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## Mutation of the inhibitory ethanol site in GABA<sub>A</sub> ρ1 receptors promotes tolerance to ethanol-induced motor incoordination

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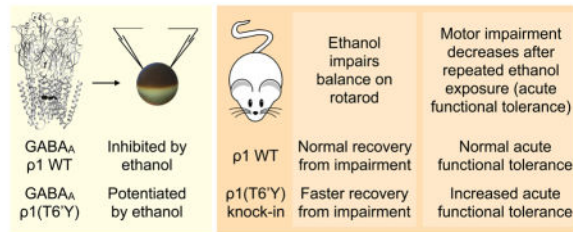
### Abstract

Genes encoding the ρ1/2 subunits of GABA<sub>A</sub> receptors have been associated with alcohol (ethanol) dependence in humans, and ρ1 was also shown to regulate some of the behavioral effects of ethanol in animal models. Ethanol inhibits GABA-mediated responses in wild-type (WT) ρ1, but not ρ1(T6'Y) mutant receptors expressed in *Xenopus laevis* oocytes, indicating the presence of an inhibitory site for ethanol in the second transmembrane helix. In this study, we found that ρ1(T6'Y) receptors expressed in oocytes display overall normal responses to GABA, the endogenous GABA modulator (zinc), and partial agonists (β-alanine and taurine). We generated ρ1 (T6'Y) knockin (KI) mice using CRISPR/Cas9 to test the behavioral importance of the inhibitory actions of ethanol on this receptor. Both ρ1 KI and knockout (KO) mice showed faster recovery from acute ethanol-induced motor incoordination compared to WT mice. Both KI and KO mutant strains also showed increased tolerance to motor impairment produced by ethanol. The KI mice did not differ from WT mice for other behavioral actions, including ethanol intake and preference, conditioned taste aversion to ethanol, and duration of ethanol-induced loss of righting reflex. WT and KI mice did not differ in levels of ρ1 or ρ2 mRNA in cerebellum or in ethanol clearance. Our findings indicate that the inhibitory site for ethanol in GABA<sub>A</sub> ρ1 receptors regulates acute functional tolerance to moderate ethanol intoxication. We note that low sensitivity to alcohol intoxication has been linked to risk for development of alcohol dependence in humans.

### Graphical Abstract

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## Keywords

GABA<sub>A</sub> rho1 receptor; ρ1 knockin and knockout mice; ethanol-induced motor impairment; acute functional tolerance; *Xenopus* oocytes

## 1.1 Introduction

GABA<sub>A</sub> receptors are molecular targets for the action of many drugs in the brain, including barbiturates, benzodiazepines, neurosteroids, and intravenous/volatile anesthetics. Like these drugs, alcohol (ethanol) enhances the function of classical GABA<sub>A</sub> heteromeric receptors (composed mainly of two  $\alpha$ , two  $\beta$ , and one  $\gamma$  or  $\delta$  subunit), but the response of GABA<sub>A</sub>  $\rho$  receptors to ethanol is distinct. Homomeric receptors formed from  $\rho1$  subunits are inhibited by low concentrations of ethanol (Mihic and Harris, 1996), and introduction of a T6'Y mutation in the second transmembrane helix of  $\rho1$  eliminates the inhibitory effect (Borghese et al., 2016).

Although roles for some GABA<sub>A</sub> subunits in the *in vivo* effects of ethanol have been examined (Blednov et al., 2013; Blednov et al., 2011; Boehm et al., 2004; Kumar et al., 2009; Lobo and Harris, 2008),  $\rho$  subunits have not been well studied in brain. In contrast to the retina, where they were first discovered and are highly expressed (Cutting et al., 1992; Cutting et al., 1991),  $\rho$  receptors are present in low levels in the central nervous system (Boue-Grabot et al., 1998; Johnston, 2002). Alcohol addiction researchers became increasingly interested in GABA<sub>A</sub>  $\rho$  receptors when a family-based association analysis linked *GABRR1* and *GABRR2* (genes encoding  $\rho1$  and  $\rho2$ , respectively) with alcohol dependence in humans (Xuei et al., 2010). In addition, a genetic correlation was found between the level of  $\rho1$  expression in the nucleus accumbens and ethanol consumption and motor activation in recombinant inbred mice [genenetwork.org; based on (Demarest et al., 2001; Gill et al., 1996)]. Furthermore, genetic deletion of the  $\rho1$  subunit in mice altered several ethanol-related behaviors (Blednov et al., 2014). For example, reduced ethanol consumption and preference and longer duration of ethanol-induced loss of righting reflex (LORR) were observed in  $\rho1$  knockout (KO) male mice. Interestingly, both male and female KO mice showed faster recovery from acute ethanol-induced motor incoordination and were less sensitive to the development of ethanol-induced conditioned taste aversion (CTA). Some of these behavioral phenotypes were also observed in wild-type (WT) mice after administration of the  $\rho1$  antagonist, (*S*)-4-amino-cyclopent-1-enyl butylphosphinic acid (Blednov et al., 2014).

Based on the discovery of a specific ethanol site in GABA<sub>A</sub> ρ1 receptors and *in vivo* evidence for ρ1 in ethanol responses in animal models, we generated ρ1 (T6'Y) knockin (KI) mice and examined the ethanol-related behaviors that were altered in ρ1 KO mice (Blednov et al., 2014). This allows a comparative study of ethanol's behavioral effects in a genetic model targeting a known ethanol site without the loss of receptor function and potential compensatory changes in other genes that may complicate the KO model. We determined that levels of ρ1 and ρ2 are not altered in the cerebellum of WT and ρ1 KI mice. In addition, our findings show that mutation of the inhibitory ethanol site in ρ1 does not alter normal channel function, but is important for the development of acute tolerance to ethanol-induced motor impairment.

## 1.2 Materials and Methods

### 1.2.1 Two-Electrode Voltage Clamp in *Xenopus* Oocytes

The materials and methods were described in detail in a previous publication (Borghese et al., 2016). Briefly, the responses of human WT and mutant GABA<sub>A</sub> ρ1 receptors expressed in oocytes were studied using two-electrode voltage clamp. Oocytes were placed in a chamber perfused with ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.5), and voltage-clamped at -70 mV. GABA applications lasted for 30–150 seconds and the interval between applications was 5–15 minutes. Drug effects were reversible, and control GABA responses remained relatively constant between applications.

Modulators (ethanol, zinc) were pre-applied for 1 minute and then co-applied with GABA. The application sequence for the WT and mutant ρ1 receptors was as follows: maximal GABA concentration (to obtain maximal response, EC<sub>100</sub>), EC<sub>20</sub> GABA, EC<sub>20</sub> GABA, pre-application of modulator immediately followed by a co-application with EC<sub>20</sub> GABA, EC<sub>20</sub> GABA, repeat with different concentration/modulator. The response to GABA in the presence of the modulator was expressed as a % change compared to the mean of the previous and subsequent GABA responses (either EC<sub>20</sub> GABA or maximal GABA responses). GABAergic partial agonists (β-alanine and taurine) were applied alone at maximal concentrations and the responses induced were expressed as the percentage of the maximal GABA response observed in that oocyte. Statistical analysis was performed using Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA), and statistical significance was determined using t-tests or two-way ANOVA.

### 1.2.2 ρ1 Knockout Mice

Mice lacking the ρ1 subunit of the GABA<sub>A</sub> receptor, B6;129S4-*Gabbr1*<sup>tm1Llu/J</sup> (stock # 010535), were purchased from The Jackson Laboratory (Bar Harbor, ME), and the colony was maintained by heterozygous breeding. After weaning, mice were housed in the Animal Resources Center at The University of Texas at Austin with *ad libitum* access to rodent chow and water with 12-h light/dark cycles (lights on at 7:00 AM). Male and female mice between 8 and 12 weeks of age were used. Experiments were approved by the university's Institutional Animal Care and Use Committee and adhered to NIH guidelines for the care and use of laboratory animals. Mouse tail tips were shipped to the GenoTyping Center of America (Ellsworth, ME) where DNA was extracted and genotypes were determined using

SYBR-labeled PCR followed by melting curve analysis. Two different assays were used to detect each allele [WT primer set 1: ATGTTGGCTGTCCAGAATATG (F), CTTTCCTAGATGGCTCATGAAC (R); WT primer set 2: CAAGTTAAGCCGAGGTTGCC (F), GACTCCTCCAAGCTGATTTCA (R); KO primer set 1: TTGGGTGGAGAGGCTATTTCG (F), CTTCCCGCTTCAGTGACAAC (R); KO primer set 2: AGACAATCGGCTGCTCTGAT (F), AGTGACAACGTCGAGCACAG (R).

### 1.2.3 CRISPR/Cas9 and Repair Template Production

An sgRNA targeting  $\rho 1$  in exon 9 near the intended mutation site was identified using the CRISPR Design Tool (Hsu et al., 2013). Two overlapping PCR primers (F: GAAATTAATACGACTCACTATAGGTTTATGGCAGGCATCACCAAGTTTTAGAGCTAGAAATAGC; R: AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATTTCTAGCTCTAAAAC) were used to generate a T7 promoter containing sgRNA template as described (Bassett et al., 2013). This template was transcribed *in vitro* using a MEGAshortscript kit (Ambion, Inc., Austin, TX). The Cas9 coding sequence was amplified from pX330 (Cong et al., 2013) using a T7 promoter containing forward primer (tattacgactcactataggGAGAATGGACTATAAGGACCACGAC) and reverse primer (GCGAGCTCTAGGAATTCTTAC) and subcloned into pCR2.1-TOPO. This plasmid was linearized with EcoRI, *in vitro* transcribed, and polyA-tailed using the mMessage mMachine T7 Ultra Kit (Ambion). Following synthesis, the sgRNA and Cas9 mRNA were purified using the MEGAclean Kit (Ambion), ethanol precipitated, and resuspended in DEPC-treated water. A 120-nucleotide single stranded DNA repair template oligo harboring the desired mutations in exon 9 of  $\rho 1$  was purchased as Ultramer DNA (Integrated DNA Technologies, Coralville, IA).

### 1.2.4 Production of $\rho 1$ Knockin Mice

sgRNA (25 ng/ $\mu$ l), Cas9 mRNA (50 ng/ $\mu$ l), and repair oligo (100 ng/ $\mu$ l) were combined in embryo injection buffer (10 mM Tris, pH 7.4, 0.1mM EDTA), aliquoted, and stored at  $-80^{\circ}\text{C}$  until use. C57BL/6J one-cell embryos were collected from super-ovulated females and cultured in KSOM at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2/95\%$  air. Embryos were briefly transferred to M2 medium and the nucleic acid mixture was injected into the cytoplasm as described (Yang et al., 2014). Embryos that survived injection were transferred to the oviduct of day 0.5 postcoitum pseudopregnant CD-1 recipient females. Pups resulting from injected embryos were screened for DNA sequence changes in exon 9 of the  $\rho 1$  gene by PCR/DNA sequence analysis. Briefly, a 388 bp amplicon spanning exon 9 was PCR amplified with forward (AGATGAGGGGCAGAGAACCT) and reverse (CTTCTCCCGAAGCTTCCGTT) primers. PCR products were sequenced directly or subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced to verify that the T298Y (T6'Y) mutation was present.

### 1.2.5 Off-Target Analysis

The sgRNA sequence (GTTTATGGCAGGCATCACCA) was run through the Off-Targets tool of the Cas9 Online Designer site (Guo et al., 2015). The top 8 predicted off-targets (Supplemental Table S1) were amplified from male Founder mouse #4110 DNA and sequenced.

### 1.2.6 Mouse Genotype Analysis

Mice were genotyped in-house using PCR followed by HincII restriction fragment length polymorphism analysis or mouse tail tips were shipped to the GenoTyping Center of America, where DNA was extracted and the KASP genotyping system (LGC Genomics, Middlesex, UK) was used with a proprietary assay.

### 1.2.7 RT-PCR and Sequence Analysis of Transcripts

Cerebellar tissue from 16 WT (n=8 females, n=8 males) and 16 p1 Kl (n=8 females, n=8 males) mice was dissected, flash-frozen in liquid N<sub>2</sub>, and stored at -80°C. Total RNA was isolated using the MagMax-96 for microarrays kit (Ambion). RNA concentration and purity were determined by UV spectrometry (Nanodrop; Thermo Scientific), and overall RNA integrity was assessed using a 2200 TapeStation (Agilent Technologies, Santa Clara, CA). Each RNA sample was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Portions of the p1 transcript were PCR-amplified from cDNA of one male and one female of each homozygous genotype using five different primer sets (Supplemental Table S2). PCR products were analyzed on 2% agarose, excised from the gel with a clean razor blade and purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Purified PCR products were sequenced in both directions using the amplification primers.

### 1.2.8 RT-PCR Measurement of $\rho$ Receptors in Cerebellum

qPCR was performed in triplicate for 90 ng of each cDNA (see above) using SsoAdvanced Universal Probes Supermix, according to manufacturer's instructions (Bio-Rad, Hercules, CA). FAM-labeled TaqMan Gene Expression Assays (Applied Biosystems) were used to amplify *Gabrr1* (Mm01212386\_m1), *Gabrr2* (Mm00433507\_m1) and *Gusb* (Mm01197699\_s1). RT-qPCR results were analyzed with CFX Manager, version 3.1 (Bio-Rad), using the single threshold Cq determination and  $\Delta\Delta Cq$  method (Pfaffl, 2001). Data were normalized to the reference gene *Gusb*, which demonstrated minimal variation among mean sample Cq values (range of 0.26). Data from WT and mutant mice were compared using an unpaired t-test.

### 1.2.9 Recovery from Ethanol-Induced Motor Incoordination

Mice were trained on a fixed-speed rotarod (Economex; Columbus Instruments, Columbus, OH) at 10 rpm, and training was considered complete when mice were able to remain on the rotarod for 60 seconds. Every 15 minutes 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 seconds.

### 1.2.10 Acute Functional Tolerance

Acute functional tolerance (AFT) to the motor ataxic effects of ethanol was measured using the two-dose method originally designed by (Erwin and Deitrich, 1996). Ethanol-naïve mice were trained to balance on a fixed-speed rotarod (10 rpm) for a 60-second period. After basal training, mice were injected with ethanol (1.75 g/kg, i.p.) and placed back on the rotarod until they fell off. Mice were tested in 5-minute intervals until they regained the

ability to balance on the rotarod for 60 seconds. Once this was achieved (t1), a retro-orbital blood sample was collected to measure blood ethanol concentration (BEC1). Mice were then immediately given a second ethanol injection (2 g/kg, i.p.). After losing the ability to remain on the rotarod, mice were tested in 5-minute intervals until they regained the ability to balance for 60 seconds (t2). Then a second blood sample was collected for BEC determination (BEC2). BECs were measured as described below. AFT was defined as the difference in BEC at t2 versus t1 (BEC2 - BEC1).

### 1.2.11 Two-Bottle Choice Ethanol Drinking

The 24-hour two-bottle choice protocol was carried out as previously described (Blednov et al., 2014; Blednov et al., 2003). Mice were allowed to acclimate for 1 week to individual housing. Two drinking tubes were continuously available, food was available *ad libitum*, and mice were weighed every 4 days. After 4 days of water consumption (both tubes), mice were given access to water and a series of increasing ethanol concentrations (3–15% ethanol (v/v), each concentration was given for 4 days). Tube positions were changed daily to control for potential side preferences, and tubes were weighed daily before and after removal from the experimental cages. The quantity of ethanol consumed (g/kg body weight/24 hours) was calculated for each mouse, and values were averaged for each concentration of ethanol tested. Evaporation/spillage estimates were calculated daily from 2 bottles placed in an empty cage (1 bottle contained water and the other contained the appropriate ethanol solution). Statistical analysis of behavioral data was performed using Prism (GraphPad Software, Inc.), and statistical significance was determined using two-way ANOVA and Bonferroni post hoc tests.

### 1.2.12 Conditioned Taste Aversion

Mice were adapted to a water restriction schedule (2 hours of water/day) over a 7-day period. At 48-hour intervals over the next 10 days, mice had access to a solution of saccharin (0.15% w/v sodium saccharin in tap water) for 1 hour. Immediately after access to saccharin, mice received injections of saline or ethanol (2.5 g/kg, i.p.). Mice had 30-minute access to tap water 5 hours after each saccharin-access period to prevent dehydration. On intervening days, mice had 2-hour continuous access to water at standard times in the morning. Reduced consumption of the saccharin solution is used as a measure of CTA.

### 1.2.13 Loss of Righting Reflex

Sensitivity to the depressant effects of ethanol (3.8 g/kg, i.p.) and ketamine (175 mg/kg, i.p.) was determined using the 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 3 times within 30 seconds. Sleep time was defined as the time from being placed in the supine position until they regained their righting reflex.

### 1.2.14 Ethanol Clearance

Animals were injected with a single dose of ethanol (4 g/kg, i.p.), and blood samples were taken from the retro-orbital sinus 30, 60, 120, 180, and 240 minutes after injection. Samples (~20  $\mu$ l) were collected into capillary tubes and centrifuged for 6 minutes at 3100g in a

Haemospin 1400 centrifuge (Analox Instruments, London, UK). Plasma samples were stored at  $-20^{\circ}\text{C}$  until BECs were determined in 5- $\mu\text{l}$  aliquots using an AM1 Alcohol Analyzer (Analox Instruments). The machine was calibrated every 15 samples using an industry standard, and BECs were determined using commercially available reagents according to the manufacturer's instructions. Samples were averaged from duplicate runs and expressed as mg/dl.

## 1.3. Results

### 1.3.1 Ethanol Modulation of $\rho 1$ Receptors in *Xenopus* Oocytes

WT and mutated GABA<sub>A</sub>  $\rho 1$  receptors were expressed in *Xenopus laevis* oocytes, and the effects of increasing concentrations of ethanol (20–200 mM) on EC<sub>20</sub> GABA responses were recorded (Figure 1A). Ethanol inhibited GABA responses in a concentration-dependent manner in WT  $\rho 1$  receptors as previously reported (Mihic and Harris, 1996). In contrast, 20–200 mM ethanol potentiated GABA responses in a concentration-dependent manner in  $\rho 1(\text{T6}'\text{Y})$  receptors [two-way repeated measures ANOVA: effect of interaction  $F_{(2,24)} = 103.8$ ,  $p < 0.0001$ ; effect of concentration  $F_{(3, 24)} = 16.69$ ,  $p < 0.0001$ ; effect of receptor  $F_{(1,8)} = 492.1$ ,  $p < 0.0001$ ], in agreement with previous results using 100–200 mM ethanol (Borghese et al., 2016).

Although the mutation is near the pore of the channel, the sensitivity to GABA was not altered (GABA EC<sub>50</sub> = 0.69 for both  $\rho 1$  and  $\rho 1(\text{T6}'\text{Y})$ , 95% confidence intervals 0.65 to 0.75, and 0.60 to 0.79, respectively) (Borghese et al., 2016). The maximal GABA-induced current was also not different in  $\rho 1$  and  $\rho 1(\text{T6}'\text{Y})$  receptors (Figure 1B). To further examine overall receptor function, we tested the endogenous modulator, zinc, and partial agonists ( $\beta$ -alanine and taurine) that are also found *in vivo*. Unlike modulation by ethanol, the effects of zinc and  $\beta$ -alanine were similar in  $\rho 1(\text{T6}'\text{Y})$  and WT receptors (Table 1). Taurine's partial agonist activity was significantly increased in  $\rho 1(\text{T6}'\text{Y})$  receptors, but at 1 mM it only activated the channel by 3% of the response evoked by a maximal GABA concentration, suggesting that the marginal increase in mutant receptors would not impact receptor function compared to WT responses.

### 1.3.2 Production of $\rho 1$ KI Mice

The CRISPR/Cas9 RNA guided nuclease and a single stranded DNA oligonucleotide were used to mutate the threonine codon at position 298 of  $\rho 1$  to tyrosine (required ACC to TAT substitutions) as illustrated in Figure 2A. In addition, a silent single base substitution (C to T) in codon 301 was made for genotyping that did not alter the encoded leucine but did create a HincII restriction site in the KI allele. PCR/DNA sequence analysis of  $\rho 1$  exon 9 revealed that of the 24 mice derived from injected embryos, 11 were WT, 10 had indels, and 3 harbored KI alleles. The three KI founders were as follows. Founder 8.3 harbored the 3 mutations that changed T298Y but lacked the silent mutation for genotyping. Founder 4109 (a female) was heterozygous for WT and the KI allele including the silent mutation. Founder 4110 (a male) appeared to harbor 3 alleles: WT, a perfect KI, and a KI allele that lacked the silent mutation.

Analysis of the predicted top 8 sgRNA off-target mutation sites in Founder mouse #4110 revealed no off-target mutations in 7 of these sites. However, off-target site #4 (chromosome 19) was found to harbor an indel (data not shown).

Founder 4110 was mated to C57BL/6J females to establish the line reported here. All offspring were genotyped for  $\rho 1$  using the PCR/restriction fragment length polymorphism approach illustrated in Figure 2B. From 25 offspring derived from this founder, 4 were WT or harbored the KI allele that lacked the silent mutation (these cannot be distinguished by this RFLP assay), 11 were heterozygous KI, and 10 were heterozygous WT with a KI allele that also had an ~80 bp duplication (designated as WT/KI\*; see Figure 2B). Note that following digestion with HincII, WT produces 51 and 337 bp fragments. In contrast, digestion of the KI allele with HincII produces fragments of 51, 130, and 207 bp. The KI with duplication produced bands of 51, 207, and ~417 bp. All WT/KI animals were also analyzed for off-target site #4. Five of 11  $\rho 1$  WT/KI animals were found to be WT at off-target site #4; 6 animals were mutated at this locus and were discarded. The  $\rho 1$  exon 9 containing amplicon from all WT/KI F1 animals that were shipped from Pittsburgh to the University of Texas at Austin for establishment of a breeding colony were sequenced to verify the fidelity of the mutated locus. Exemplar DNA sequence chromatograms are shown in Figure 2C. In the KI sample, note the presence of the double peaks (marked with green \*), demonstrating heterozygosity where the 4 substitutions were made. Heterozygotes were interbred to produce homozygous and WT mice.

Homozygotes of both sexes were overtly normal and grossly indistinguishable from littermates. RT-PCR products spanning exon 3 through most of exon 10 of  $\rho 1$  were sequenced in a male and female of each homozygous genotype. All samples produced the expected sequence with no additional alterations (data not shown).

### 1.3.3 Recovery from Ethanol-Induced Motor Impairment

When mice were trained to balance on a fixed-speed rotarod, we observed no group differences in basal training before ethanol injection and no differences in initial responses measured 15 minutes after injection. However, both male (effect of genotype:  $F_{(1,80)} = 125$ ,  $p < 0.001$ ; effect of time:  $F_{(7,80)} = 203$ ,  $p < 0.001$ ; time x genotype interaction:  $F_{(7,80)} = 21$ ,  $p < 0.001$ ) and female (effect of genotype:  $F_{(1,80)} = 26$ ,  $p < 0.001$ ; effect of time:  $F_{(7,80)} = 129$ ,  $p < 0.001$ ; time x genotype interaction:  $F_{(7,80)} = 5.8$ ,  $p < 0.001$ )  $\rho 1$  KI mice demonstrated faster recovery than WT mice from the acute motor incoordination produced by ethanol (2 g/kg, i.p.; Figure 3A, B).

### 1.3.4 Acute Functional Tolerance to Ethanol-Induced Motor Impairment

A rotarod test was also used to assess acute, rapid tolerance to the ataxic effects of ethanol. The time to recover from motor incoordination after the first and second ethanol injections was significantly shorter in  $\rho 1$  KI compared with WT mice (effect of genotype:  $F_{(1,27)} = 103$ ,  $p < 0.001$ ; Figure 4A). The BEC measured after the first ethanol exposure (BEC1) did not differ between genotypes, whereas BEC2 was significantly higher in  $\rho 1$  KI compared with WT mice (effect of genotype:  $F_{(1,27)} = 21.5$ ,  $p < 0.001$ ; Figure 4B). The difference in recovery time between the second and first ethanol injections was significantly shorter in  $\rho 1$



KI mice compared to controls ( $p < 0.001$ ; Figure 4C). This resulted in greater AFT (defined as BEC2 - BEC1) to ataxia in KI mice compared to controls ( $83.7 \pm 8.2$  versus  $36.3 \pm 7.9$ , respectively,  $p < 0.001$ ; Figure 4D). No sex differences were found, and data from male and female mice were combined.

In our previous study of  $\rho 1$  KO mice (Blednov et al., 2014), we did not measure AFT to ethanol-induced motor impairment so we performed that test using KO mice in the current study. Similar to  $\rho 1$  KI mice, the time to recover after the first and second ethanol exposures was significantly shorter in  $\rho 1$  KO compared with WT mice (effect of genotype:  $F_{(1,41)} = 594$ ,  $p < 0.001$ ; Figure 4E). The BEC measured after the first ethanol injection (BEC1) did not differ between genotypes, but BEC2 was significantly higher in KO compared with WT mice (effect of genotype:  $F_{(1,41)} = 11.4$ ,  $p < 0.01$ ; Figure 4F). The difference in recovery time between the second and first ethanol injections was significantly shorter in KO mice compared to controls ( $p < 0.001$ ; Figure 4G). Thus, AFT (BEC2 - BEC1) increased in KO mice compared to controls ( $61.5 \pm 8.4$  versus  $37.4 \pm 7.3$ , respectively,  $p < 0.001$ ; Figure 4H). No sex differences were found, and data from male and female mice were combined.

### 1.3.5 Ethanol Consumption

In the two-bottle choice paradigm in which mice could drink either water or increasing concentrations of ethanol, no differences in the amount of ethanol consumed or preference for ethanol were found between WT and  $\rho 1$  KI mice of either sex (Figure 5). Female mice drank more ethanol than males, but there were no genotype differences.  $\rho 1$  KI female mice demonstrated slightly reduced total fluid intake compared to WT (effect of genotype:  $F_{(1,18)} = 7.8$ ,  $p < 0.05$ ; genotype x concentration interaction effect:  $F_{(5,90)} = 2.4$ ,  $p < 0.05$ ; Figure 5F). There was also a trend toward reduced total fluid intake in  $\rho 1$  male mice, but this did not reach significance ( $F_{(1,18)} = 3.1$ ,  $p = 0.09$ ; Figure 5C).

### 1.3.6 Conditioned Taste Aversion

There were no differences in consumption of saccharin during trial 0 (before conditioning) between WT and  $\rho 1$  KI mice, respectively ( $94.2 \pm 5.3$  and  $99.1 \pm 3.7$  g/kg body weight for female mice;  $84 \pm 3.8$  and  $81.1 \pm 2.9$  g/kg body weight for male mice). In order to minimize initial fluctuations in saccharin intake and any small differences between sexes, consumption 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). Ethanol-saccharin pairings reduced saccharin intake across trials compared with saline-saccharin pairings, indicating the development of CTA in male mice of both genotypes (effect of treatment in WT males:  $F_{(1,14)} = 13.9$ ,  $p < 0.01$ ; effect of treatment in KI males:  $F_{(1,18)} = 4.4$ ,  $p < 0.05$ ; Figure 6A), as well as in female mice of both genotypes (effect of treatment in WT females:  $F_{(1,12)} = 24.7$ ,  $p < 0.001$ ; effect of treatment in KI females  $F_{(1,14)} = 19.9$ ,  $p < 0.001$ ; Figure 6B). No genotype differences were found between saline- or ethanol-treated groups for either sex.

### 1.3.7 Loss of Righting Reflex

In our previous study, ethanol and ketamine significantly prolonged the duration of LORR in  $\rho 1$  KO compared to WT mice (Blednov et al., 2014). However in the current study, the

duration of ethanol- or ketamine-induced LORR did not differ in WT or  $\rho 1$  KI mice (Figure 7A, B). Because there were no sex differences in the sedative effects of these drugs, data from male and female mice were combined.

### 1.3.8 Ethanol Clearance

There were no genotype differences in BECs measured over 4 hours after injection of ethanol (4 g/kg, i.p.) in male or female mice (Figure 8A, C) and no differences in the slopes of the curves (Figure 8B, D).

### 1.3.9 $\rho 1/\rho 2$ mRNA Levels

To examine potential expression changes that could contribute to the behavioral differences between genotypes, we measured mRNA levels of  $\rho 1$  and  $\rho 2$  GABA<sub>A</sub> receptor subunits in the cerebellum. Compared with most other brain regions, this area contains higher levels of  $\rho$  subunits (Boue-Grabot et al., 1998), and is also important for motor coordination. The ratios (KI/WT) of  $\rho 1$  and  $\rho 2$  mRNA levels were 0.98 and 0.93, respectively, and showed no statistical difference from 1.0, indicating that the  $\rho 1$  mutation does not alter  $\rho 1$  or  $\rho 2$  mRNA abundance in cerebellum.

## 1.4 Discussion

In contrast to classical heteromeric GABA<sub>A</sub> receptors, homomeric  $\rho 1$  receptors are inhibited by ethanol (Mihic and Harris, 1996) and have other distinctive functions (Naffaa et al., 2017). The  $\rho 1$  subunit was shown to have a specific inhibitory site for ethanol (Borghese et al., 2016). Introduction of a T6'Y mutation in the second transmembrane helix of  $\rho 1$  transforms the inhibitory ethanol effects into potentiated GABA responses, analogous to conventional GABA<sub>A</sub> receptors. In this study, we show that  $\rho 1$ (T6'Y) GABA<sub>A</sub> responses are potentiated by ethanol in a concentration-dependent manner, but the point mutation does not alter channel modulation by GABA, the endogenous modulator (zinc) or the partial agonist  $\beta$ -alanine. Although taurine activation of  $\rho 1$ (T6'Y) receptors increased slightly (3% of the maximal GABA-induced current), it is unlikely that this would be physiologically significant and would not explain the differential effects of ethanol on these receptors. We then generated  $\rho 1$ (T6'Y) KI mice and examined the behavioral effects of ethanol that were altered in  $\rho 1$  KO mice (Blednov et al., 2014). Compared to KO mice, KI mice allow us to distinguish the importance of loss of receptor function from the ethanol inhibition of function. Considering our evidence that the mutation does not alter normal channel function and does not alter levels of  $\rho 1$  or  $\rho 2$  receptors in cerebellum, the KI mice constructed here provide a valuable animal model to study the effects of ethanol on  $\rho 1$  function *in vivo*.

We previously reported increased hypnotic effects of ethanol but faster recovery from ethanol-induced motor incoordination in  $\rho 1$  KO mice (Blednov et al., 2014). Other changes, which were sex-specific, included reduced ethanol intake and preference in male  $\rho 1$  KO mice and reduced development of ethanol-induced CTA in female  $\rho 1$  KO mice. The main behavioral differences between WT and  $\rho 1$  KO mice (faster recovery from acute ethanol intoxication and increased hypnotic effects of ethanol) were also observed in WT mice after administration of a  $\rho 1$  antagonist (Blednov et al., 2014).

Our current study shows that a point mutation (T6'Y) in the second transmembrane helix of  $\rho 1$  promotes recovery from acute motor incoordination likely by promoting the development of tolerance to ethanol (but not clearance of ethanol).  $\rho 1$  KI mice developed higher AFT to ethanol-induced motor impairment than WT mice, as previously observed in global  $\rho 1$  KO mice. In contrast, lower AFT to ethanol-induced motor ataxia was found in KI mice harboring ethanol-insensitive  $\alpha 1$  GABA<sub>A</sub> receptor subunits (Werner et al., 2009). Thus, specific subtypes of GABA<sub>A</sub> receptors may regulate acute tolerance to ethanol intoxication. It is somewhat surprising that both deletion of the  $\rho 1$  subunit (KO mice) and elimination of ethanol inhibition of  $\rho 1$  (KI mice) produced an increase in the development of tolerance. One might expect elimination of a site of ethanol action to reduce the drive for tolerance and that removal of function (KO) would produce opposite effects from removal of inhibition of function (KI). This paradox may reflect our lack of understanding of the role of  $\rho 1$  receptors in brain circuitry and function.

Male and female  $\rho 1$  KI mice did not differ from WT mice in ethanol consumption, CTA to ethanol, or the hypnotic effects of ethanol or ketamine. Because these effects of ethanol were altered in the KO mice, it is likely that the  $\rho 1$  subunit is important for these behaviors, not because ethanol is acting on the receptor to inhibit function, but because of an intrinsic role of this receptor in neuronal inhibition. Alternatively, the phenotypes that are observed in KO mice could be due to compensatory changes in other ethanol targets that are unchanged in the KI mice.

Alcohol use disorder (AUD) has a strong genetic component (Kendler et al., 2011; Merikangas et al., 1998), and an individual's sensitivity and tolerance to alcohol are important factors in AUD risk. Sensitivity to an adverse effect of alcohol, such as ataxia and sedation, may influence the amount an individual consumes and predict future patterns of alcohol use. A classic study by (Schuckit, 1985) found that non-alcohol dependent men with a positive family history for alcoholism were significantly less sensitive to alcohol compared to those with a negative family history. A low level of response and positive family history were both found to be significant predictors of future alcohol problems (Schuckit, 1994), presumably because reduced sensitivity would allow individuals to consume larger quantities of alcohol before experiencing adverse effects such as ataxia. Reduced sensitivity to alcohol in humans, as in our mouse studies, may result from greater development of acute tolerance (Newlin and Thomson, 1991).

Our findings that genetic manipulations of  $\rho 1$  receptors regulate acute tolerance to the ataxic effects of ethanol may provide a phenotypic explanation for the study showing that genes encoding  $\rho 1/2$  subunits are associated with alcohol dependence (Xuei et al., 2010). Although overall changes in ethanol consumption were not different in  $\rho 1$  mutant mice, with the exception of  $\rho 1$  KO male mice, (Blednov et al., 2014), it is important to note that changes in AFT to ethanol-induced motor impairment are not always accompanied by changes in ethanol intake in rodents (Erwin et al., 2000; Fritz et al., 2014; Fritz et al., 2013; Shram et al., 2004; Wallace et al., 2007). Furthermore, in this study we only examined ethanol consumption using the two-bottle free choice assay. Other tests of ethanol consumption may reveal differences that are not detected with the two-bottle free choice assay, although we

note that there were no changes in two other drinking tests (two-bottle choice intermittent and drinking-in-the-dark) in  $\rho 1$  KO mice (Blednov et al., 2014).

In conclusion, our collective studies using  $\rho 1$  mutant mice point to a key role for this GABA<sub>A</sub> receptor subunit in the central actions of ethanol and the development of acute tolerance. The KI mice described here offer a new genetic model for future *in vitro* and *in vivo* studies of ethanol action and drug discovery. Considering the genetic association of  $\rho 1/2$  subunits with alcohol dependence in humans (Xuei et al., 2010), our findings that genetic manipulation of  $\rho 1$  produces increased acute tolerance to intoxication may explain how this receptor contributes to AUD risk.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

<b>KO</b>	knockout
<b>KI</b>	knockin
<b>WT</b>	wild-type
<b>CTA</b>	conditioned taste aversion
<b>LORR</b>	loss of righting reflex
<b>AFT</b>	acute functional tolerance
<b>BEC</b>	blood ethanol concentration

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**Highlights**

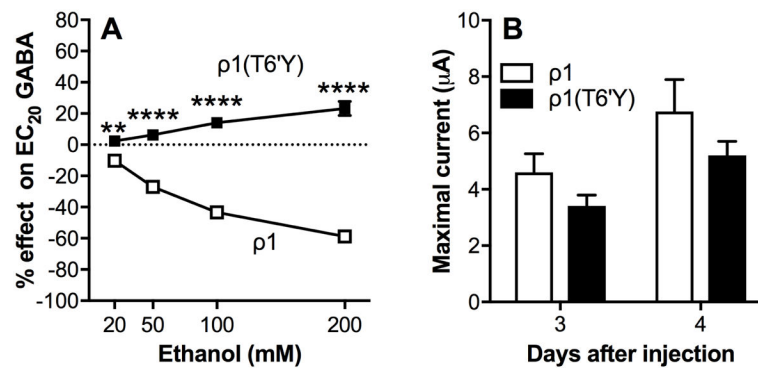
$\rho 1$  and  $\rho 1(T6'Y)$  receptors differ in ethanol but not GABA responses

$\rho 1(T6'Y)$  knockin mice were generated to test the *in vivo* role of the ethanol site

$\rho 1$  knockin and knockout mice recover faster from ethanol-induced ataxia

$\rho 1$  knockin and knockout mice show increased acute functional tolerance

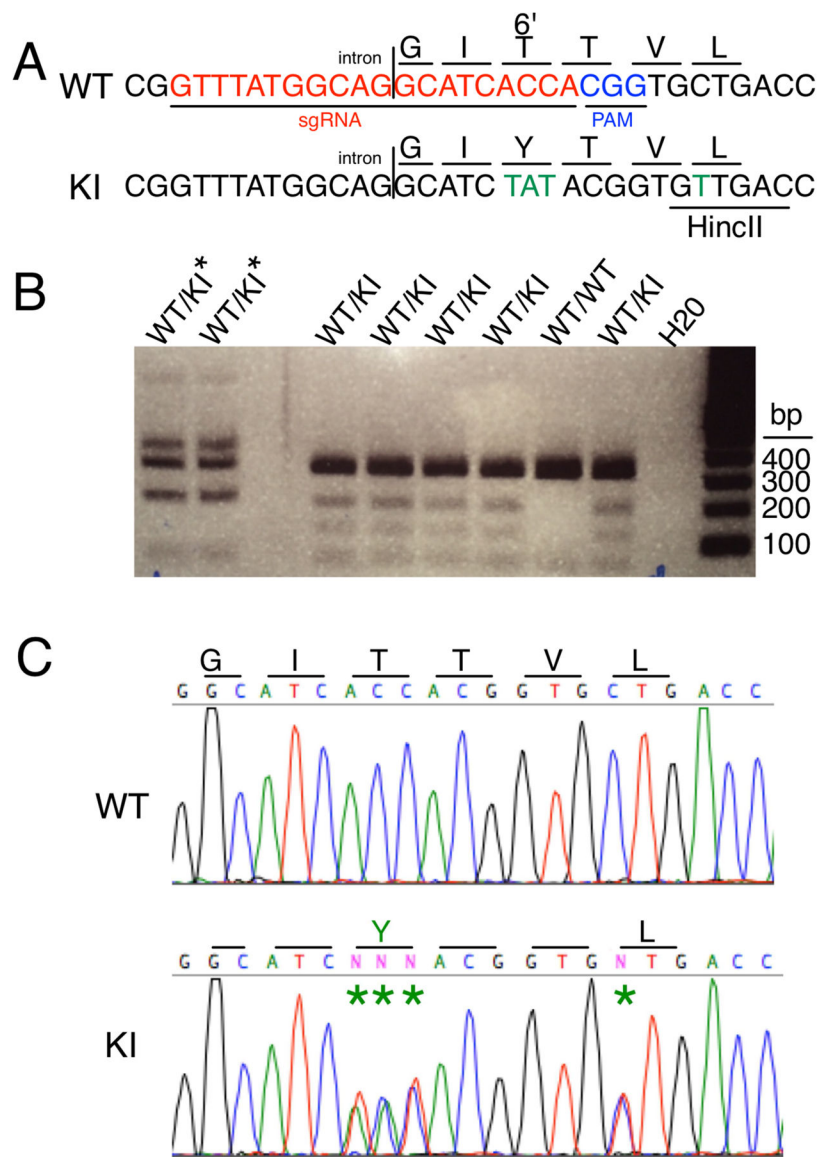
$\rho 1$  regulates tolerance to ethanol intoxication, which may increase ethanol abuse



**Figure 1. Ethanol concentration-response curves and maximal GABA-induced currents in  $\rho 1$  and  $\rho 1(T6'Y)$  receptors**

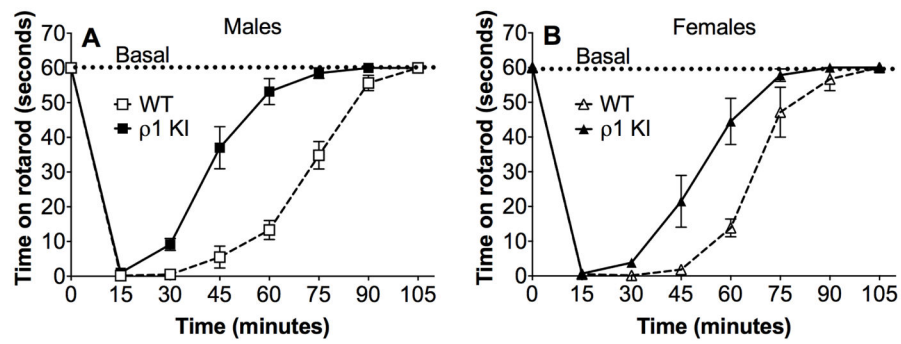
**A.** Increasing concentrations of ethanol were applied to WT and mutated  $\rho 1$  receptors expressed in oocytes, and effects on EC<sub>20</sub> GABA responses were recorded. Data are presented as mean  $\pm$  SEM (error bars smaller than symbols). Data were analyzed using two-way ANOVA with Sidak's multiple comparisons post hoc test: \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  versus WT ( $n = 5$  oocytes). **B.** Maximal GABA-induced currents at 3–4 days after injection ( $n = 13$ –18). Two-way ANOVA did not indicate a significant interaction or a difference between WT and mutant receptors.



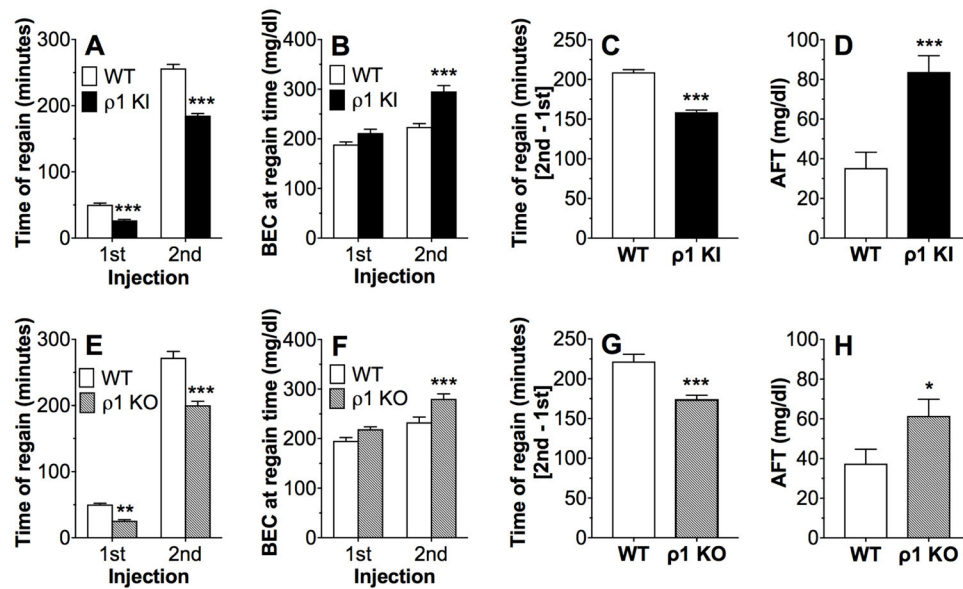


**Figure 2.  $\rho 1$  KI mouse strategy and characterization**

**A.** Partial  $\rho 1$  WT and KI mouse genomic DNA sequence. The sequence used to create the sgRNA sequence for CRISPR/Cas9 gene targeting is shown in red. The CRISPR protospacer adjacent motif (PAM) is shown in blue. Note that the KI allele harbors 4 nucleotide substitutions (shown in green). An ACC to TAT substitution changes the threonine (T) codon at the 6' position in the second transmembrane domain to a tyrosine (Y) in the KI. A silent substitution (C to T change) does not alter the leucine (L) that is encoded at the 9' position, but it does create a HincII restriction endonuclease recognition site in the KI that is used for genotype analysis. **B.** Ethidium bromide stained agarose gel of  $\rho 1$  PCR products from WT (WT/WT), heterozygous KI (WT/KI), and heterozygous KI mice that also have an indel (WT/KI\*). **C.** Exemplar DNA sequence chromatograms from PCR products derived from a WT and a heterozygous KI mouse. Altered nucleotides are marked with a green asterisk.

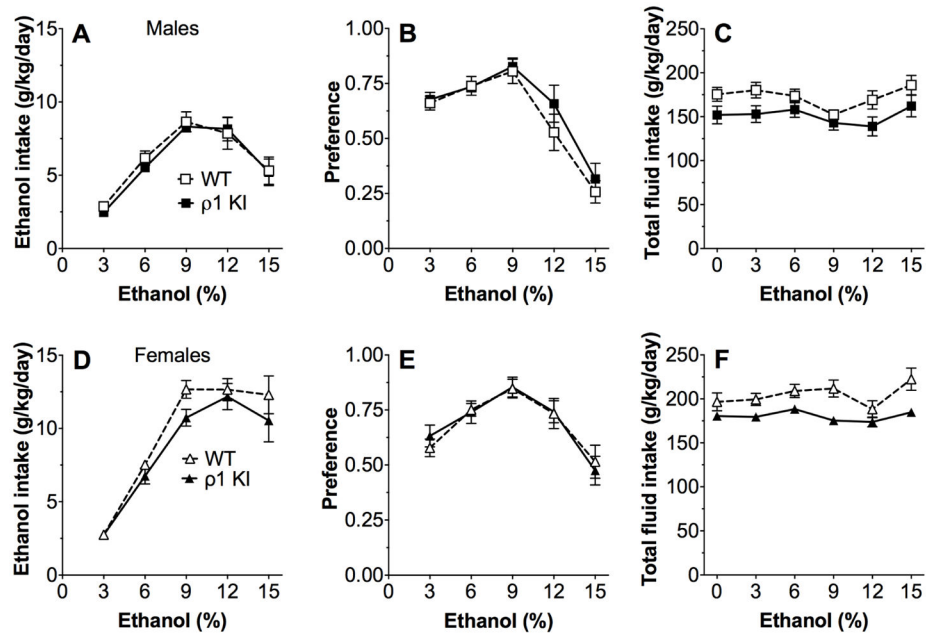


**Figure 3. Recovery from ethanol-induced motor incoordination in  $\rho 1$  KI and WT mice**  
 Time (in seconds) on the rotarod after injection of ethanol (2 g/kg, i.p.) in (A) male and (B) female mice. Data are presented as mean  $\pm$  SEM (n = 6 per genotype and sex).



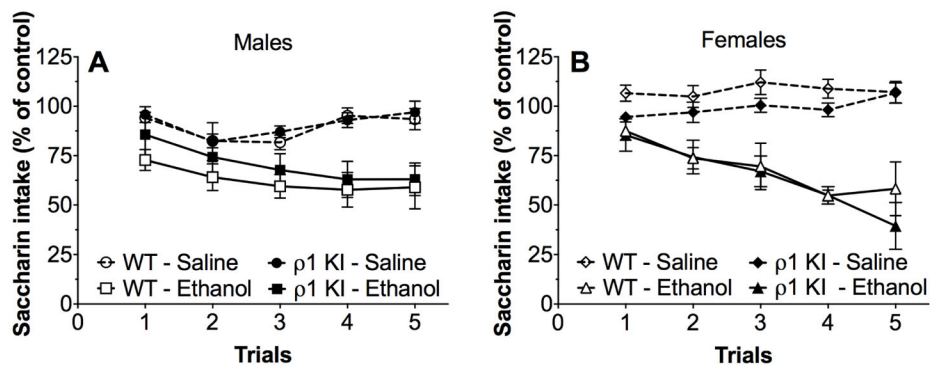
**Figure 4. Acute functional tolerance to ethanol-induced motor incoordination in  $\rho 1$  KI,  $\rho 1$  KO, and WT mice**

Time (in minutes) needed to regain the ability to remain on the rotarod for 60 seconds after two ethanol injections in  $\rho 1$  KI (A) and  $\rho 1$  KO (E) mice compared to controls. BECs (mg/dl) measured at the time of regain after each ethanol injection in  $\rho 1$  KI (B) and  $\rho 1$  KO (F) mice compared to controls. Differences in the time of regain (in minutes) between the second and first ethanol injections in  $\rho 1$  KI (C) and  $\rho 1$  KO (G) mice compared to control mice. Acute functional tolerance (AFT) measured as the differences in BECs (BEC2 - BEC1) after each ethanol injection in  $\rho 1$  KI (D) and  $\rho 1$  KO (H) mice compared to controls. Data are presented as mean  $\pm$  SEM ( $n = 14-15$  for WT and  $\rho 1$  KI mice;  $n = 20-23$  for WT and  $\rho 1$  KO mice; \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to corresponding WT). Data from male and female mice were combined because no sex differences were found.



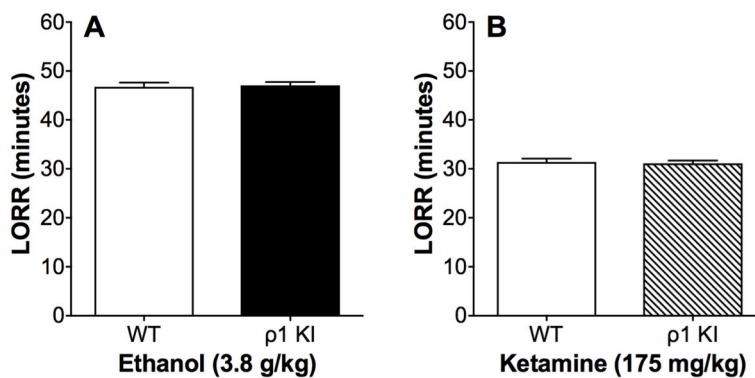
**Figure 5. Ethanol intake in  $\rho 1$  KI and WT mice**

Fluid intake was measured every 24 hours using a two-bottle choice drinking protocol. Each concentration of ethanol was offered for 4 days. **A.** Ethanol intake, **B.** preference for ethanol, and **C.** total fluid intake in male KI and WT mice ( $n = 10$  per genotype). **D.** Ethanol intake, **E.** preference for ethanol, and **F.** total fluid intake in female KI and WT mice ( $n = 10$  per genotype). Data are presented as mean  $\pm$  SEM.



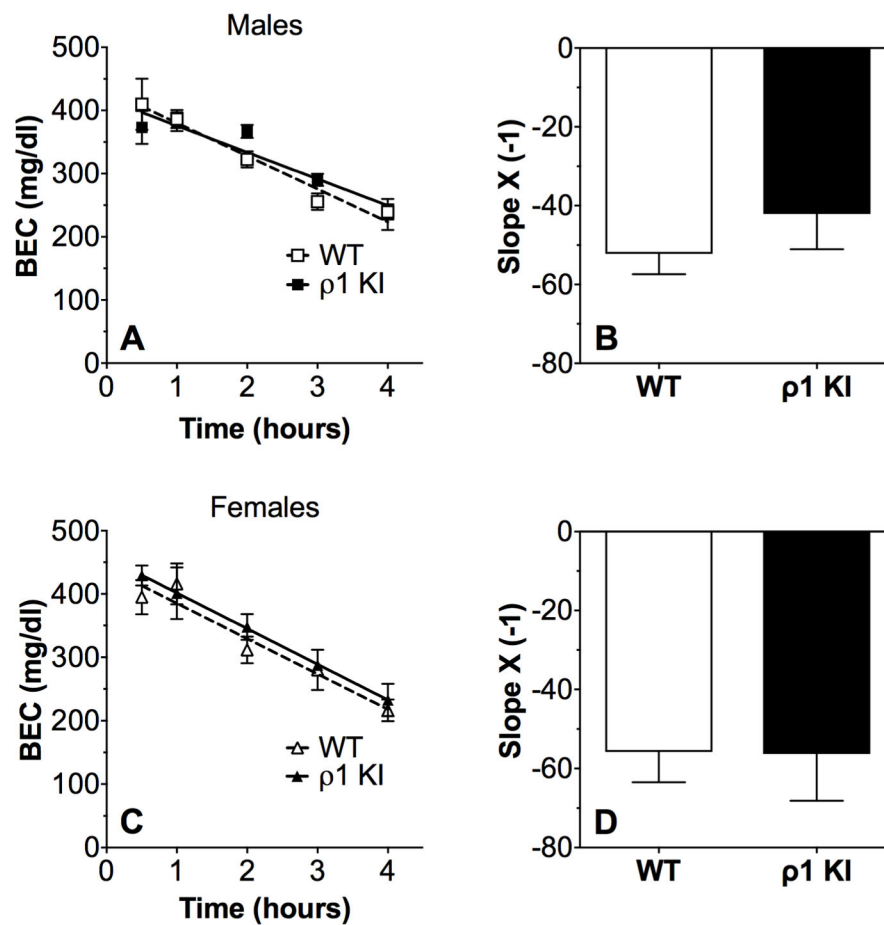
**Figure 6. Conditioned taste aversion in p1 KI and WT mice**

Saccharin intake in (A) male ( $n = 8-11$  per group) and (B) female ( $n = 7-8$  per group) mice was calculated as a percentage of the consumption before conditioning (trial 0). Each trial consists of 1-hour access to saccharin solution (0.15% w/v) followed by saline or ethanol (2.5 g/kg, i.p.) injections on the first day and then limited access to water on the following day (see Methods). Data are presented as mean  $\pm$  SEM.



**Figure 7. Duration of loss of righting reflex in ρ1 KI and WT mice**

**A.** Duration of loss of righting reflex (LORR) after ethanol (3.8 g/kg, i.p.) injection in male and female mice. **B.** Duration of LORR after ketamine (175 mg/kg, i.p.) injection in male and female mice. Data are presented as mean  $\pm$  SEM ( $n = 10-12$  per genotype). Data from male and female mice were combined because no sex differences were found.



**Figure 8. Clearance of blood ethanol in p1 KI and WT mice**

Blood ethanol concentration (BEC) measured over 4 hours in male (A) and female (C) mice after injection of ethanol (4 g/kg, i.p.). Slopes of the curves in male (B) and female (D) mice. Data are presented as mean  $\pm$  SEM (n = 5 per genotype and sex).

**Table 1**

## Channel Modulation by Zinc and Activation by Partial Agonists

Modulator/Partial Agonist	$\rho 1$	$\rho 1(T6'Y)$	n
Zinc (4 $\mu$ M)	$-49 \pm 2$	$-47 \pm 6$	7
$\beta$ -Alanine (1 mM)	$37 \pm 5$	$52 \pm 6$	6-7
Taurine (1 mM)	$1.7 \pm 0.3$	$3.0 \pm 0.4^*$	6-7

Zinc inhibition of GABA responses (shown as % change of the EC<sub>20</sub> GABA responses) and responses evoked by the partial agonists,  $\beta$ -alanine and taurine (shown as % of maximal GABA response), in WT and mutant  $\rho 1$  receptors expressed in oocytes (n = number of oocytes tested; unpaired t-test, \*p < 0.05).

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