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Behavioral Characterization of Knockin Mice with Mutations M287L and Q266I in the Glycine Receptor α1 Subunit

Yuri A. Blednov, Jill M. Benavidez, Gregg E. Homanics, and R. Adron Harris

Waggoner Center for Alcohol and Addiction Research, University of Texas, Austin, Texas (Y.A.B., J.M.B., R.A.H.); and Departments of Anesthesiology and Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania (G.E.H.)

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ABSTRACT

We used behavioral pharmacology to characterize heterozygous knockin mice with mutations (Q266I or M287L) in the α1 subunit of the glycine receptor (GlyR) (*J Pharmacol Exp Ther* **340:**304–316, 2012). These mutations were designed to reduce (M287L) or eliminate (Q266I) ethanol potentiation of GlyR function. We asked which behavioral effects of ethanol would be reduced more in the Q266I mutant than the M287L and found rotarod ataxia to be the behavior that fulfilled this criterion. Compared with controls, the mutant mice also differed in ethanol consumption, ethanol-stimulated startle response, signs of acute physical dependence, and duration of loss of righting response produced by ethanol, butanol, ketamine, pentobarbital, and flurazepam. Some of these behavioral changes were mimicked in wild-type mice by acute injections of low, subconvulsive doses of strychnine. Both mutants showed increased acoustic startle response and increased sensitivity to strychnine seizures. Thus, in addition to reducing ethanol action on the GlyRs, these mutations reduced glycinergic inhibition, which may also alter sensitivity to GABAergic drugs.

Introduction

Glycine receptors (GlyRs) are the primary mediators of inhibitory neurotransmission in the brain stem and spinal cord (Legendre, 2001), but GlyRs are also found in a number of higher brain regions, including the hippocampus, nucleus accumbens (NAcc) (Molander and Soderpalm, 2005; Hernandes and Troncone, 2009), cerebellum (Takahashi et al., 1992), and olfactory bulb (van den Pol and Gorcs, 1988). GlyR function is potentiated in vitro by ethanol, longer chain alcohols, and volatile anesthetics (Celentano et al., 1988; Aguayo and Pancetti, 1994; Mascia et al., 1996; Eggers et al., 2000; Yamakura and Harris, 2000). Behavioral tests also suggest involvement of the GlyR with the acute effects of ethanol. Glycine and the glycine precursor, serine, can enhance the ethanol-induced loss of righting reflex (LORR) in mice, and this effect can be blocked by strychnine (Williams et al., 1995). Ethanol also inhibits strychnine seizures in mice (McSwigan et al., 1984). GlyRs expressed in adult rat amygdala modulate amygdala-associated anxiety-like behavior in a manner dependent on the basal "emotional state" of the animal (McCool and Chappell, 2007). In addition, GlyRs in the NAcc may regulate voluntary consumption of ethanol (Molander et al., 2005). In line with these findings, the glycine reuptake inhibitor *cis*-*N*-methyl-*N*-(6-methoxy-1-phenyl-1,2,3,4-tetrahydronaphthalen-2-ylmethyl)aminomethylcarboxylic acid hydrochloride (Org 25935) decreased ethanol, but not water, intake as well as ethanol preference (Molander et al., 2007) and reduced "relapse-like" drinking (Vengeliene et al., 2010). Transgenic expression of S267Q mutant GlyR α1 subunits carrying an ethanol-resistant S267Q point mutation led to the reduction of sensitivity to ethanol in three behavioral tests: ethanol inhibition of strychnine seizures, motor incoordination (rotarod), and LORR (Findlay et al., 2002).

Our recent studies of alcohol actions on recombinant GlyRs (Borghese et al., 2012) showed that mutations M287L in the transmembrane 3 region and Q266I in the transmembrane 2

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ABBREVIATIONS: GlyR, glycine receptor; BEC, blood ethanol concentration; CTA, conditioned taste aversion; EtOH, ethanol; HIC, handling-induced convulsion; KI, knockin; LORR, loss of righting reflex; NAcc, nucleus accumbens; nAChR, nicotinic acetylcholine receptor, NMDA, *N*-methyl-D-aspartic acid; PTZ, pentylenetetrazole; Org 25935, *cis*-*N*-methyl-*N*-(6-methoxy-1-phenyl-1,2,3,4-tetrahydronaphthalen-2-ylmethyl) aminomethylcarboxylic acid hydrochloride.

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region of the α1 subunit lead to a reduction in ethanol potentiation of glycine-induced current. Construction of knockin (KI) mice with each of these mutations allowed behavioral testing to determine the influence of these changes on behavioral effects of ethanol and other drugs.

Materials and Methods

Mouse Breeding and Genotyping. Two separate gene-targeting experiments were conducted to create two independent GlyR α1 subunit KI mouse lines harboring mutations that changed glutamine at position 266 to isoleucine (Q266I KI) or methionine at position 287 to leucine (M287L KI) (Borghese et al., 2012). All mice were derived from a 129S1/X1 × C57BL/6J hybrid genetic background that was subsequently backcrossed to C57BL/6J for two generations at the University of Pittsburgh (Pittsburgh, PA). Breeding pairs of mice from this generation of backcrossing were sent to the University of Texas (Austin, TX). Wild-type mice ($\alpha1^{Q/Q}$ and $\alpha1^{M/M}$) and KI mice (heterozygous, $\alpha1^{Q/I}$ and $\alpha1^{M/L}$) used for these experiments were produced at the University of Texas from heterozygous ($\alpha1^{Q/I}$ and $\alpha1^{M/L}$) and correspondent wild-type ($\alpha1^{Q/Q}$ and $\alpha1^{M/M}$) breeding pairs. After weaning, mice were housed 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). Both male and female mice between 8 and 12 weeks of age were used for behavioral experiments. 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 the Institutional Animal Care and Use Committees at each university and conducted in accordance with National Institutes of Health guidelines with regard to the use of animals in research.

Alcohol Preference Drinking, 24-H 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 (gram per kilogram of body weight per 24 h) was calculated for each mouse, and these values were averaged for every concentration of ethanol. Immediately after 3% ethanol, a choice between 6% (v/v) ethanol and water was offered for 4 days, then 9% (v/v) ethanol for 4 days, 12% (v/v) ethanol for 4 days, 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-H Access. Wild-type or KI 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 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.

Loss of Righting Reflex. Sensitivity to depressant effects of ethanol (3.4 or 3.8 g/kg) and other drugs such as flurazepam (225 mg/kg), pentobarbital (50 mg/kg), *n*-butanol (1 g/kg), and ketamine (175 mg/kg) was determined by using the standard duration of LORR (sleep time) assay in mice. When mice 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. Sleep time was

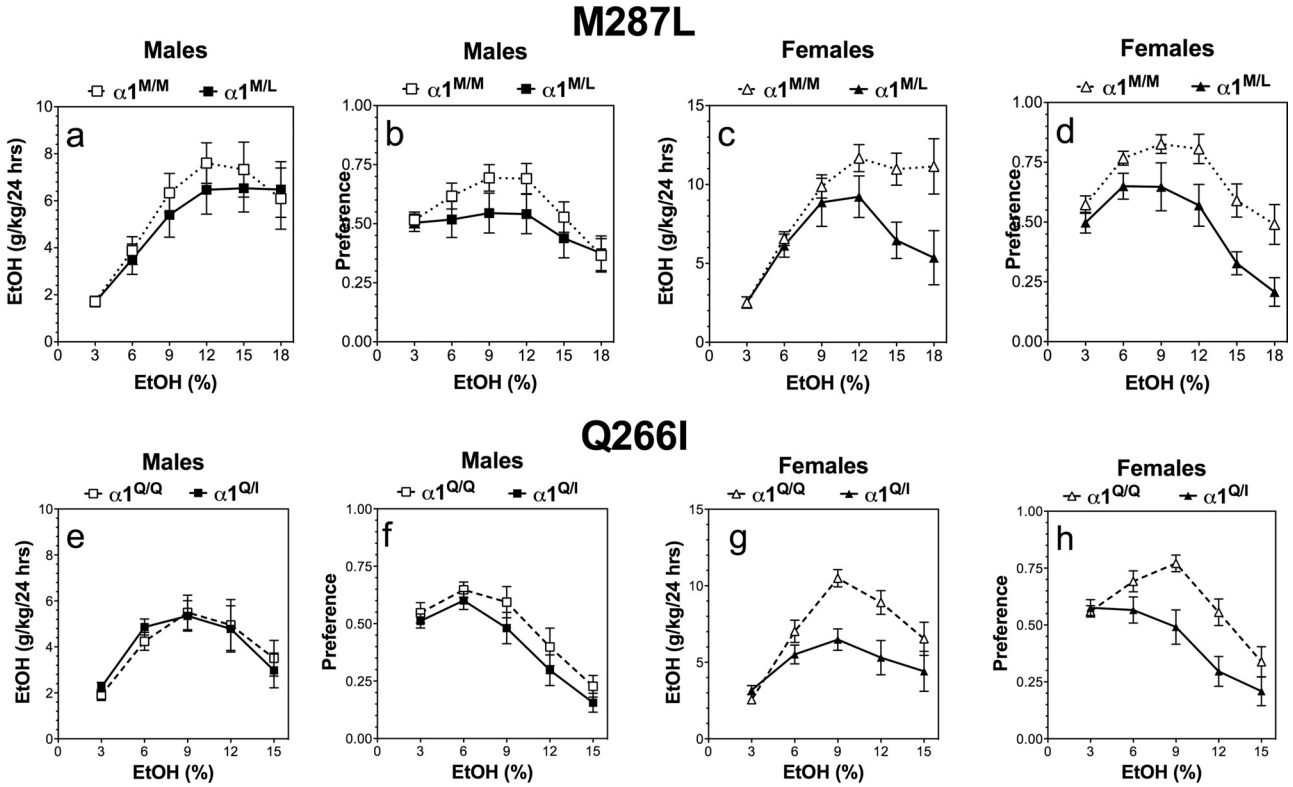

Fig. 1. Decreased ethanol preference and intake in M287L and Q266I KI mice depends on sex. a to d, M287L KI mice. Ethanol consumption (g/kg/24 h) in males (n = 10 per genotype) (a) and females (n = 8–10 per genotype) (c). Ethanol preference in males (b) and females (d). e to h, Q266I KI mice. Ethanol consumption (g/kg/24 h) in males (n = 10 per genotype) (e) and females (n = 10 per genotype) (g). Ethanol preference in males (f) and females (h). See *Ethanol Consumption* for statistical analysis. Values represent mean ± S.E.M. for 4 days of consumption.

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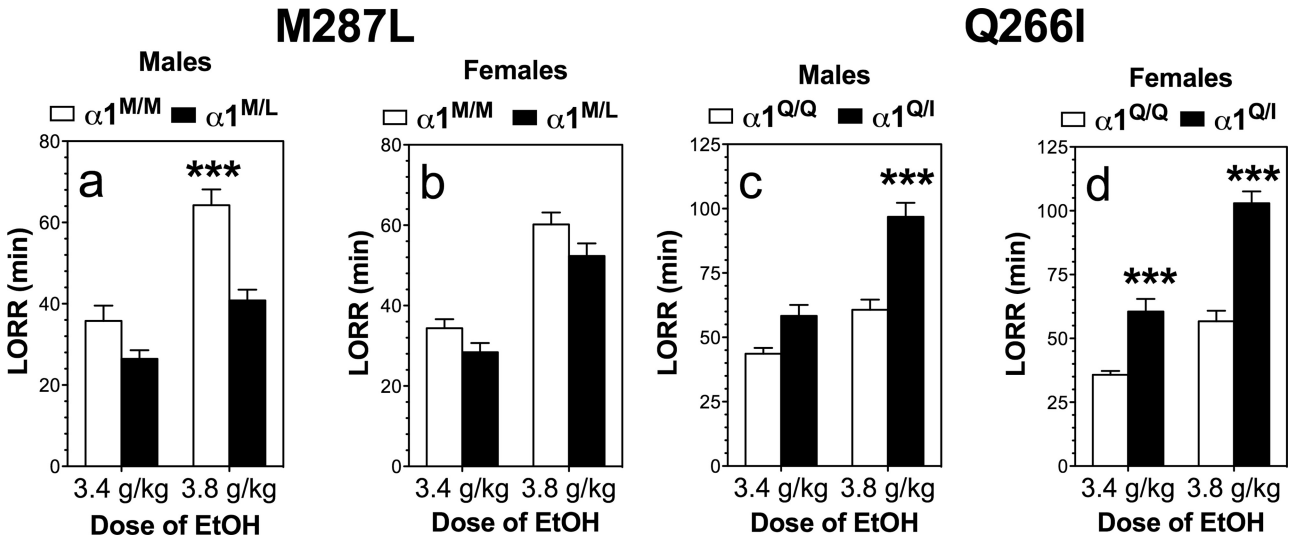

Fig. 2. M287L mutation reduced and Q266I increased the duration of ethanol-induced LORR. a and b, M287L KI mice: duration of LORR (min) in males (n = 9–11 per dose and genotype) (a) and females (n = 8–17 per genotype) (b). c and d, Q266I KI mice: duration of LORR (min) in males (n = 8–10 per dose and genotype) (c) and females (n = 8–10 per genotype) (d). ***, $P < 0.001$, significant difference relative to wild-type mice for the same dose of ethanol (Bonferroni post-test). Values represent mean ± S.E.M.

defined as the time the mice were placed in the supine position until they regained their righting reflex.

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 of continuous access to water at standard times in the morning (days 2, 4, 6, 8, and 10). Reduced consumption of the saccharin solution was used as a measure of CTA.

Ethanol-Induced Acute Withdrawal. Mice were scored for handling-induced convulsion (HIC) severity 30 min before and immediately before intraperitoneal ethanol administration. The two predrug baseline scores were averaged. A dose of 4 g/kg ethanol in saline was injected intraperitoneally, and the HIC score was tested every hour until the HIC level reached baseline. Acute withdrawal was quantified as the area under the curve but above the predrug baseline level (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,

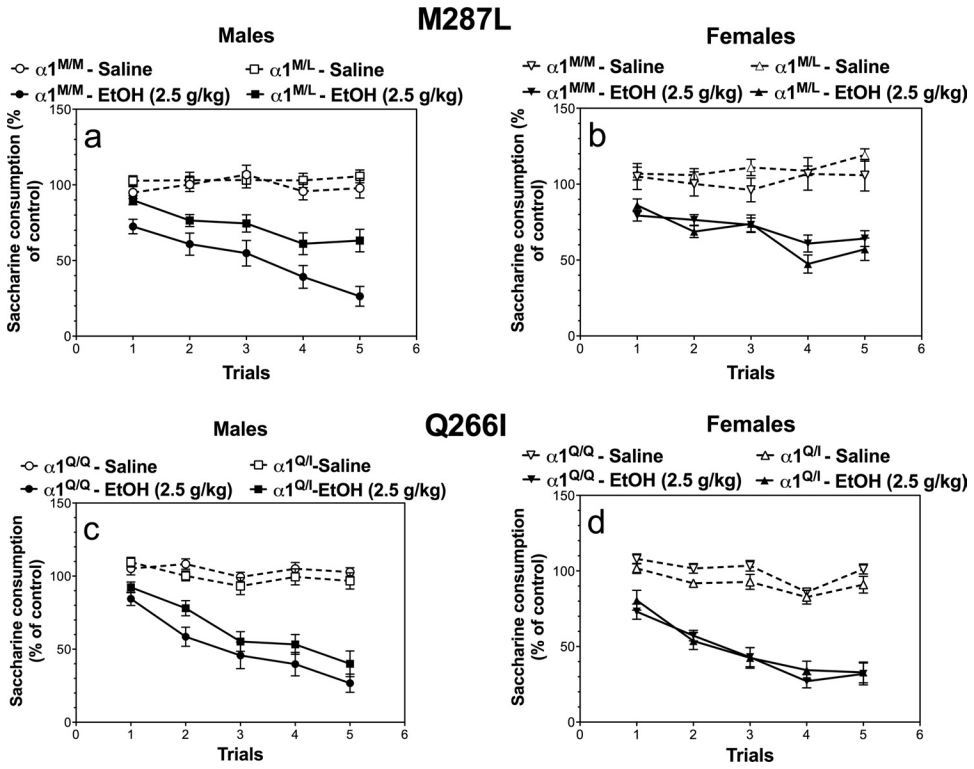

Fig. 3. Both M287L and Q266I male KI mice showed decreased ethanol conditioned taste aversion. Data represent the changes in saccharin consumption produced by injection of saline or ethanol expressed as percentage from control trial (trial 0). a and b, M287L KI mice: males (n = 9 for saline injection for both genotypes; n = 11–12 for groups with ethanol injection) (a) and females (n = 8 for saline injection for both genotypes; n = 11 for groups with ethanol injection) (b). c and d, Q266I KI mice: males (n = 8 for saline injection for both genotypes; n = 10–11 for groups with ethanol injection) (c) and females (n = 10 for saline injection for both genotypes; n = 15 for groups with ethanol injection) (d). See *Conditioned Taste Aversion* for statistical analysis. Values represent mean ± S.E.M.

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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 when lifted and after a gentle spin.

Rotarod. Mice were trained on a fixed speed rotarod (Economex; Columbus Instruments, Columbus, OH; speed of rod 5.0 rpm), and training was considered complete when mice were able to remain on the rotarod for 60 s. 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.

Startle Reflex. Acoustic startle responses were measured by using SR-LAB test stations and software (San Diego Instruments, San Diego, CA). Startle responses were recorded as described previously (Findlay et al., 2003). In brief, test sessions began by placing the mouse in the Plexiglas holding cylinder for a 5-min acclimation period. Over the next 8 min, mice were presented with each of seven trial types across five discrete blocks of trials for a total of 30 trials. The intertrial interval was 10 to 20 s. One trial type measured the response to no stimulus (baseline movement). The other six trial types measured the response to a startle stimulus alone, consisting of a 40-ms sound burst of 90, 95, 100, 105, 110, or 115 dB. Startle amplitude was measured every 1 ms over a 65-ms period beginning at the onset of the startle stimulus. The six trial types were presented in pseudorandom order such that each trial type was presented once within a block of six trials. The maximum startle amplitude (V_{max}) over this sampling period was taken as the dependent variable. A background noise level of 70 dB was maintained over the duration of the test session.

Chemically Induced Convulsions. *N*-methyl-D-aspartic acid (NMDA), pentylenetetrazole (PTZ), strychnine, and nicotine were purchased from Sigma-Aldrich (St. Louis, MO). All of these drugs (except nicotine) were dissolved in normal saline and administered intraperitoneally at a dosing volume of 10 ml/kg body weight. Nicotine was also dissolved in saline but administered subcutaneously at a dosing volume of 5 ml/kg body weight. All testing was performed during approximately the same time of the day (i.e., between the hours of 10:00 AM and 5:00 PM) to lessen potential variability caused by diurnal rhythms. During testing, the mice were placed into observation chambers (20 × 10 × 10-cm Plexiglas cages) individually. For each dose of convulsants, a group of five animals was used. After injection of any convulsant (except nicotine), the animals were continuously observed (up to 30 min after administration) for first latency to clonic and tonic convulsions. Nicotine in a low subconvulsive dose (2 mg/kg) (Korkosz et al., 2006) also was used in experiments for modification of ketamine-induced LORR. Data were recorded and tabulated as to the number of animals exhibiting each of the behavioral signs as well as the latency to first occurrence (mean ± S.E.M.). Effective dose (ED_{50}) was calculated from linear regression of dose-response curve for each convulsant.

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. Flurazepam (Sigma-Aldrich; 225.0 mg/kg i.p.) and pentobarbital (Sigma/RBI, Natick, MA; 50.0 mg/kg i.p.) were dissolved in 0.9% saline and injected at 0.01 ml/g of body weight. *n*-Butanol (Sigma-Aldrich; 1.0 g/kg i.p.) solution was prepared in 0.9% saline (5% 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-

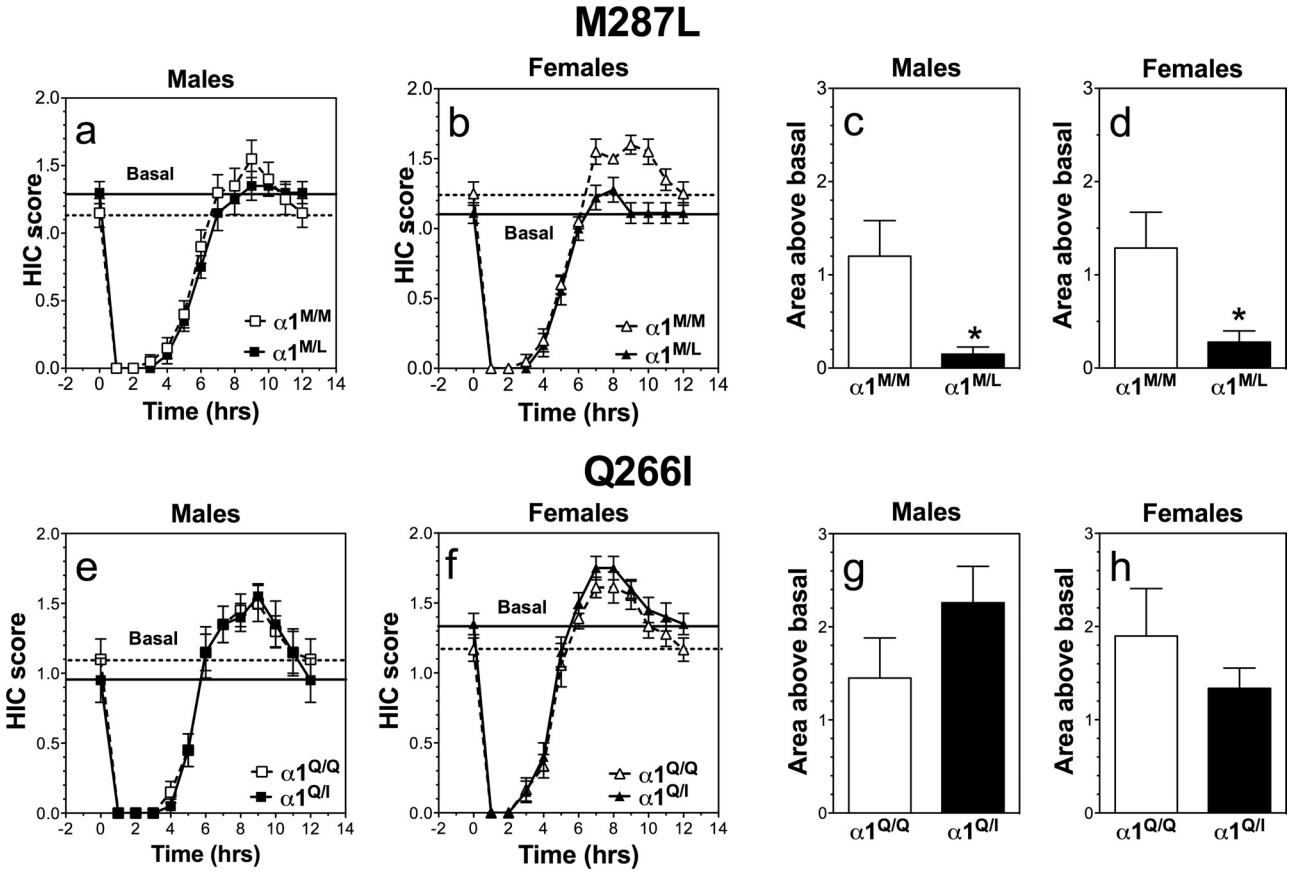

Fig. 4. Severity of acute ethanol withdrawal was reduced in M287L KI mice. Data represent the mean HIC score as a function of time after injection of ethanol (a, b, e, and f) and area above the basal HIC level (c, d, g, and h). a to d, M287L KI mice: males (n = 10 per genotype) (a and c) and females (n = 9–10 per genotype) (b and d). e to h, Q266I KI mice: males (n = 10 per genotype) (e and g) and females (n = 9–10 per genotype) (f and h). *, $P <$ 0.05, significant difference between wild-type and KI mice (Student's t test). Values represent mean ± S.E.M.

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orbital sinus 30, 60, 120, 180, and 240 min after injection. Blood ethanol concentration (BEC) 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 ± S.E.M. The statistics software program GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used. Analysis of variance (two-way analysis of variance with Dunnett's or Bonferroni's post hoc tests) and Student's *t* test were carried out to evaluate differences between groups.

Results

Ethanol Consumption. In a two-bottle free-choice paradigm in which mice could drink either water or a series of increasing ethanol concentrations, KI male mice from both colonies showed a slightly lower preference for ethanol compared with wild-type males ($F_{1,108} = 4.5$, $P < 0.05$, main effect of genotype for M287L KI and $F_{1,90} = 4.4$, $P < 0.05$, main effect of genotype for Q266I KI), although the amount of alcohol consumed was similar for mutant and wild-type mice (Fig. 1). Female mice from both colonies consumed a lower amount of ethanol ($F_{1,96} = 14.2$, $P < 0.001$, main effect of genotype for M287L KI and $F_{1,90} = 17.2$, $P < 0.001$, main effect of genotype for Q266I KI) with lower preference ($F_{1,96} = 28.5$, $P < 0.001$, main effect of genotype for M287L KI and $F_{1,90} = 19.9$, $P < 0.001$, main effect of genotype for Q266I KI) in comparison with wild-type females (Fig. 1).

Preference for Nonalcohol Tastants. There was no difference between M287L KI and wild-type mice in preference for saccharin in the 24-h two-bottle choice test (data not shown), but mutant M287L KI mice demonstrated a slightly higher avoidance of quinine solutions ($F_{1,36} = 8.6$, $P < 0.01$ for males and $F_{1,32} = 10.9$; $P < 0.01$ for females) (data not shown). In contrast, mutant Q266I male mice showed a lower preference for saccharin compared with wild-type mice ($F_{1,54} = 12.0$; $P < 0.01$) (data not shown). KI Q266I female mice and wild-type females consumed saccharin with similar preference. No differences in avoidance of quinine solution were found between KI Q266I mice of both sexes and wild type of corresponding gender (data not shown). Thus, the decreased

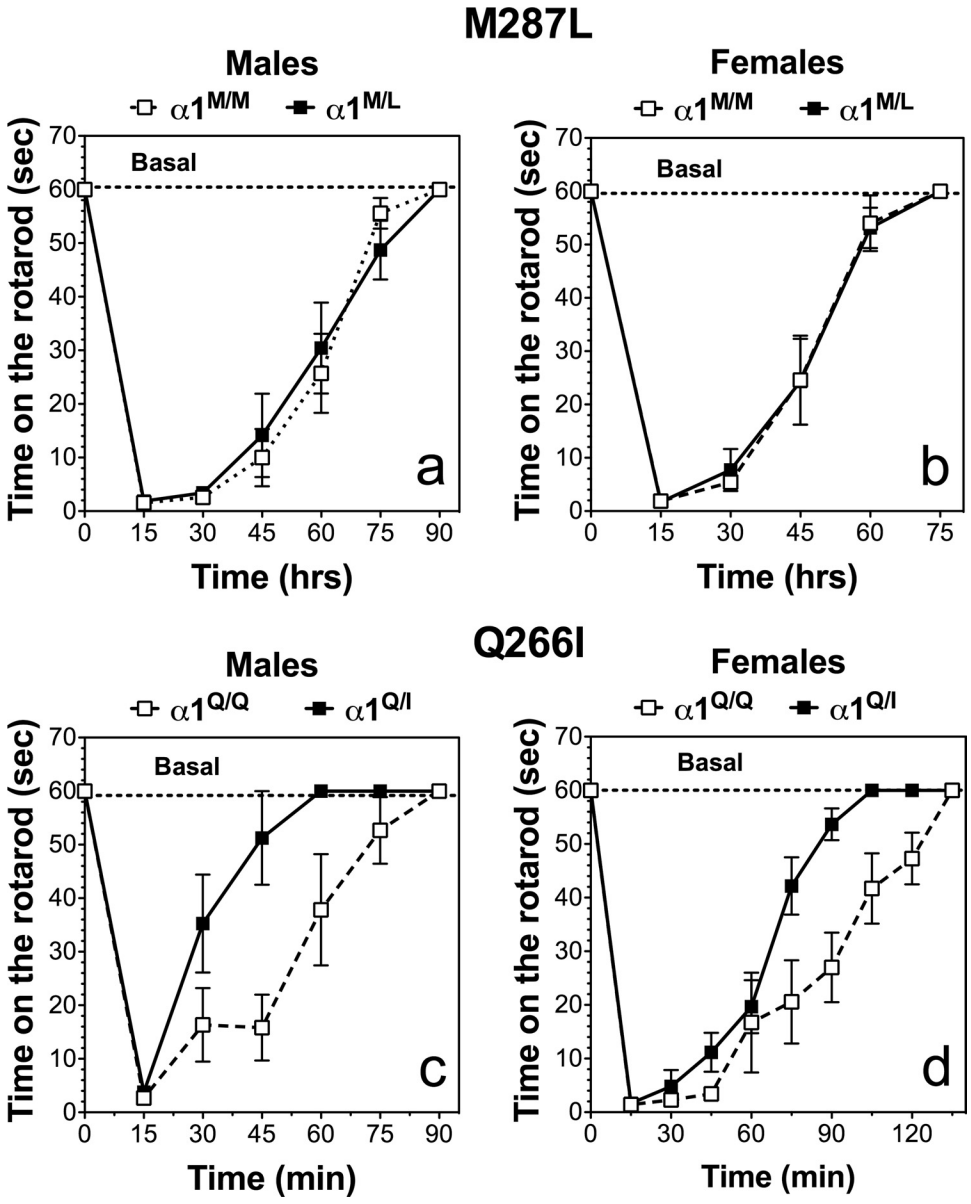

Fig. 5. Faster recovery from motor incoordinating effect of ethanol in Q266I KI mice. Data represent time on the rotarod (s) after injection of ethanol (2 g/kg). a and b, M287L KI mice: males ($n = 7$ per genotype) (a) and females ($n = 7$ per genotype) (b). c and d, Q266I KI mice: males ($n = 4–6$ per genotype) (c) and females ($n = 6–7$ per genotype) (d). See *Motor-Incoordinating Effect of Ethanol* for statistical analysis. Values represent mean ± S.E.M.

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preference for ethanol seen in the KI mice is unlikely to be caused by changes in preference for sweet or bitter tastes.

Depressant Effects of Ethanol. An effect of ethanol dose on ethanol-induced LORR was demonstrated in mice of both sexes and genotypes (M287L KI colony, main effect of genotype: $F_{1,35} = 25.6$, $P < 0.001$ for male mice and $F_{1,47} = 4.9$, $P < 0.05$ for female mice; Q266I KI colony, main effect of genotype: $F_{1,31} = 33.6$, $P < 0.001$ for male mice and $F_{1,32} = 77.8$, $P < 0.001$ for female mice) (Fig. 2). Post hoc analyses further supported the differences between wild-type and KI mice except M287L KI female mice (Fig. 2b). Overall, M287L mice demonstrated shorter duration of LORR than their wild-type littermates. In contrast, Q266I mice showed a significantly longer duration of LORR compared with wild-type mice.

Conditioned Taste Aversion. Consumption of saccharin during the 1-h period varied slightly in wild-type and mutant mice of both sexes (M287L colony: 84.3 ± 3.8 and 90.9 ± 3.2 g/kg body weight for wild-type and KI male mice, respectively; 116.6 ± 4.1 and 112.6 ± 4.2 g/kg body weight for wild-type and KI female mice, respectively; Q266I colony: 85.4 ± 3.1 and 83.8 ± 2.6 g/kg body weight for wild-type and KI male mice, respectively; 77.2 ± 3.6 and 85.2 ± 5.7 g/kg body weight for wild-type and KI female 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 subsequent conditioning trials by the amount of saccharin solution consumed on trial 0 (before conditioning). Figure 3 depicts saccharin intake (as a percentage of trial 0) for each genotype and sex over the course of the five saccharin access periods. Ethanol produced trial-dependent reductions in saccharin intake over trials, indicating the development of conditioned taste aversion in mice of both sexes and all genotypes (M287L colony main effect of treatment: $F_{1,95} = 131$, $P < 0.001$ and $F_{1,90} = 80$, $P < 0.001$ in wild-type and KI male mice, respectively; $F_{1,85} = 58$, $P < 0.001$ and $F_{1,85} = 169$, $P < 0.001$ in wild-type and KI female mice, respectively; Q266I colony main effect of treatment: $F_{1,80} = 191$, $P < 0.001$ and $F_{1,85)} = 87$, $P < 0.001$ in wild-type and KI male mice, respectively; $F_{1,115} = 279$, $P < 0.001$ and $F_{1,114} = 128$, $P < 0.001$ in wild-type and KI female mice, respectively). No differences between wild-type and KI mice were found in saline-treated groups. KI male mice from both colonies developed weaker CTA than wild-type male mice ($F_{1,1055} = 30$, $P < 0.001$, main effect of genotype for M287L colony and $F_{1,95} = 8.9$, $P < 0.01$, main effect of genotype for Q266I colony) (Fig. 3, a and c). There were no differences in the development of CTA (comparison of ethanol-treated groups) between wild-type and KI female mice (Fig. 3, b and d). Thus, the decreased ethanol CTA was found in male but not female KI mice.

Acute Ethanol Withdrawal Severity. A single 4 g/kg ethanol dose suppressed basal HIC in both the KI and wild-type mice from both colonies for approximately 5 h, followed by increased HIC (Fig. 4, a, b, e, and f). All animals demonstrated signs of withdrawal (HIC scores higher than the basal level). Both M287L male and female KI mice showed weaker withdrawal ($P < 0.05$, Student's t test) compared with their corresponding wild-type littermates (Fig. 4, c and d), but there were no differences in withdrawal for male and female mice from the Q266I mutant colony (Fig. 4, g and h).

Motor-Incoordinating Effect of Ethanol. Acute administration of ethanol (2 g/kg) produced incoordination as mea-

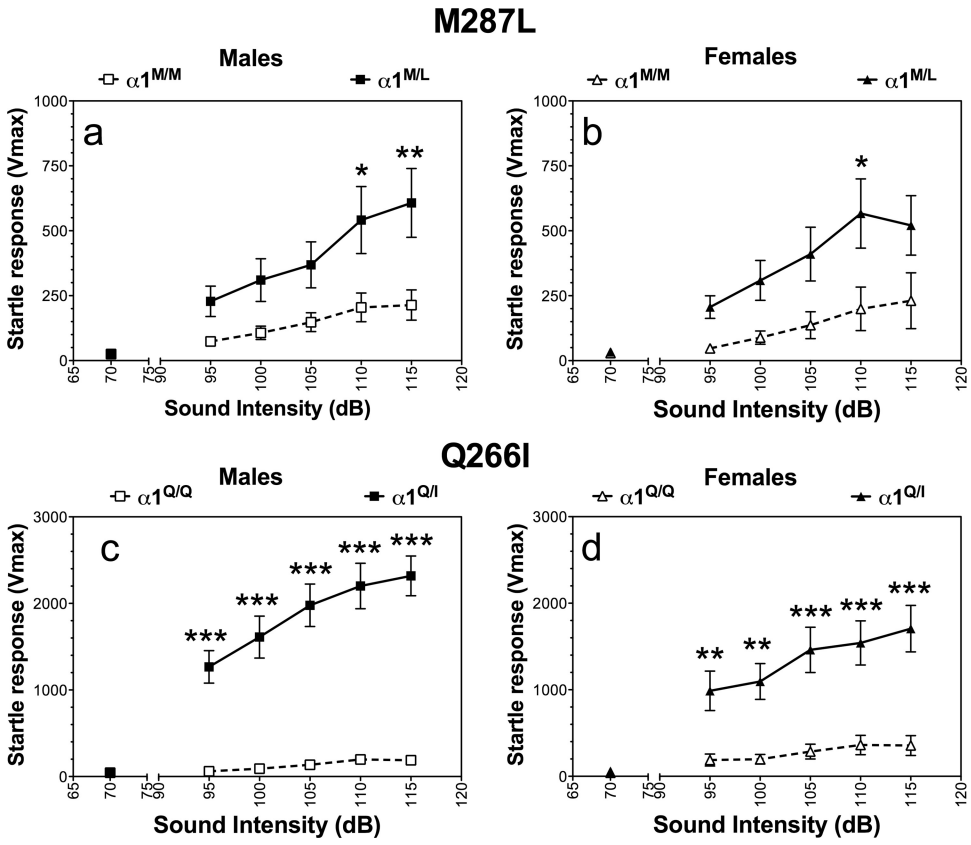

Fig. 6. Acoustic startle response was increased in both M287L and Q266I KI mice. Data represent the maximum startle amplitude (V_{max}) as a function of sound intensity (dB). a and b, M287L KI mice: males ($n = 13$ per genotype) (a) and females ($n = 13$–14 per genotype) (b). c and d, Q266I KI mice: males ($n = 13$–16 per genotype) (c) and females ($n = 15$–17 per genotype) (d). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant difference relative to wild-type mice for the same sound intensity (Bonferroni post-test). Values represent mean ± S.E.M.

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sured by the rotarod test, and there were no differences between wild-type and M287L KI mice in recovery from this motor incoordination ($F_{1,80}$ = 115, $P > 0.05$, dependence on genotype; $F_{7,80}$ = 115, $P < 0.001$, dependence on time) (Fig. 5, a and b). In contrast, male and female Q266I KI mice recovered from ethanol-induced motor-incoordination faster than their wild-type littermates of corresponding sex (males: $F_{1,56}$ = 15.6, $P < 0.001$, dependence on genotype; $F_{6,56}$ = 27.5, $P < 0.001$, dependence on time; females: $F_{1,110}$ = 22.9, $P < 0.001$, dependence on genotype; $F_{9,110}$ = 59.3; $P < 0.001$, dependence on time) (Fig. 5, c and d).

Startle Response. Because changes in GlyR function are accompanied by changes in acoustic startle response (Findlay et al., 2003), we studied this behavior in the KI mice. A dramatic increase in the acoustic startle responses was observed in KI mice of both sexes (M287L, main effect of genotype: $F_{1,120}$ = 28, $P < 0.001$ for males and $F_{1,125}$ = 26, $P < 0.001$ for females; Q266I, main effect of genotype: $F_{1,135}$ = 219, $P < 0.001$ for males and $F_{1,150}$ = 94, $P < 0.001$ for females) (Fig. 6). The increased startle response was much greater in mice carrying the Q266I mutation.

In the next series of experiments, we tested a series of depressant and convulsant drugs to determine whether the GlyR mutations could change the sensitivity of other neurotransmitter receptors.

LORR Induced by Different Depressant Drugs. The duration of sleep time induced by *n*-butanol (Fig. 7, a and e), pentobarbital (Fig. 7, b and f), ketamine (Fig. 7, c and g), and flurazepam (Fig. 7, d and h) was significantly increased in M287L KI mice of both sexes. However, in Q266I KI mice only ketamine (Fig. 8, c and g) and flurazepam (Fig. 8, d and h) increased the duration of LORR compared with wild-type littermates of the same sex. No differences between mutant Q266I KI and wild-type mice were found in the duration of LORR induced by *n*-butanol (Fig. 8, a and e) or pentobarbital (Fig. 8, b and f).

Chemically Induced Seizures. No differences in sensitivity (ED_{50}) for tonic convulsions induced by PTZ ($GABA_A$ receptor blocker) were found between wild-type and M287L KI male mice (74 and 72 mg/kg, respectively; Fig. 9a). There were also no differences between wild-type and M287L KI mice for NMDA (207 and 208 mg/kg, respectively; Fig. 9b). However, M287L KI male mice were significantly more sensitive than wild-type mice in the development of tonic convulsions induced by strychnine, a GlyR antagonist (1.05 and 0.67 mg/kg, respectively; Fig. 9c). No differences in NMDA-induced tonic convulsions were found between Q266I KI male mice and their wild-type littermates (244 and 222 mg/kg, respectively; Fig. 9e). However, Q266I KI male mice were significantly more sensitive than wild-type mice in the development of tonic convulsions induced by strychnine (0.93 and 0.52 mg/kg, respectively; Fig. 9f) as well as by PTZ (72 and 46 mg/kg, respectively; Fig. 9d). Thus, both KI mice were more

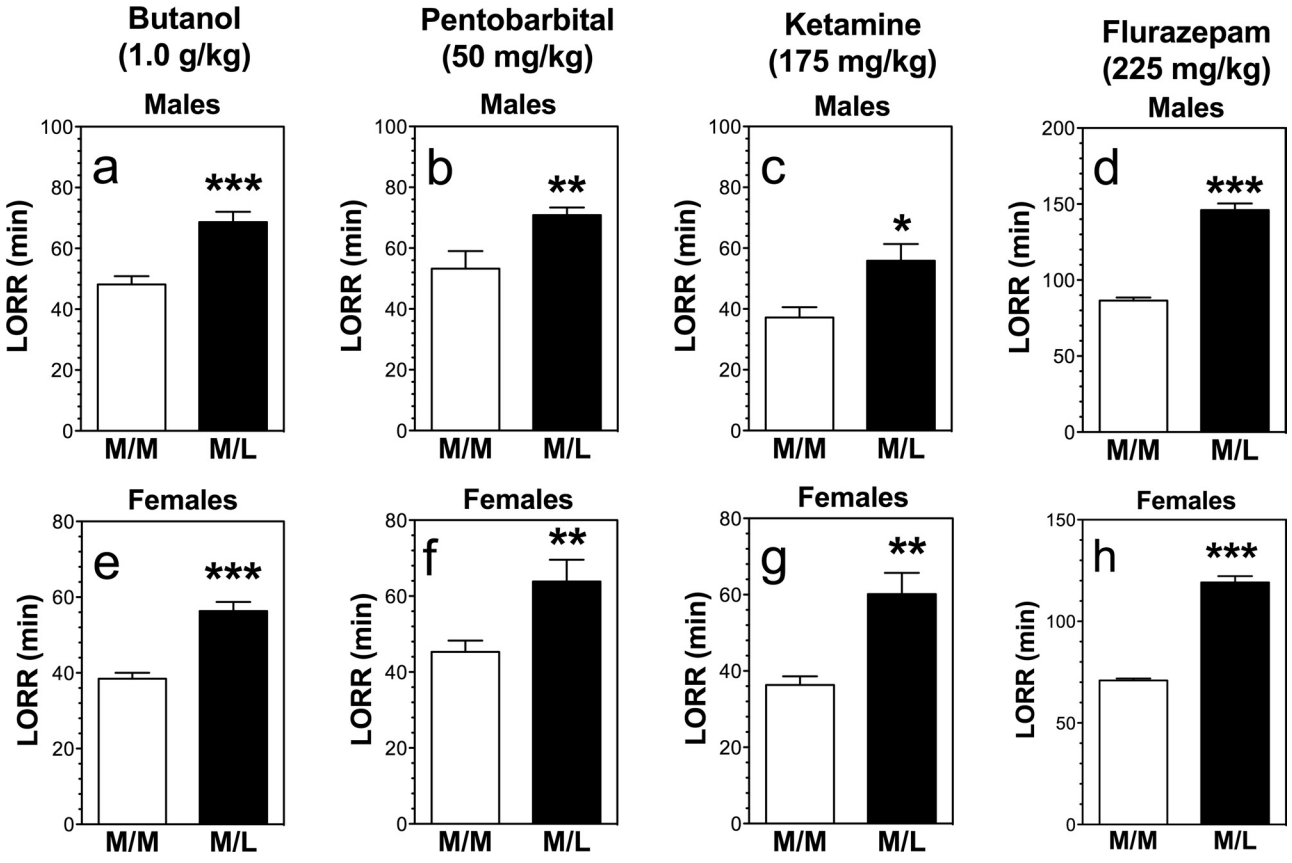

Fig. 7. Depressant effect of *n*-butanol, pentobarbital, flurazepam, and ketamine in M287L KI mice. Data represent duration of LORR (min). a to d, males: *n*-butanol (n = 7–10 per genotype) (a), pentobarbital (n = 7–10 per genotype) (b), ketamine (n = 6–10 per genotype) (c), and flurazepam (n = 10 per genotype) (d). e to h, females: *n*-butanol (n = 12–14 per genotype) (e), pentobarbital (n = 7–9 per genotype) (f), ketamine (n = 7–8 per genotype) (g), and flurazepam (n = 9–10 per genotype) (h). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant difference relative to wild-type mice (Student's *t* test). Values represent mean ± S.E.M. M/M, wild-type mice; M/L, M287L KI mice.

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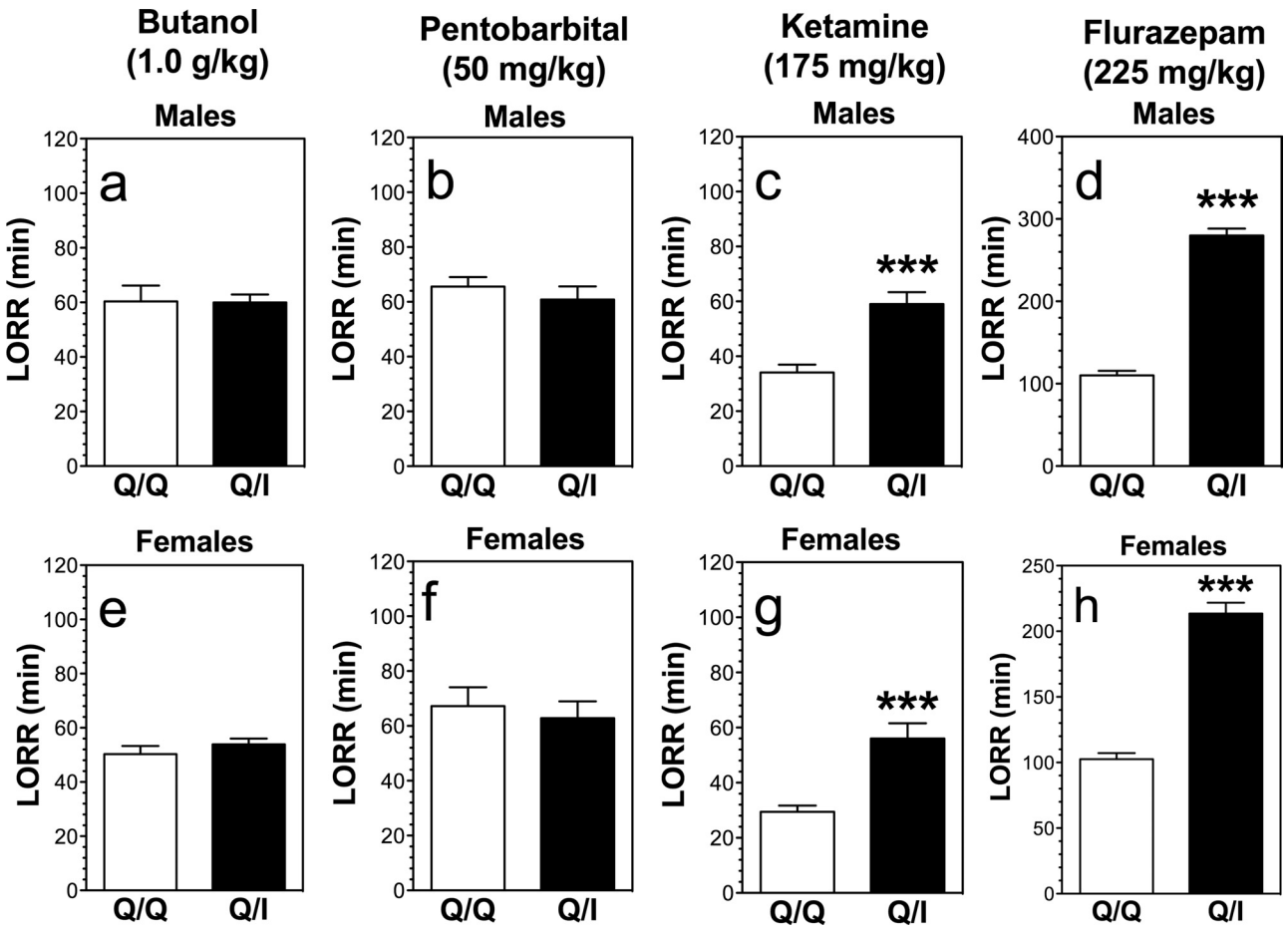

Fig. 8. Depressant effect of *n*-butanol, pentobarbital, flurazepam, and ketamine in Q266I KI mice. Data represent duration of LORR (min). a to d, males: *n*-butanol (n = 7–11 per genotype) (a), pentobarbital (n = 12–15 per genotype) (b), ketamine (n = 7–11 per genotype) (c), and flurazepam (n = 11–16 per genotype) (d). e to h, females: *n*-butanol (n = 9–10 per genotype) (e), pentobarbital (n = 8–9 per genotype) (f), ketamine (n = 9–10 per genotype) (g), and flurazepam (n = 9 per genotype) (h). ***, $P < 0.001$, significant difference relative to wild-type mice (Student's t test). Values represent mean ± S.E.M. QQ, wild-type mice; Q/I, Q266I KI mice.

sensitive to the convulsant actions of strychnine, but not NMDA or PTZ.

Ethanol Effect on Acoustic Startle Response. We next studied the effect of ethanol on the acoustic startle response in M287L and Q266I KI male mice. In wild-type mice from both colonies, significant main effects of ethanol dose and sound intensity were observed (M287L KI mice: $F_{2,216}$ = 21.4, $P < 0.001$ and $F_{5,216}$ = 13.2, $P < 0.001$, respectively; Q266I KI mice: $F_{3,168}$ = 4.9, $P < 0.01$ and $F_{5,168}$ = 8.1, $P <$ 0.001, respectively) (Fig. 10, a and c). Overall, low doses of ethanol increased the amplitude of startle response in wild-type mice from both colonies. We observed significant main effects of dose and sound intensity in M287L KI male mice ($F_{2,216}$ = 20.5, $P < 0.001$ and $F_{5,216}$ = 16.7, $P < 0.001$, respectively) (Fig. 10b). However, low doses of ethanol reduced the amplitude of startle response in M287L mutant mice compared with its activation in wild-type mice. In contrast, dependence on sound intensity but not on the effect of ethanol was found in Q266I KI male mice ($F_{5,168}$ = 21.1, $P <$ 0.001, effect of intensity) (Fig. 10d).

Pharmacological Replication of Behavioral Phenotypes Seen in M287L and Q266I KI Mice. The changes in drug sensitivity described above could be the result of compensation for reduced GlyR function that may occur during development or may represent more dynamic changes in response to impairment of GlyR function. To determine whether acute impairment of GlyR function could mimic any of the effects seen in the KI mice, we injected low, subconvulsive doses of strychnine (0.2–0.4 mg/kg s.c.) to C57BL/6J male mice.

Administration of strychnine substantially increased the duration of LORR induced by a low dose of flurazepam as well as a low dose of ketamine (Fig. 11, a and b). Duration of LORR induced by a low dose of ethanol (3.2 g/kg) was also significantly increased after injection of a subconvulsive dose of strychnine (0.4 mg/kg s.c.) (Fig. 11d). Duration of pentobarbital-induced LORR was also increased after an injection of strychnine ($F_{2,12}$ = 5.2, $P < 0.05$, one-way analysis of variance) (Fig. 11e). Post hoc analyses showed that the statistically significant increase of duration of pentobarbital-induced LORR occurred only after the injection of a very low dose of strychnine (0.2 mg/kg s.c.).

The absence of an ethanol-induced activation of startle response in M287L and Q266I KI mice could be a result of activation of nicotinic acetylcholine receptor (nAChR) function in these mutant mice (Lewis and Gould, 2003). In addition, such changes could explain the increase of ketamine-induced LORR observed in both KI mice, because ketamine blocks nAChRs (Harrison and Simmonds, 1985). To model activation of nAChR in vivo we injected a low dose of nicotine

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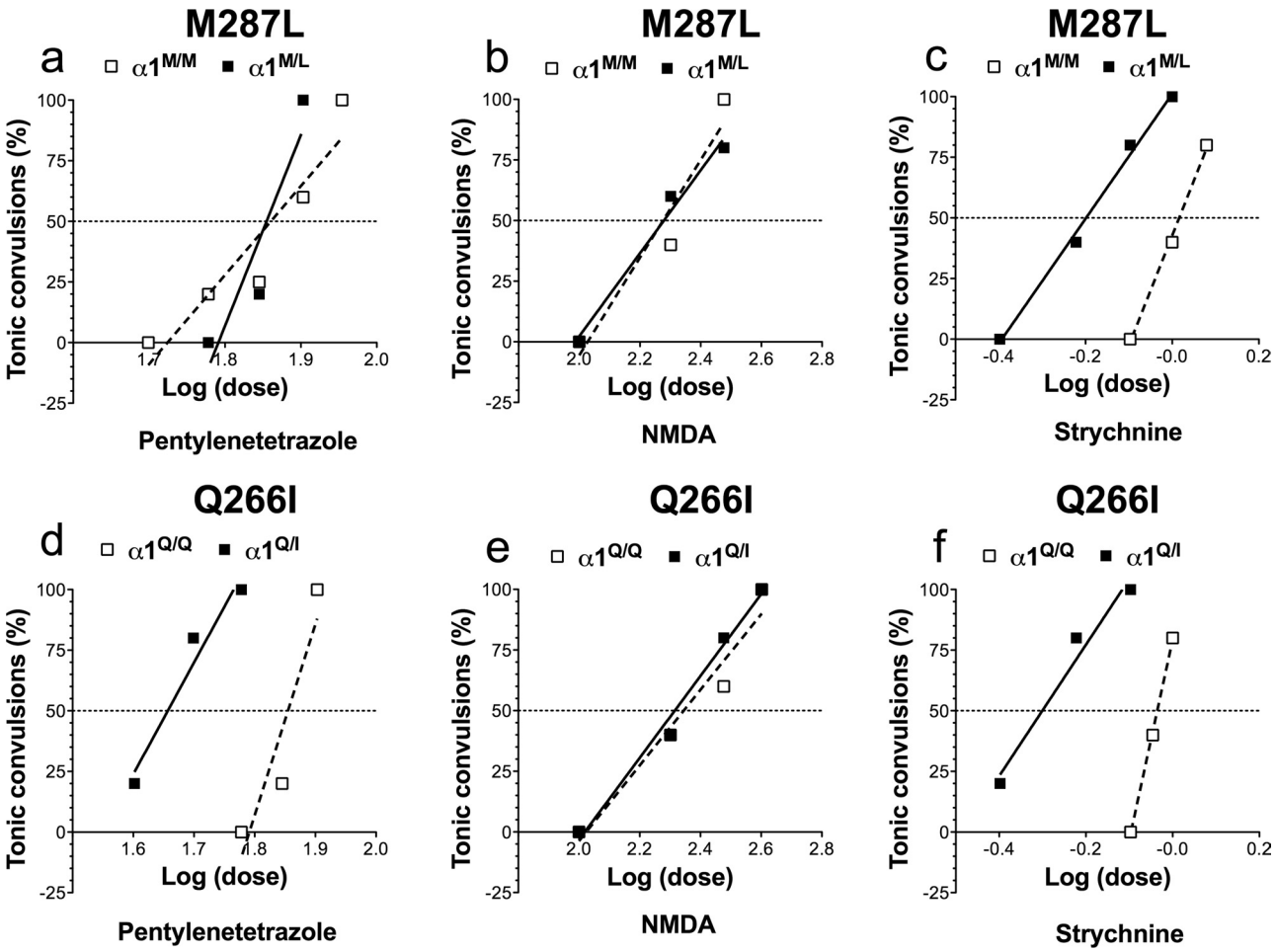

Fig. 9. Effects of pentylenetetrazole (a and d), NMDA (b and e), and strychnine (c and f) on tonic convulsions in M287L (a–c) and Q266I (d–f) male mice. Data represent the percentage of mice that showed tonic convulsions for each of the drug doses. Five mice were used for each dose.

(2.0 mg/kg s.c.) in C57BL/6J mice. Indeed, duration of ketamine-induced LORR was significantly increased after an injection of nicotine (Fig. 11c), mimicking the effect of the KI mutations.

Finally, pretreatment of C57BL/6J mice with subconvulsive doses (0.4 or 0.6 mg/kg) of strychnine significantly increased the startle response ($F_{2,132} = 24.6$, $P < 0.0001$, main effect of treatment; $F_{5,132} = 41.3$, $P < 0.0001$, main effect of sound intensity) (Fig. 11f).

Ethanol Metabolism. There were no differences in ethanol metabolism between wild-type and KI mice (data not shown). The slopes of the regression lines were: -0.79 ± 0.03 and -0.87 ± 0.04 for wild-type and M287L KI male mice, respectively; -0.94 ± 0.04 and -0.85 ± 0.04 for wild-type and M287L KI female mice, respectively; -0.81 ± 0.04 and -0.95 ± 0.06 for wild-type and Q266I KI male mice, respectively; and -0.86 ± 0.06 and -0.94 ± 0.07 for wild-type and Q266I KI female mice, respectively.

Discussion

The first goal of this study was to define the behavioral effects of alcohol that would be reduced or eliminated by introduction of two ethanol-resistant mutations in the GlyR α1 subunit. Five such behaviors were identified: acute alcohol withdrawal, ethanol-induced LORR, alcohol consumption, recovery from rotarod ataxia, and alcohol-induced taste aversion (for summary of behavioral phenotypes; see Table 1). Studies with recombinant receptors and neuronal electrophysiology showed that the Q266I mutation produces a much larger suppression of alcohol action than M287L (Borghese et al., 2012); therefore, we will focus on behavioral actions of alcohol that are more affected in the Q266I mutant mice. Only the recovery from ethanol ataxia (rotarod test) meets this criterion, and it is thus the best candidate for a behavioral action of ethanol mediated by enhancement of GlyR function. However, we should also consider the role of taurine in the behavioral responses to ethanol. Taurine is an amino acid abundant in mammalian brain and a partial agonist of the GlyR (Albrecht and Schousboe, 2005), and there is evidence that ethanol can release taurine (Olive, 2002). Ethanol-induced dopamine release in NAcc of rats seems to require taurine action on GlyR (Ericson et al., 2006, 2011). It is possible that some of the reduced behavioral actions of ethanol seen in the α1(M287L) mice were caused by the absence of taurine action on this mutant GlyR (Borghese et al., 2012).

As noted by Borghese et al. (2012), both KI mutations are far from silent and reduce the function of GlyRs. The mutations were lethal for homozygous mice, and the heterozygous mice used in the behavioral studies showed an increased startle response, probably reflecting impairment of GlyR function (Harvey et al., 2008). The increased sensitivity of both mutants to strychnine-induced convulsions also supports the conclusion that GlyR function is impaired in M287L

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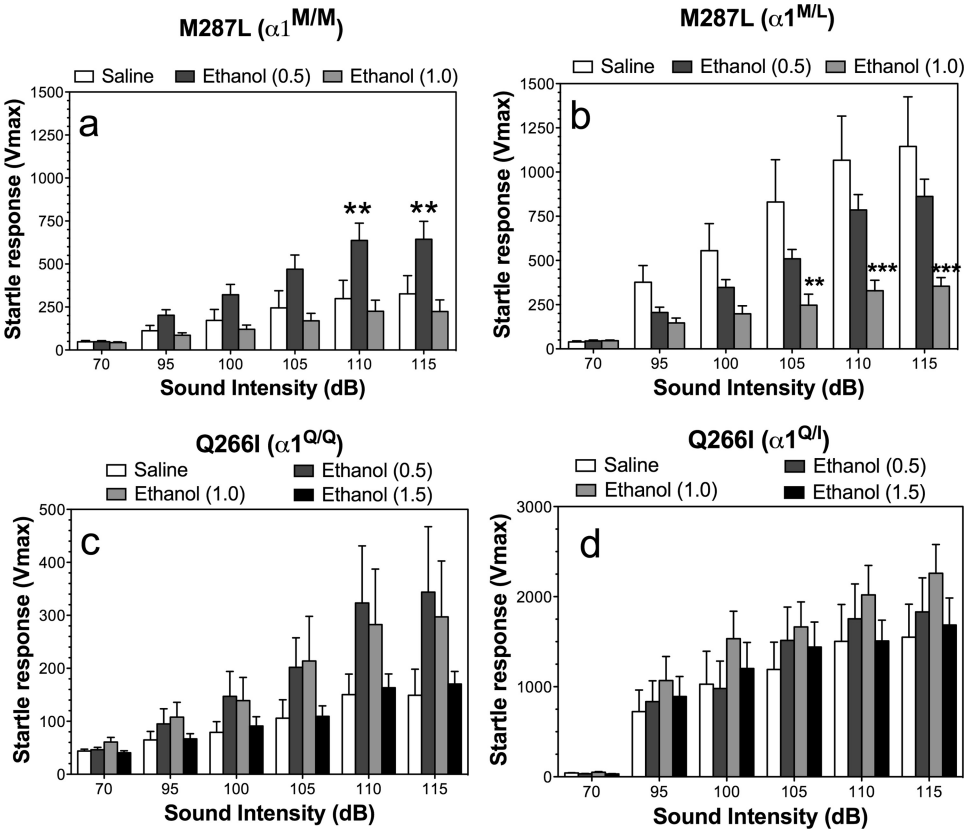

Fig. 10. Effect of ethanol on acoustic startle response in M287L KI (a and b) and Q266I KI (c and d) male mice. Data represent the maximum startle amplitude (V_{max}) as a function of sound intensity (dB). a and b, M287L mouse colony: wild-type mice (n = 13 per dose) (a) and M287L KI mice (n = 13 per dose) (b). c and d, Q266I KI mouse colony: wild-type mice (n = 8 per dose) (c) and Q266I KI mice (n = 8 per dose) (d). Note difference in scale between c and d because of the heightened startle response in Q266I KI mice. **, $P < 0.01$; ***, $P < 0.001$, significant difference between ethanol-treated and saline mice for the same sound intensity (Bonferroni post-test). Values represent mean ± S.E.M.

and Q266I KI mice. The increased sensitivity to startle and seizures is similar to the phenotype of mice carrying the S267Q mutation in the α1 subunit of GlyR (Findlay et al., 2003). The behavioral effects seen in our mutants were consistent with the decreased conductance of the α1(Q266I)β GlyR compared with α1β wild-type and α1(M287L)β GlyRs and the greater reduction of glycine-evoked currents in neurons from heterozygous Q266I mice compared with M287L heterozygotes (Borghese et al., 2012). There are several other differences between the two receptors in glycine and taurine pharmacology, but most of them would result in M287L-containing GlyRs showing greater deficiency in glycinergic function (i.e., higher glycine EC_{50}, insensitivity to taurine), so these changes do not explain the increased startle response in Q266I.

In addition to the impairment of GlyR function, our pharmacological studies suggest changes in several other neurotransmitter systems in these mutants. The increase in flurazepam- and pentobarbital-induced LORR could be related to changes in $GABA_A$ function because both of these drugs are allosteric modulators of $GABA_A$ receptors (Sigel and Luscher, 2011). Differences in flurazepam effects were seen in both M287L and Q266I mutants, whereas differences in pentobarbital were found only in M287L KI mice. This may be explained by the observation that the M287L mutation (but not Q266I) increases the sensitivity of the GlyR to modulation by pentobarbital, allowing pentobarbital to produce some behavioral effects through GlyR in addition to $GABA_A$ receptors (Borghese et al., 2012). Q266I mutant mice were more sensitive to PTZ-induced convulsions, again suggesting changes in $GABA_A$ receptor function. However, the absence of any differences in flunitrazepam radioligand binding in spinal cord and brainstem between the Q266I mutant and wild-type mice suggests that changes in GABAergic function are not caused by an altered number of receptors (Borghese et al., 2012).

Ketamine is an antagonist of NMDA receptor function (Akk et al., 2008). However, it is not clear whether the depressant effects of high doses of ketamine that produce LORR are mediated solely by NMDA receptor inhibition. There are several reports that ketamine may inhibit nAChRs in a behaviorally relevant concentration range (Harrison and Simmonds, 1985; Flood and Krasowski, 2000). Nicotine and ethanol increase the acoustic startle response, and mecamylamine, a nAChR antagonist, attenuates the effects of both drugs (Lewis and Gould, 2003). Our data showing that the activation of startle response produced by low doses of ethanol is absent in both mutants may indicate an activation of nicotinic acetylcholine neurotransmission in the mutants. In addition to its effect on nAChRs, Hevers et al. (2008) showed that at pharmacologically relevant concentrations (10–100 μM) ketamine enhances $GABA_A$ receptor function. However, it should be noted that ketamine potentiation of GABAergic function was specific for α6-containing $GABA_A$ receptors.

Taken together, these data suggest that the M287L and Q266I mutations induce changes in sensitivity to multiple drugs, including some acting on $GABA_A$ and nAChRs. This is a potential complication to interpretation of behavioral effects of ethanol in these mutants because of the role of $GABA_A$ and nAChRs in alcohol actions. For example, deletion of α7 nAChRs increases the duration of ethanol-induced LORR (Bowers et al., 2005), and another nAChR (α4β2) can modulate the effects of both ethanol and nicotine on acoustic

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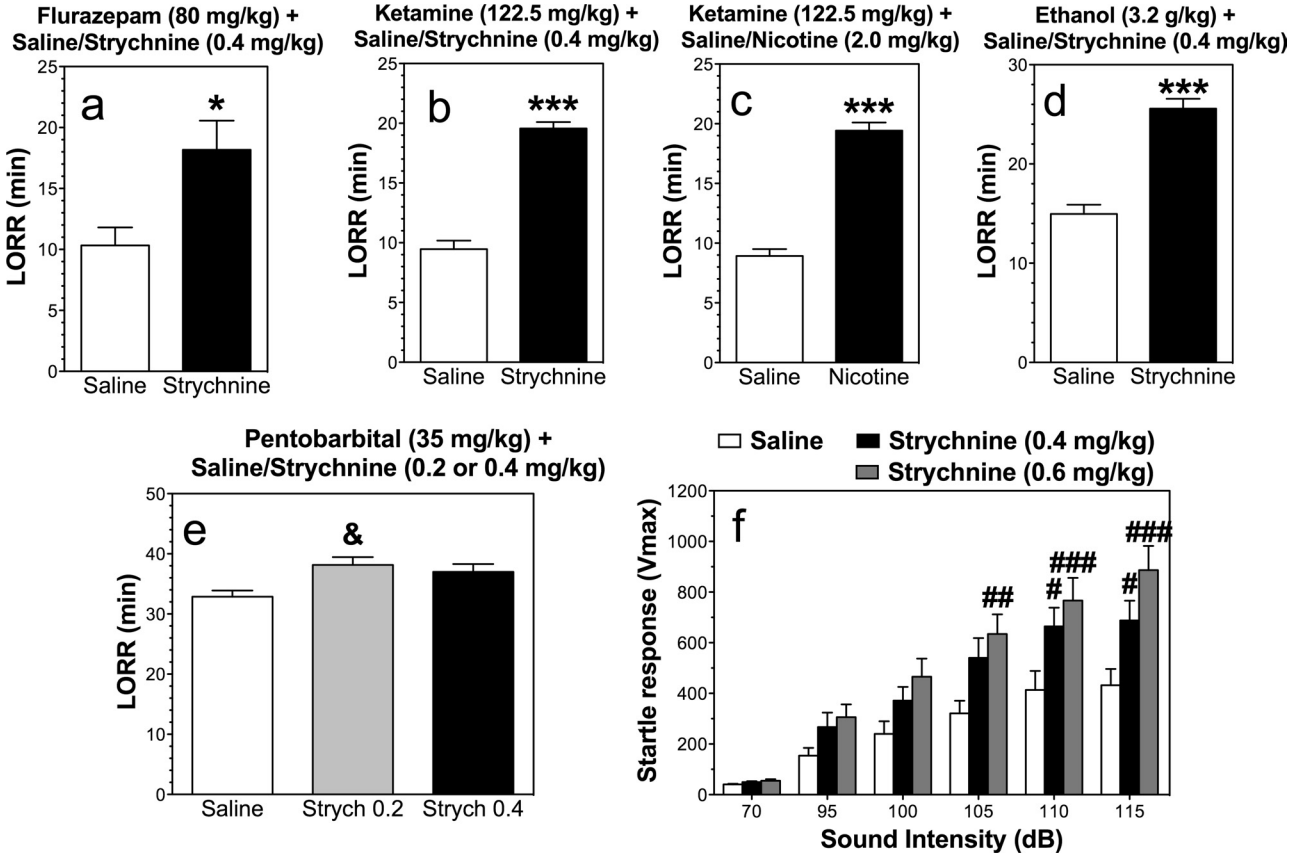

Fig. 11. Pharmacological reproduction of behavioral phenotypes seen in KI mice. C57BL/6J mice were used for these studies. a to e, data represent duration of LORR (min) for several depressant drugs after pretreatment with saline, strychnine, or nicotine. a, flurazepam (80 mg/kg) after strychnine (0.4 mg/kg) ($n = 5$ per group). b, ketamine (122.5 mg/kg) after strychnine (0.4 mg/kg) ($n = 5$ per group). c, ketamine (122.5 mg/kg) after nicotine (2.0 mg/kg). d, ethanol (3.2 g/kg) after strychnine (0.4 mg/kg) ($n = 5$ per group). e, pentobarbital (35 mg/kg) after strychnine (0.2 mg/kg and 0.4 mg/kg) ($n = 5$ per group). f, data represent the maximum startle amplitude (V_{max}) as a function of sound intensity (dB). *, $P < 0.05$; ***, $P < 0.0001$, significant difference relative to saline-treated control (Student's t test). &, $P < 0.05$, significant difference relative to saline-treated control (post hoc Dunnett's test for multiple comparison). #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, significant difference relative to saline-treated control (post hoc Bonferroni's test for multiple comparison). Values represent mean ± S.E.M.

TABLE 1

Summary of the behavioral phenotypes found in heterozygous α1 GlyR M287L and Q266I KI mice ($\alpha1^{M/L}$ and $\alpha1^{Q/I}$, respectively)

The signs reflect comparison with wild type: ↓, decreased behavior; ↑, increased behavior; ←, left shift in time-dependent recovery curve; 0, no change. The arrows were assigned based on statistical analyses as noted in the figure legends.

Test	Drug	Behavior	Dose	M287L		Q266I	
				Male	Female	Male	Female
Two-bottle choice	Ethanol	Ethanol intake, g/kg/24 h		0	↓	0	↓
		Preference		↓	↓	↓	↓
		Fluid intake, g/kg/24 h		↑	↑	↑	↑
Two-bottle choice	Saccharin	Preference		0	0	↓	0
		Fluid intake, g/kg/24 h		0	0	↓	0
Two-bottle choice	Quinine	Preference		↓	↓	0	0
		Fluid intake, g/kg/24 h		0	0	↑	↑
LORR	Ethanol	Sleep time	3.8 g/kg	↓	0	↑	↑
CTA, saccharin	Ethanol		2.5 g/kg	↓	0	↓	0
Acute withdrawal	Ethanol	HIC score	4 g/kg	↓	↓	0	0
Rotarod	Ethanol	Recovery	2 g/kg	0	0	←	←
Startle reflex	Ethanol	Increased startle	0.5–1.0–1.5 g/kg	↓		↓	
LORR	Butanol	Sleep time	1.0 g/kg	↑	↑	0	0
LORR	Pentobarbital	Sleep time	50 mg/kg	↑	↑	0	0
LORR	Ketamine	Sleep time	175 mg/kg	↑	↑	↑	↑
LORR	Flurazepam	Sleep time	225 mg/kg	↑	↑	↑↑	↑↑
Seizures	PTZ	EC_{50}		0		↓	
	NMDA	EC_{50}		0			
	Strychnine	EC_{50}		↓		↓	
Startle reflex				↑	↑	↑↑	↑↑
Metabolism	Ethanol	BEC	4 g/kg	0	0	0	0

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TABLE 2

Summary of behavioral phenotypes observed in heterozygous α1 GlyR M287L, Q266I, and S267Q KI mice, and heterozygous *oscillator* (*Glra1*$^{spd\text{-}ot}$) mice

The signs reflect comparison with wild type: ↓, decreased behavior; ↑, increased behavior; ←, left shift in time-dependent recovery curve; 0, no change. Ethanol doses (in g/kg): 3.8 for LORR, 4 for acute withdrawal, and 2 for rotarod test. Sources for S267Q and *oscillator* data: Findlay et al., 2003, Kling et al., 1997, and Y. A. Blednov, R. D. Mayfield, and R. A., Harris, manuscript submitted for publication. The startle reflex response for *oscillator* mice was increased in Kling et al. (1997) and showed no change in Findlay et al. (2003).

Test	Behavior	M287L		Q266I		S267Q		*oscillator*	
		Male	Female	Male	Female	Female	Male	Male	Female
Startle reflex		↑	↑	↑↑	↑↑	↑↑	↑↑	↑, 0	↑, 0
LORR	Sleep time	↓	0	↑	↑	0	↑	↑	0
Acute withdrawal	HIC score	↓	↓	0	0	0	0	0	0
Two-bottle choice, ethanol	Ethanol intake, g/kg/24 h	0	↓	0	↓	↑	0	0	0
	Preference	↓	↓	↓	↓	0	0	0	↓
	Fluid intake, g/kg/24 h	↑	↑	↑	↑	↑	↑	0	↑
Two-bottle choice, saccharin	Preference	0	0	↓	0	↓	0	0	0
	Fluid intake, g/kg/24 h	0	0	↓	0	↑	↑	0	0
Two-bottle choice, quinine	Preference	↓	↓	0	0	0	↓	0	0
	Fluid intake, g/kg/24 h	0	0	↑	↑	↑	↑	0	↓
Rotarod	Recovery	0	0	←	←	←	←		

startle (Owens et al., 2003). Furthermore, a number of behavioral effects of ethanol have been attributed to actions at the $GABA_A$ receptors (Boehm et al., 2006; Enoch, 2008).

A significant shortcoming of null mutant mice is that extensive compensation may occur in response to the deletion and may involve genes that are distinct from the deletion. One assumption underlying construction and use of KI mice is that they will display either no or limited compensation compared with knockout mice, thus providing an advantage over null mutants (Werner et al., 2006; Blednov et al., 2011; Harris et al., 2011). However, the mutations used in this study clearly impair the function of the GlyR, and we asked whether some of the changes in behavior that we observed were the result of compensation occurring during development or from the acute impairment in GlyR function. We used strychnine as the prototypical GlyR antagonist, although it does have effects on some nAChRs (Matsubayashi et al., 1998). We found that the changes in LORR and startle response induced by several drugs in the mutants can be duplicated by injection of strychnine in normal mice, and they are not likely to be the result of developmental compensations. Therefore, inhibition of GlyR function acutely perturbs a complex neuronal network, indicating that glycinergic inhibition is a key regulator of this network. This is consistent with emerging evidence for a key role for tonic glycinergic inhibition in tuning inhibitory control and plasticity in the spinal cord (Rajalu et al., 2009; Takazawa and MacDermott, 2010). Although most of the work on glycinergic networks has been restricted to the spinal cord, there is increasing evidence for an important role of GlyRs in forebrain structures (Hernandes and Troncone, 2009). For many alcohol phenotypes, such as reward, aversion, and physical dependence, these brain regions are critical, and it will be of interest to determine whether mutations in GlyR also influence inhibitory control and plasticity in those structures.

It is interesting to note that a low dose of strychnine increased ethanol-induced LORR, thus duplicating the phenotype observed in Q266I mutant mice. These data suggest that the increases in ethanol-induced LORR in Q266I mice are the result of reduced glycinergic function, whereas the reduction of ethanol-induced LORR observed in M287L mutants may result from the ethanol-insensitive mutation.

It is of interest to compare the behavioral profile of the two KI mutant mice used in this study with the closely related GlyR α1 subunit S267Q KI mouse described previously (Findlay et al., 2003). In addition, we have carried out some behavioral testing with heterozygous *oscillator* mice, which lack the GlyR α1 subunit protein (Findlay et al., 2003; Y. A. Blednov, R. D. Mayfield, and R. A., Harris, manuscript submitted for publication). The results allow the comparison of a null mutant with the three lines of KI mice (Table 2). All four of these lines of mutant mice have impaired function of GlyRs, and it is of interest to ask which behaviors are similarly altered in all mutants. Of the behaviors measured, only increased startle reflex and fluid intake in the ethanol consumption test were consistent across the mutants. Increased startle reflex is the hallmark of reduced GlyR function (Harvey et al., 2008); the increased fluid consumption in the ethanol test is difficult to interpret because it was not seen consistently in the saccharin and quinine consumption tests. Because the Q266I and S267Q mutations eliminated the action of alcohol on GlyR, whereas the M287L mutation (or the *oscillator* deletion) did not, effects of alcohol that are reduced in Q266I and S267Q, but not in M287L or *oscillator*, are of particular interest. The only behavior that fit that pattern was the more rapid recovery from rotarod incoordination found in Q266I and S267Q, but not in M287L (not tested in *oscillator*) (Table 2).

In summary, our results show the ability of a single amino acid change in the GlyR α1 subunit to decrease specific behavioral actions of ethanol and alter other nonethanol behaviors, demonstrating the importance of GlyR function in diverse neuronal systems.

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Authorship Contributions

Participated in research design: Blednov, Homanics, and Harris.
Conducted experiments: Blednov and Benavidez.
Performed data analysis: Blednov, Benavidez, and Harris.
Wrote or contributed to the writing of the manuscript: Blednov, Homanics, and Harris.

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Address correspondence to: Dr. R. Adron Harris, Waggoner Center for Alcohol and Addiction Research, University of Texas, 1 University Station, A4800, Austin, TX 78712-0159. E-mail: harris@mail.utexas.edu

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