. The two-bottle choice test was used to measure voluntary consumption of two different concentrations of saccharin or quinine solutions versus water. There were no differences between genotypes in preference for saccharin (A) or avoidance of quinine (C). Main effects of genotype were observed for both saccharin (B,  $p < 0.05$ ) and quinine (D,  $p < 0.005$ ) in total fluid intake. Pair-wise comparisons indicate no difference in genotype at individual concentrations of saccharin (B), but HA/HA mice had increased total fluid intake (quinine + water) at 0.06 mM quinine compared with SL/SL mice (D). Values are mean  $\pm$  S.E.M.,  $n = 9-10$ . \*,  $p < 0.05$ .



**Fig. 7.** Ethanol-induced hypothermia. Ethanol (2 or 3 g/kg) caused a dose-dependent decrease in body temperature ( $p < 0.0001$ ) that was similar for both genotypes. Values are mean  $\pm$  S.E.M.,  $n = 10$ –11 per genotype per dose. Male and female mice were used, but no gender differences were observed.

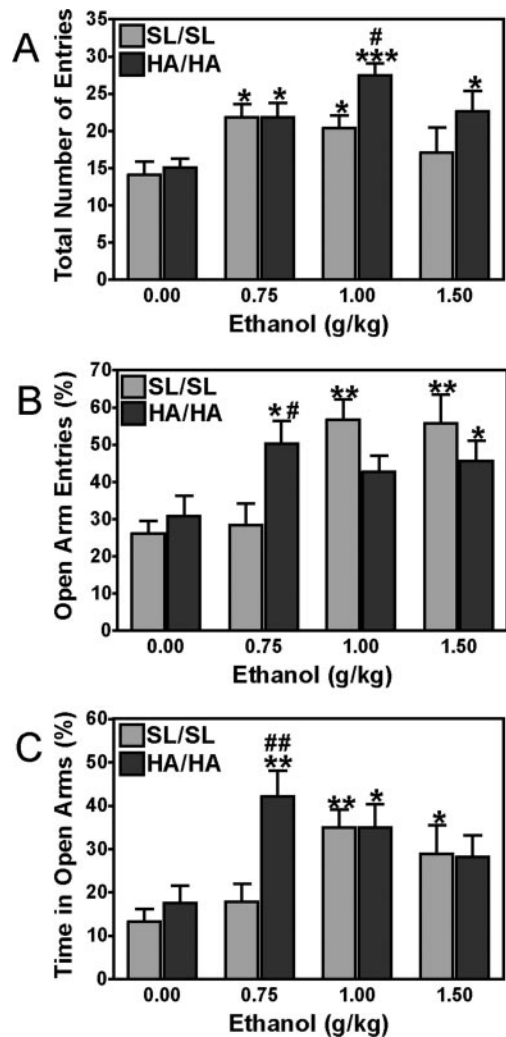


**Fig. 8.** Effect of ethanol on spatial memory. Latency (seconds) for mice to escape the water on the last training day and the effects of ethanol impairment. Acute ethanol administration significantly impaired spatial memory ( $p < 0.001$ ), but the impairment was not dependent upon genotype. Data represent mean  $\pm$  S.E.M.,  $n = 26$  SL/SL mice and 12 HA/HA mice. Male and female mice were used, but no gender differences were observed.

ANOVA indicated a significant main effect of dose ( $F_{3,73} = 7.0$ ;  $p < 0.001$ ) and interaction of genotype  $\times$  dose ( $F_{3,73} = 4.3$ ,  $p < 0.01$ ) but no main effect of genotype in the percentage of open arm entries. For percent time spent in open arms, the statistical analysis indicated a significant effect of dose ( $F_{3,73} = 6.0$ ,  $p < 0.01$ ), genotype ( $F_{3,73} = 4.0$ ,  $p < 0.05$ ), and interaction of genotype  $\times$  dose ( $F_{3,73} = 2.9$ ,  $p < 0.05$ ).

Because the anxiolytic effects observed at 1.0 and 1.5 g/kg may be potentially complicated by the genotypic differences observed in total arm entries, we limited our analysis and interpretation to the 0.75 g/kg dose. At this dose, ethanol decreased anxiety-like behavior in knockin mice but not in controls. This is evidenced by the increase in percent open arm entries in HA/HA mice (Fig. 9B,  $p < 0.05$ ) and percent time in open arms (Fig. 9C,  $p < 0.01$ ). Therefore, HA/HA mice appear to be more sensitive to the anxiolytic effects of ethanol.

**Metabolism and Clearance.** To determine whether ethanol pharmacokinetics differed between wild-type and knockin mice, BECs were measured every 30 min for 2 h following injection of 3.5 g/kg i.p. ethanol BECs (e.g., at 30 min postinjection,  $415 \pm 17$  mg/dl for SL/SL;  $414 \pm 13$  mg/dl for HA/HA) and clearance (SL/SL,  $1.13 \pm 0.22$  mg/dl/min,  $n = 5$ ; HA/HA,  $1.22 \pm 0.19$  mg/dl/min,  $n = 6$ ) did not differ with respect to genotype, thus allowing valid comparisons between genotypes for ethanol-related behaviors. Clearance



**Fig. 9.** Evaluation of anxiety and activity using the elevated plus maze. Total arm entries (A), percentage of open arm entries (B), and percent total time spent in open arms (C) are shown. Saline or ethanol was administered 10 min prior to testing. The locomotor stimulant effect was dependent on ethanol dose and genotype. The anxiolytic effect of ethanol was detected in both genotypes; ethanol increased the percentage of open arm entries relative to total number of entries and percentage of time spent in open arms relative to the total time. HA/HA mice had increased responses compared with SL/SL mice at specific doses (0.75 for anxiolytic effect; 1.0 for locomotor-stimulant effect). Data represent mean  $\pm$  S.E.M.,  $n = 10$ –11 per group per genotype. Male and female mice were used for experiments. No effect of gender was observed. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$  represent comparison with the saline groups (within genotype). #,  $p < 0.05$ ; and ##,  $p < 0.01$  represent differences between genotypes at each ethanol dose.

rates are similar to previously published rates for mice with a similar genetic background (Mihalek et al., 2001).

## Discussion

In this study, we used mice in which double-mutant  $\alpha 1$  subunits of GABA<sub>A</sub>-Rs replaced the wild-type subunit (knockin mice). We chose mutations that render the receptor resistant to ethanol (and also to isoflurane) but provide a near normal response to GABA and normal modulation by flunitrazepam (Borghese et al., 2006b). This allowed us to test the importance of  $\alpha 1$ -containing GABA<sub>A</sub>-Rs to specific cellular and behavioral responses to ethanol.

We first determined the effects of the knockin mutations on

synaptic sensitivity to ethanol using hippocampal electrophysiological analysis. We recorded from proximal GABAergic synapses onto CA1 pyramidal neurons because these synapses are thought to express high levels of the  $\alpha 1$  subunit (Nusser et al., 1996), and the ethanol sensitivity of these synapses has been extensively characterized (for review, see Siggins et al., 2005; Weiner and Valenzuela, 2006). Interestingly, the knockin mutation did not alter the potentiating effect of ethanol on evoked IPSCs at low concentrations (20 or 40 mM) but markedly attenuated the effect at a relatively high ethanol concentration (80 mM). These data suggest that, at least in the hippocampal CA1 region, ethanol facilitation of GABAergic synapses at low concentrations is mediated primarily by mechanisms that do not require the  $\alpha 1$  subunit (i.e., facilitation of GABA release, for review, see Siggins et al., 2005; Weiner and Valenzuela, 2006) but that a direct interaction with postsynaptic GABA<sub>A</sub>-Rs contributes to the potentiation observed at higher ethanol concentrations.

We also carried out a limited characterization of some of the other pharmacological properties of CA1 GABA<sub>A</sub> IPSCs from wild-type and knockin mice. Importantly, flunitrazepam and pentobarbital potentiation were not affected by the double mutation in the  $\alpha 1$  subunit, suggesting that the mutations did not alter other pharmacological properties of  $\alpha 1$ -containing GABA<sub>A</sub>-Rs in hippocampal synapses. These findings are not entirely consistent with the results of similar experiments in recombinant receptors (Borghese et al., 2006b): in  $\alpha 1\beta 2/3\gamma 2s$  GABA<sub>A</sub>-Rs expressed in *Xenopus* oocytes, flunitrazepam potentiation was not modified in mutated  $\alpha 1$ -containing GABA<sub>A</sub>-Rs, but pentobarbital potentiation was decreased by approximately 30%. However, it should be emphasized that only a single concentration of each drug was tested in the initial studies reported here. A more thorough analysis of the concentration dependence of the effects of benzodiazepines, barbiturates, and other subunit-selective modulators will be needed to confirm that the mutations only disrupted the pharmacological effects of ethanol on synaptic GABA<sub>A</sub>-Rs. Moreover, even though  $\alpha 1$  may be present in high levels in these hippocampal synapses, other  $\alpha$  subunits present could participate and result in a normal response to pentobarbital. In addition, the mutation did not change paired-pulse facilitation or paired-pulse depression (data not shown), suggesting that the  $\alpha 1$  HA/HA knockin did not result in dramatic compensatory alterations in the presynaptic properties of these GABAergic synapses.

Behaviorally, knockin mice displayed a faster recovery from the ataxic effects of ethanol or etomidate than wild-type mice, but the recovery from pentobarbital ataxia was not affected by the mutation. Ataxia is due, at least in part, to drug effects on cerebellar function, and it is important to note that normal  $\alpha 1$  protein levels were observed in the cerebellum of mutant animals (Borghese et al., 2006b). Knockin mice also displayed decreased behavioral responses to the hypnotic actions of etomidate, but hypnotic effects of ethanol were normal. The decreased behavioral sensitivity to etomidate is consistent with previously observed electrophysiological recordings (Borghese et al., 2006b). The discrepancy in the responses to etomidate and ethanol may be due to the fact that etomidate has a more selective mechanism of action than ethanol. A single mutation in the  $\beta 3$  subunit of the GABA<sub>A</sub>-R markedly reduced the LORR duration after etomi-

date administration (Jurd et al., 2003), whereas the ethanol effects are likely determined by its action on multiple targets, including non-GABA<sub>A</sub>-R targets (Markel et al., 1996; Crabbe et al., 2006).

There were no differences between genotypes in preference toward ethanol, saccharine, and quinine. However, knockin mice showed an increased total consumption of fluids in each of these tests. The GABA<sub>A</sub>-R has been strongly implicated in appetitive behaviors: administration of positive modulators such as benzodiazepines induces hyperdipsia and hyperphagia, whereas inverse agonists of the benzodiazepine site induce hypophagia, possibly through the modulation of palatability (Cooper, 2005). This would suggest that GABAergic transmission in the knockin mice is enhanced in the centers that control appetitive behaviors, probably due to compensatory changes, since the  $\alpha 1$ -containing GABA<sub>A</sub>-Rs do not seem to mediate the hyperphagic effect of benzodiazepines (Cooper, 2005).

In contrast, knockin mice were observed to be more sensitive to the anxiolytic effects of ethanol as tested on the elevated plus maze assay. This effect was dependent on dose and was detected in both measurements of anxiety-like behavior at an ethanol dose of 0.75 g/kg. If  $\alpha 1$ -containing receptors were responsible for this effect of ethanol, then the knockin mice would be predicted to be less sensitive, not more sensitive. However, the anxiolytic effects of benzodiazepines have been linked to GABA<sub>A</sub>-Rs containing the  $\alpha 2$  and  $\alpha 3$  subunits, not the  $\alpha 1$  subunit (Atack, 2005). We found that the  $\alpha 3$  and possibly  $\alpha 2$  subunits are increased in the forebrain of these knockin mice (Borghese et al., 2006b). Therefore, we posit that increased expression of other ethanol-sensitive  $\alpha$  subunits may contribute to the observed phenotype and may represent molecular targets for the anxiolytic effects of ethanol.

Comparing these results with studies of the  $\alpha 1$  null mutant (knockout) mice (Blednov et al., 2003a,b; Kralic et al., 2003, 2005), the knockout mice showed differences in more behavioral effects of ethanol, including consumption, tremor suppression, acute withdrawal, and hypnotic effects. However, the knockout mice displayed substantial and widespread compensatory changes in synaptic physiology, gene expression, and GABAergic circuitry (Kralic et al., 2006; Ponomarev et al., 2006), and it is likely that these secondary changes, in response to deletion of a large fraction of brain GABA<sub>A</sub>-Rs, contribute to phenotypes seen in the knockout, but not the knockin mice.

It is somewhat surprising that the behavioral actions of ethanol that are changed by the knockin mutation are observed with moderate doses (e.g., 1 g/kg) of ethanol, whereas the electrophysiological differences were only seen with high concentrations of ethanol. It is possible that the electrophysiological methods used in this study were not sensitive enough to detect differences in the effects at low concentrations of ethanol. For example, analysis of ethanol effects on mIPSC frequency and kinetics in SL/SL and HA/HA mice may help to clarify the relative contribution of pre- and postsynaptic mechanisms to ethanol's overall facilitatory effects on GABAergic synapses in these mice. Alternatively, GABA<sub>A</sub>-Rs that do not contain the  $\alpha 1$  subunit may mediate the effects of low concentrations of ethanol via extrasynaptic GABA<sub>A</sub>-Rs (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Hancher et al., 2005), although these low-dose effects

have not been observed in all studies (Borghese et al., 2006a). Lastly, because ethanol is known to affect multiple systems in the brain, it is possible that non-GABAergic systems may be responsible for certain behavioral effects or the combined effect of ethanol on multiple systems. Multiple genes mediate each of ethanol's effects (Crabbe et al., 2006), and modifying one of them may not be sufficient to alter the behavioral endpoint.

In summary, we demonstrated that incorporation of two specific mutations into the  $\alpha 1$  subunit that had been shown to abolish GABA<sub>A</sub>-R sensitivity to ethanol in vitro significantly decreased ethanol-induced potentiation of synaptic responses at high ethanol concentrations in vitro. Moreover, using these knockin animals, we also demonstrated that  $\alpha 1$ -containing GABA<sub>A</sub>-Rs may play a role in recovery from ethanol's ataxic effects, indicating that  $\alpha 1$ -containing GABA<sub>A</sub>-Rs are important for the mediation of specific ethanol-induced behavioral responses.

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