Effects of *Gabra2* point-mutations on alcohol intake: Increased binge-like and blunted dependence-inducing drinking by mice

Emily L. Newman, M.S. [1]; Georgia Gunner, B.S. [1]; Polly Huynh, B.S. [1]; Darrel Gachette, B.S. [1]; Stephen Moss, Ph.D. [2]; Trevor Smart, Ph.D.[3]; Uwe Rudolph, M.D.[4,5]; Joseph F. DeBold, Ph.D.[1]; Klaus A. Miczek, Ph.D.[1,2]

[1] Dept. of Psychology, Tufts University; [2] Dept. of Neuroscience, Tufts University;
[3]Dept. of Neuroscience, Physiology and Pharmacology, University College London;
[4] Laboratory of Genetic Neuropharmacology, McLean Hospital;
[5]Dept. of Psychiatry, Harvard Medical School

Communicating author: Emily L. Newman
Tufts University Department of Psychology,
530 Boston Avenue,
Medford, MA 02155, USA
e-mail: emily.newman@tufts.edu, or ELnewman@gmail.com
Phone: 617-627-2792

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Abstract

Background*:* Alcohol use disorders are associated with single nucleotide polymorphisms in *GABRA2*, the gene encoding the $GABA_A$ receptor α2-subunit in humans. Deficient GABAergic functioning is linked to impulse control disorders, intermittent explosive disorder, and to drug abuse and dependence, yet it remains unclear if α2-containing $GABA_A$ receptor sensitivity to endogenous ligands is involved in excessive alcohol drinking.

Methods*:* Male wild-type C57BL/6J and point-mutated mice rendered insensitive to GABAergic modulation by benzodiazepines (H101R), allopregnanolone or THDOC (Q241M), or high concentrations of ethanol (S270H/L277A) at α2-containing $GABA_A$ receptors were assessed for their binge-like, moderate or dependence-inducing drinking using drinking in the dark, continuous access and intermittent access to alcohol protocols, respectively. Social approach by mutant and wild-type mice in withdrawal from intermittent access to alcohol was compared to approach by water-drinking controls. Social deficits in withdrawal were treated with allopregnanolone (0, 3.0, 10.0 mg/kg, i.p.) or midazolam (0, 0.56, 1.0 mg/kg, i.p.).

Results*:* Mice with benzodiazepine-insensitive α2-containing $GABA_A$ receptors (H101R) escalated their binge-like drinking. Mutants harboring the Q241M point-substitution in *Gabra2* showed blunted chronic intake in the continuous and intermittent access protocols. S270H/L277A mutants consumed excessive amounts of alcohol but, unlike wild-types, they did not show withdrawal-induced social deficits.

Conclusions*:* These findings suggest a role for: 1.) H101 for species-typical binge-like drinking, 2.) Q241 for escalated dependence-inducing drinking, and 3.) S270 and/or L277 for the development of withdrawal-associated social deficits. Clinical findings report reduced BZD-binding sites in the cortex of dependent patients; the present findings suggest a specific role for

BZD-sensitive α2-containing receptors. In addition, amino acid residue 241 in *Gabra2* is necessary for positive modulation and activation of $GABA_A$ receptors by allopregnanolone and THDOC; we postulate that neurosteroid action on α2-containing receptor may be necessary for escalated chronic ethanol intake.

Key words: *Gabra2*, alcohol use disorder, binge-like drinking, alcohol withdrawal, chronic alcohol drinking

More than half of American adults consume alcohol at least once a year; yet, only 7% of the population will receive a diagnosis of an alcohol use disorder (AUD). Deficits in inhibitory transmission, particularly in the prefrontal cortex, may increase the risk of developing an AUD and have been linked to impulse control disorders, intermittent explosive disorder, and to drug abuse and dependence (Volkow et al., 1993; Best et al., 2002; Coccaro et al., 2007; Davidson et al., 2000; Heinz et al., 2011). Within the central nervous system, fast synaptic inhibition is mediated, in part, by $GABA_A$ receptors comprised of 2α, 2β, and 1γ subunits surrounding a ligand-gated chloride ion channel (Olsen and Sieghart 2008). Heterogeneity in $GABA_A$ receptor composition can determine sensitivity to endogenous and exogenous receptor modulators including benzodiazepines, neurosteroids, ethanol and general anesthetics (Belelli and Lambert 2005; Farrant and Nusser 2005; Olsen and Sieghart 2008).

Numerous human genetics studies identify a link between alcohol dependence and single-nucleotide polymorphisms (SNPs) in *GABRA2*, the gene encoding the $GABA_A$ receptor α2-subunit protein (Covault et al., 2004; Edenberg et al., 2004; Bierut et al., 2010; Li et al., 2014). Minor allelic variants of these SNPs appear to be inherited together within haplotype blocks in the *GABRA2* gene (Covault et al., 2004; Fehr et al., 2006; Enoch et al., 2009). Guided by research on the association between allelic variants in the human genome and alcohol dependence, the present work employs preclinical genetic mouse models to clarify whether alterations in α2-containing $GABA_A$ receptor sensitivity to alcohol, select neurosteroids, or to benzodiazepines may play a functional role in escalating binge-like or dependence-inducing alcohol consumption.

A major limitation in the field of alcohol research is the enigmatic site of action for clinically relevant doses of alcohol. Using *in vitro* techniques, studies have identified the α4βδ or α6βδ $GABA_A$ subtypes for potentiation of inhibitory currents by low, physiologically relevant concentrations of alcohol: yet, to date, there is no universal agreement on these findings (Suzdak et al., 1986; Mehta and Ticku 1988; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Borghese et al., 2006a; but White et al., 1990; Mihic et al., 1994; Homanics et al., 1997; Borghese and Harris 2007). A second approach is to use rodent models of voluntary alcohol consumption to evaluate the behavior of mice harboring targeted mutations. Although behavior is far-removed from the possible receptor site of action, in the absence of selective pharmacological tools, behavioral studies can reveal which receptor domains may be necessary for non-selective drugs like alcohol to elicit specific behavioral effects.

By introducing targeted point-substitutions in the α2-subunit protein sequence, three mutant mouse strains have been generated with α2-containing $GABA_A$ receptors that are insensitive to modulation by benzodiazepines (*in vitro:* Wieland et al., 1992; Benson et al., 1998; *in vivo:* Low et al., 2000), modulation and activation by allopregnanolone and tetrahydrodeoxycorticosterone (*in vitro:* Hosie et al., 2006, 2009), or modulation by high concentrations of ethanol (*in vitro:* Mihic et al., 1997; Borghese et al., 2006b; *in vivo:* Homanics et al., 2005; Blednov et al., 2011; Werner et al., 2011). Assessing these mutant mice for binge-like, moderate and dependence-inducing alcohol consumption may clarify the relationship between α2-containing $GABA_A$ receptor sensitivity to positive modulators and escalated alcohol consumption.

Methods

Animals

Mutant H101R mice were homozygous for a histidine to arginine point-substitution in *Gabra2*, conferring insensitivity to benzodiazepines at α2-containing $GABA_A$ receptors. H101R mutants were initially backcrossed for fifteen generations to a wild-type C57BL/6J (WT; Jackson Laboratories, Bar Harbor, ME) background to establish a line that is congenic with WT mice. Therefore, experimental H101R mutants and WT mice were generated from filial homozygous breeding pairs.

Experimental mutant S270H/L277A mice were homozygous for a serine to histidine mutation and a gain-of-function leucine to alanine point-substitution in *Gabra2*, rendering them insensitive to some effects of ethanol while maintaining near-normal GABA-responding (Werner et al., 2011). Mutant S270H/L277A mice were bred to a C57BL/6J background for at least six generations at Jackson Laboratories (stock number: 012942), and for two generations in the Tufts Psychopharmacology lab (Medford, MA). Experimental neurosteroid-insensitive Q241M mutants, bred to a C57BL/6J background, were homozygous for a glutamine to methionine point-substitution in *Gabra2*. Homozygous S270H/L277A and Q241M point-mutants and their WT counterparts were bred from heterozygous pairs. Tail samples were collected for genotyping by PCR (Transnetyx, Inc.). Data analyses revealed no differences between ethanol consumption by WT mice generated from heterozygous crosses and WT mice bred from homozygous pairs; therefore all WT groups were collapsed for subsequent analyses and for data portrayal. See **Table 1** for all experimental group *n*s.

At eight-to-ten weeks, experimental mutant and WT males were housed singly to assess individual alcohol or tastant solution intake. Adult wild-type female C57BL/6J mice (n=20) were ovariectomized (OVX) and used as social stimulus mice for social approach testing during withdrawal from alcohol. All mice were housed in clear polycarbonate cages (28x17x14 cm) lined with pine shavings within a temperature-controlled mouse vivarium (21±1 ºC, 30-40% humidity) that was kept on a 12-h photocycle (lights off 0700h). Experimental males and OVX mice received unrestricted access to rodent chow (Purina LabDiet 5001, PMI Nutrition International, Brentwood, MO). With the exception of males assigned to the drinking in the dark protocol, mice received continuous access to tap water. During assessments of fluid intake, solutions were presented in 50 mL centrifuge tubes (Nalgene). Each centrifuge tube was fitted with a rubber stopper (No. 5, Fisher Scientific, Agawam, MA) and a sipper-tube containing two ball bearings (Ancare Corp., Bellmore, NY) to prevent unintentional fluid loss. All animals were cared for in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* (8th ed., 2011) and protocols were approved by the Institutional Animal Care and Use Committee of Tufts University.

Binge-like drinking: Drinking in the dark

Adult mutant H101R, S270H/L277A, Q241M and WT males were assessed for binge-like ethanol intake in their home cages according to the four-day, drinking in the dark (DID) procedure outlined by Rhodes et al., (2005). Three hours into the dark photoperiod, water bottles were replaced with a single 50 mL centrifuge tube containing 20% EtOH (w/v). On days 1-3, mice received 2-hr access to 20% EtOH after which EtOH was replaced with water for the remaining 22-hr. On day 4, binge-like intake was measured over the course of an extended, four-hr access period. Blood samples were then promptly collected from the submandibular vein, centrifuged at 4ºC and plasma (5 µL) was analyzed for blood ethanol concentration (BEC) using the AM-1 Analox Analyzer (Analox Instruments USA; Lunenburg, MA). Binge-like drinking was operationally defined as a pattern of alcohol consumption resulting in a BEC exceeding 80 mg/dL within 4 h.

In an adaptation of the DID protocol, H101R, S270H/L277A, Q241M and WT males were evaluated for their pattern of 20% EtOH binge-like intake using a contact lickometer setup. Each experimental male's home cage was fitted with a custom-made stainless steel panel; sipper-tubes were lowered through a hole in the right side of each panel for fluid presentation. Stainless steel mesh flooring was secured to the bottom of each panel to form a raised platform. To drink, mice stood on this mesh platform and made tongue-contact with the metal sipper-tube, thereby closing a circuit. The mesh platform and sipper-tube were each connected to a contact lickometer controller (MedAssociates; model ENV-250B) which transmitted signals to a MED-PC interface; a lick was recorded each time a closed circuit was detected (detection threshold: >0.001 ms interlick interval). All mice were habituated to the lickometer setup for three days with free access to tap water and rodent chow prior to the four-day DID procedure. Blood was collected immediately after 4-hour access to 20% EtOH on the fourth day of the DID protocol for BEC analysis.

In a second adaptation of DID, we aimed to determine if escalated binge-like drinking in the classic protocol was due to involuntary alcohol intake in the absence of water. According to this adaptation, mice had access to two centrifuge tubes of water for 22h or 20h per day. For the first three days, one water tube was exchanged with 20% EtOH, signifying the beginning of the two-hour, two-bottle choice access period. On the final, binge day, mice received four-hour access to water and 20% EtOH. Because only H101R mutants escalated their binge-like drinking in the one-bottle DID test, only these mutants and WT mice were assessed in this protocol.

For all drinking experiments, mice were weighed daily and centrifuge tubes were weighed prior to and after the EtOH or tastant access period to determine intake volume (mL;

assuming 1g=1mL). Alcohol consumption was calculated as grams of EtOH consumed according to body weight (g/kg) and as percent preference. To control for unintentional fluid loss, bottle measurements were recorded from an empty cage. These values were subtracted from each mouse's mL intake to account for leakage during the fluid access period.

Intermittent and continuous access* to *ethanol

Eight-to-ten-week old mutant H101R, S270H/L277A, Q241M and WT males were assessed for dependence-inducing, voluntary ethanol consumption according to the intermittent access procedure as explained previously by Hwa et al., (2015). In short, three hours into the dark phase on Mondays, Wednesdays and Fridays, mice received two-bottle choice, 24-hr access to tap water and 20% EtOH. On all other days, mice were presented with two centrifuge tubes filled with tap water. To control for side-preference, EtOH presentation alternated between the right and left side of the cage lid. This intermittent schedule of alcohol presentation has been shown to induce escalated EtOH consumption by C57BL/6J WT males (20-25 g/kg/24h; Hwa et al., 2011). To contrast with the escalated levels of alcohol intake observed using the intermittent access procedure, separate adult mutant and WT males either received continuous access to 20% EtOH and water for six weeks (**Fig S1**).

Ascending concentrations of ethanol, sucrose or quinine

Ascending concentrations of EtOH (3, 6, 10, 20% w/v) and water were presented to adult male mutant and WT mice. Each concentration was made available for four consecutive days with presentation alternating sides daily (3% EtOH and water on days 1-4, 6% on days 5-8, 10%

on days 9-12, and 20% on days 13-16). Four-day individual intake averages were calculated for each concentration to account for any side preference.

To determine if preference for palatable and aversive tastants differed between mutant lines, male mutant and wild-type mice were tested for their sucrose (10, 30, 100 mM) and quinine (0.1, 0.3 mM) intake. Ascending concentrations of the tastant solution and water were presented for four days per concentration as detailed for EtOH above. After the final day of access to the highest concentration of sucrose, mice received two weeks of water prior to receiving the lowest concentration of quinine.

Social approach in forced alcohol abstinence

Alcohol withdrawal severity was assessed as an indicator of dependent-like behavior in chronic, alcohol –drinking mice. To do this, we chose to measure deficits in social approach (Knapp et al., 2005; Newman et al., 2015) and defined alcohol dependence as a pattern of drinking that produced withdrawal symptoms in the form of reduced social approach behavior by mice in forced ethanol abstinence as compared to ethanol-naïve, genotype-matched controls (**Fig S1, S2**).

Male mutant and WT mice either received intermittent access to 20% EtOH or access to two bottles of tap water for 16 consecutive weeks (**Fig S1**). During week seven, mice were habituated to intraperitoneal (i.p.) injections and were evaluated for side preference in a three-chamber apparatus. During side preference screens, the male mouse was placed in the center chamber of the three-chamber apparatus; after a 5-min habituation, the two side doors were opened and the mouse was permitted to explore all three chambers for 10-min. Two ethanol-drinking experimental mice (one WT, one H101R) and two EtOH-naïve control mice (one

H101R, one Q241M) were excluded from subsequent social approach testing because they showed a >60% side preference in the absence of a social stimulus animal during these 15-min screening sessions (**Table 1**).

From weeks eight to sixteen, mice were tested weekly for social approach toward a novel OVX stimulus mouse six-to-eight hours after 20% EtOH was replaced with water for EtOH-drinking animals. During social approach testing, the male experimental or control mouse was first held within the central chamber of a three-chamber apparatus for a 5-min habituation period (**Fig S2**). Males were randomly assigned a novel OVX female each social approach test day and no two males received the same female stimulus animal on the same day. As the male habituated to the central chamber, his assigned OVX stimulus mouse was placed in a wire mesh cage in either the right or left chamber. Following habituation, the male received an injection and was returned to the central chamber. Thirteen minutes later, the doors on either side of the central chamber were lifted, allowing the male to move freely between the central chamber, the chamber with the OVX stimulus mouse, and a third chamber with an empty stimulus cage during a 10-min social approach test. EthoVision XT software tracked the male and recorded his total distance travelled (cm) and the duration of time he spent within the social approach zone. The social approach zone was defined as the region extending 2.25 cm past the radius of the occupied stimulus cage.

For the initial social approach test, ethanol-drinking experimental males in forced abstinence and ethanol-naïve controls were treated systemically with vehicle (half received 20% βCD, half received 0.9% saline). To establish which genotypes demonstrated social deficits in withdrawal, two-way analyses of variance (ANOVA) were conducted within each genotype (Forced EtOH abstinence vs. EtOH-naïve; saline vs. 20% BCD vehicle on the first day).

To treat alcohol withdrawal symptoms and recover social approach behavior, mice were tested weekly in the three-chamber apparatus following intraperitoneal injections of midazolam (0, 0.56, 1.0 mg/kg) or allopregnanolone (0, 3.0, 10.0 mg/kg) in an injection volume of 1 mL/100 grams of body weight. Allopregnanolone (3α-hydroxy-5α-pregnan-20-one; Steraloids, Inc.) was dissolved in a vehicle of 20% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich) and midazolam HCl (Sigma-Aldrich) was dissolved in 0.9% NaCl vehicle. EtOH-naïve (Naïve) and EtOH-withdrawn (WD) mutant and WT mice received drug doses in a randomized order according to a mixed, factorial design; each mouse was tested six times for social approach following injection of vehicle and doses of allopregnanolone (ALLO) and midazolam (MDZ). Stable levels of drinking were maintained throughout social approach testing from weeks eight to sixteen (**Fig S5**).

Statistical analyses

Ethanol intake (g/kg) data collected from DID experiments were analyzed using two-way repeated measures analyses of variance (2-way RM ANOVA; genotype x day). For mice receiving continuous (CA) or intermittent access (IA) to alcohol, individual mean intake (g/kg) and percent ethanol intake data for 18 ethanol-access days were analyzed by 2-way ANOVA to detect interactions between protocol and genotype. To identify genotype-associated differences in the progression of IA or CA drinking, 2-way RM ANOVA (genotype x week) were also run on individual daily intake (g/kg) and alcohol preference values averaged by week.

For all significant 2-way ANOVA, Dunnett's test was used to compare treatment levels to a control condition (for DID, CA, IA drinking experiments: wild type x mutant; for social approach and locomotion in withdrawal: wild type x mutant, vehicle x drug dose; for ascending concentrations of EtOH: 10% EtOH x all other concentrations, wild type x mutant; for sucrose or quinine concentrations: lowest concentration x all other concentrations, wild-type x mutant).

Results

Escalated binge-like drinking by H101R mutants

Gabra2 H101R mutant mice escalated their four-hour binge-like drinking compared to wild-types in the drinking in the dark (DID) protocol (**Fig 1A, 1B)**. Two-way RM ANOVA (genotype x day) of EtOH intake (g/kg/2 or 4hr) revealed main effects of genotype (F(3,102)=77.67, p<0.001) and day (F(3, 34)=13.32, p<0.001). Compared with their two-hour access intake, mice consumed significantly more when they received four-hour access to EtOH on the final day of the DID protocol.

In separate mice, four-hour DID licking data were collected using a lickometer setup and analyzed in ten-minute time bins (**Fig S4**). Two-way RM ANOVA (time bin x genotype) identified a main effect of time bin (F(24, 600)=7.144, p<0.001) with the greatest number of licks occurring in the first ten-minutes of EtOH access. Two-way ANOVA of EtOH intake data in the lickometer setup identified an interaction between genotype and day (F(9,66)=2.28, p=0.027) driven by increased drinking by H101R mice compared to wild-types on the binge day (**Fig 1B)**, but not on prior, two-hour access days (data not shown). Although a significant time bin x genotype interaction was not apparent upon analyzing the four-hour licking data, only wild-type mice appeared to show a dip in licking at the two-hour time point when EtOH would have been removed on the preceding days (**Fig S4**).

Blood was collected from wild-type and mutant mice assigned to single-bottle DID experiments. A one-way ANOVA of BEC (mg/dL) by genotype revealed a non-significant trend (p=0.062) of reduced BECs in Q241M mutants (*M*=73.93±12.3) and slightly higher than average BECs in H101R mutants (*M*=127.43±18.72) as compared to wild-types (**Fig 1C**). With the exception of Q241M mutants, all genotypes satisfied the requirement of >80 mg/dL for binge-like drinking. A simple linear regression equation (BEC = 52.3+0.136*(licks within final hour)) was able to predict significant variability in BEC values according to the number of licks within the final hour of four-hour binge-like drinking (F(1,28) =12.01, p=0.002; R^2 = 0.3).

Two-way ANOVA (genotype x DID protocol) detected a significant interaction (F(1, 32)=9.53, p=0.004). H101R mutants in the original, single-bottle DID experiment consumed more EtOH (g/kg) as compared to those assessed for two-bottle choice DID; in contrast, WT mice consumed similar amounts regardless of DID protocol **(Fig S3)**. In contrast with the original DID protocol findings, there was no significant difference in 4-hour binge intake (g/kg) between WT and H101R mice that were evaluated in a two-bottle choice DID protocol. Despite consuming comparable g/kg EtOH, H101R mice had a significantly higher alcohol preference (%) as compared to WT controls (1-way ANOVA; (F(1, 15)=6.32, p=0.024); WT: *M*=84.30±6.53; H101R: *M*=57.81±8.04). High preference for alcohol was driven by low water consumption by H101R mice and contributed to significantly lower total fluid intake by these mutants (1-way ANOVA; (F(1,15)=6.20, p=0.025)).

Reduced chronic ethanol intake by Q241M mutants

Mice that received intermittent rather than continuous access to alcohol consumed significantly greater amounts of ethanol (F(1, 68)=157.9, p<0.001; **Fig 1D, 1E**). Two-way ANOVA also detected a significant main effect of genotype on EtOH intake (F(3, 68)=24.16, *p*<0.001); compared to WT mice, *Gabra2* Q241M mutants consumed less 20% EtOH while S270H/L277A mutants consumed more 20% EtOH (**Fig 1D**).

Daily individual EtOH intake values (g/kg) were averaged by week for mice with intermittent access to EtOH. Two-way RM ANOVA of these data revealed a significant genotype by week interaction (F(15, 185)=2.13, p=0.01), and main effects of genotype (F(3, 37)=33.27, p<0.001) and week (F(5, 185)=9.15, p<0.001). While wild-type mice consistently consumed more EtOH than Q241M mutants, both wild-type and Q241M mice consumed progressively more EtOH per day for the first three weeks **(Fig 2A)**. EtOH intake values stabilized for all genotypes following the third week of intermittent access. Similarly, Q241M mutants consumed less EtOH than wild-types in the continuous access experiment, driving a main effect of genotype (F(3, 31)=9.94, p<0.001; **Fig 2B**). In contrast with mice that received intermittent access to alcohol, those with continuous access reduced their drinking, generating significantly lower intake values following the second week of drinking (main effect of week: (F(5, 155)=5.72, p<0.001; **Fig 2B**). Therefore, it appears that, regardless of the drinking protocol, mice require 9-14 days of alcohol access for their daily EtOH intake (g/kg) to stabilize. Two-way RM ANOVA on average total daily volume intake (mL water + mL EtOH) values revealed no effect of genotype (Wt vs. mutant genotypes) or of chronic alcohol access protocol (continuous vs. intermittent).

Daily drinking data were also analyzed as % EtOH preference (calculated as: mL EtOH intake/mL total fluid intake * 100). For mice with intermittent EtOH access, 2-way RM ANOVA detected significant main effects of genotype (F(3, 37)=8.33, p<0.001) and week (F(5, 185)=5.15, p<0.001) on % EtOH preference. As revealed by post-hoc analyses, Q241M mice had significantly lower preference for EtOH as compared to wild-types, and, for all genotypes, average daily % EtOH preference stabilized following the third week of intermittent access (**Fig 2C).** Analysis of continuous access % EtOH preference data identified main effects of genotype (F(3, 31)=3.39, p=0.03) and week (F(5, 155)=2.96, p=0.014). The main effect of genotype was driven by a difference between the Q241M and S270H/L277A mutants; yet, no mutant line differed appreciably from wild-type controls. As seen with g/kg intake data from mice given continuous access to alcohol, % EtOH preference stabilized during the third week of continuous access drinking (**Fig 2D**).

Social approach in forced alcohol abstinence

On the first day of social approach testing, EtOH-withdrawing mice and EtOH-naïve controls received either 0.9% saline vehicle or 20% β-cyclodextrin vehicle. These social approach data were analyzed within genotype by two-way ANOVA (EtOH-withdrawing vs. EtOH-naïve; saline vs. 20% BCD vehicle on the first day) to establish whether withdrawing mice exhibited deficits in social approach as compared to their ethanol-naïve counterparts. This analysis revealed that wild-type mice in forced abstinence spent significantly less time in the social approach zone as compared to EtOH-naïve wild-type controls (F(1, 16)=14.347, p=0.002; **Fig 3**). Likewise, H101R mutants that were in forced abstinence from alcohol spent significantly less time in the social approach zone compared to mice with no history of EtOH consumption (F(1, 15)=9.164, p=0.008; **Fig 3**).

Conversely, there was no difference in social approach behavior between Q241M or S270H/L277A mutants in forced abstinence and their EtOH-naïve counterparts (**Fig 3**). These initial analyses guided subsequent treatments with midazolam and allopregnanolone to reverse withdrawal-associated social deficits observed in WT and H101R mice. Since two-way

ANOVA did not reveal any differences between social approach duration or distance travelled data according to vehicle (0.9% saline vs. 20% β-cyclodextrin) for any genotype, individual vehicle averages were used for data portrayal (**Fig 4** and **Fig S6**).

Because six-week intermittent access drinking differed by genotype and was predicted to impact behavior in withdrawal, social approach was analyzed with one-way RM ANOVA by genotype. Social approach after midazolam or allopregnanolone treatment was compared to behavior after 0.9% saline or 20% β-cyclodextrin administration, respectively. These analyses revealed significant treatment effects in wild-type mice with the 0.56 and 1.0 mg/kg doses of midazolam and the 3.0 and 10.0 mg/kg doses of allopregnanolone increasing social approach in withdrawal as compared to their respective vehicles (MDZ: ($F(2, 18)=8.241$, $p=0.003$); ALLO: ($F(2, 18)=5.22$, $p=0.016$); **Fig 4A**). For H101R mice, there was a significant effect of midazolam ($F(2, 16)=6.403$, $p=0.009$) with both doses reducing social approach time, which is likely due to sedation (**Fig 4B, 4D**). Since the data were not normally distributed, Friedman RM ANOVA on ranks was conducted on allopregnanolone data for H101R mice to reveal a significant effect of the drug treatment ($\chi^2(2)=13.56$, $p=0.001$). As compared to 20% β-cyclodextrin vehicle, both the 3.0 and 10.0 mg/kg doses of allopregnanolone increased social approach in withdrawal from alcohol (**Fig 4B**).

Distance travelled during social approach testing in withdrawal was used as a potential metric of withdrawal-induced motor impairments (Knapp et al. 2005) and for allopregnanolone- or midazolam-induced sedation. None of the genotypes showed motor impairment due to withdrawal as revealed by one-way ANOVA between same-genotype EtOH-naïve and EtOH-withdrawn mice. This suggests that the social approach protocol used in the present study allowed for independent measurements of social avoidance and locomotor behavior. Additional one-way RM ANOVA or Friedman RM ANOVA were run within genotype to detect drug-treatment effects on motor activity during withdrawal. There was no effect of drug treatment on locomotor behavior in wild-type mice in withdrawal (**Fig 4C**). However one-way RM ANOVA did detect a significant effect of treatment in H101R mutants ($F(2, 16)=14.771$, $p<0.001$) with reduced distance travelled following treatment with either dose of midazolam (0.56 or 1.0 mg/kg; **Fig 4D**). This suggests that reduced social approach at this dose was associated with increased sedation.

To establish whether there was an effect of genotype on social approach, a one-way ANOVA was conducted on data collected from EtOH-naïve, vehicle-treated mice. Q241M mice showed a trend toward reduced social approach compared to wild-type animals. Thus, EtOH-naïve wild-type and EtOH-naïve Q241M mice were included in subsequent social approach tests following midazolam and allopregnanolone administration to determine whether anxiolytic compounds could recover social approach by Q241M mutants.

Two-way RM ANOVA on social approach by EtOH-naïve Q241M and wild-type mice treated with midazolam revealed a significant effect of drug treatment ($F(2, 36)=4.88$, $p=0.013$) and an interaction between genotype and drug administration ($F(2, 36)=4.75$, $p=0.015$). Midazolam (1.0 mg/kg) treatment increased social approach time by EtOH-naïve Q241M mice to levels that were comparable to EtOH-naïve wild-type controls (**Fig S6A, S6B**). Conversely, there was no effect of allopregnanolone treatment or genotype on approach behavior. Midazolam treatment interacted with genotype ($F(2,36)=4.12$, $p=0.025$), producing a significant reduction in distance travelled by EtOH-naïve wild-type mice, but not by Q241M mutants (**Fig S6C, S6D**). Allopregnanolone treatment also interacted with genotype ($F(2,36)=10.584$, *p*<0.001); however, this interaction was driven by increased distance travelled by wild-type mice and reduced locomotion by Q241M mutants (**Fig S6C, S6D**).

The significant difference between ethanol intake (g/kg) by wild-type and Q241M mice remained consistent throughout the sixteen weeks of intermittent alcohol drinking ($F(3, 37) = 22.22$, $p<0.001$); drug administration and social approach testing beginning in week eight did not impact ethanol consumption by any genotype **(Fig S5).**

Concentration-dependent ethanol, sucrose or quinine preference

Two-way RM ANOVA was used to detect an interaction between genotype and either intake or percent preference for a specific concentration of ethanol. Analysis of intake data (g/kg) revealed a significant interaction ($F(9,96)=11.28$, $p<0.001$) and a main effect of concentration ($F(3, 96)=102.31$, $p<0.001$). Post-hoc comparisons were conducted as 10% vs. 3, 6, or 20% EtOH (w/v). All genotypes consumed more 10% EtOH (w/v) as compared to the 3% solution while only the H101R and S270H/L277A mutants consumed more 20% than the 10% concentration. Conversely, Q241M mice drank considerably less 20% EtOH (**Fig 5**). As compared with wild-types, H101R mice consumed significantly more 20% EtOH while Q241M mutants consumed significantly less 20% EtOH (**Fig 5**). Two-way RM ANOVA of % EtOH preference data detected a significant effect of EtOH concentration ($F(3,96)=82.77$, $p<0.001$) which was due to reduced preference for the 20% EtOH (w/v) solution regardless of genotype (**Fig 5**). Two-way RM ANOVA on total daily fluid intake (mL EtOH + mL H2O) revealed a significant main effect of concentration ($F(3, 96)=40.89$, $p<0.001$) and an interaction between genotype and EtOH concentration ($F(9,96) = 4.535$, $p<0.001$). All mice consumed the most total fluid upon receiving access to 3% EtOH and water; however, only Q241M mice did not show increased total volume intake when offered 20% EtOH and water. This suggests that mice may adjust their water intake based on their g/kg EtOH consumption; because 20% EtOH intake by Q241M mice was low, they did not increase their water intake like WT, H101R and S270H/L277A mutants.

Two-way RM ANOVA was also used to determine if there was a significant interaction between genotype and preference for ascending concentrations of sucrose solution (10, 30, 100 mM). A main effect of concentration ($F(2, 42)=44.04$, $p<0.001$; **Fig S7**) was associated with preference for the 30 and 100 mM sucrose solutions as compared to the 10 mM sucrose concentration. Two-way RM ANOVA revealed a main effect of quinine ($F(1,20)=29.13$, $p<0.001$) with all genotypes avoiding the high, 0.3 mM quinine solution **(Fig S7)**. Analysis of total daily fluid intake (mL sucrose solution + mL water) revealed a main effect of sucrose concentration ($F(2, 42)=69.98$, $p<0.001$) resulting from greater volumetric intake when mice were given access to 100 mM sucrose and water. Interestingly, a similar effect was observed when mice received 0.3 mM quinine and water; all genotypes significantly increasing their total fluid intake (mL quinine solution + mL water; $F(1, 20)=19.81$, $p<0.001$). While increased volumetric intake during sucrose testing was associated with substantial100 mM sucrose solution intake, the increase in fluid consumed when mice received access to 0.3 mM quinine was driven by elevated water consumption.

Discussion

The present study highlights the following findings: mutant mice with BZD-insensitive α2-containing $GABA_A$ receptors escalated their binge-like alcohol intake; conversely, chronic intermittent drinking by H101R mutants was indistinguishable from wild-type mice. Rendered insensitive to ALLO and THDOC at α2-containing $GABA_A$ receptors, Q241M mutants consumed less than wild-types in the chronic dependence-inducing and moderate drinking protocols. Finally, mice harboring the S270H/L277A mutations in the *Gabra2* protein sequence consumed the same amount of alcohol as wild-type mice; yet, unlike wild-type mice, these mutants did not show disrupted social approach in withdrawal from chronic, excessive alcohol intake.

Human and rodent studies have revealed a correlation between reduced $GABA_A$ receptor BZD-binding sites and alcohol-dependence (Freund 1980; Freund and Ballinger 1988; Volkow et al., 1993, 1995; Gilman et al., 1996; Lingford-Hughes et al., 1998; Laukkanen et al., 2013; but Korpi et al., 1992). By evaluating H101R mutant mice, we provide evidence to suggest that BZD-insensitivity - or a yet unknown functional change caused by the mutation - can promote excessive binge-like alcohol consumption when mice are not given the choice between ethanol and water. In a two-bottle choice drinking in the dark procedure (2BC DID), H101R mutants maintained wild-type-like levels of EtOH consumption; these values were significantly lower than those achieved by H101R mutants with access to EtOH only. In contrast with wild-types, 2BC did not elicit equal volumetric EtOH and water drinking by H101R mutant mice (EtOH preference means; H101R: 84.3%; WT: 57.81%). Interestingly, this disparity was not observed in chronic 2BC protocols despite similar EtOH (g/kg) intake between H101R and wild-type mice. These findings may indicate that, in H101R mutants, the presentation of a non-EtOH-containing bottle is sufficient to diminish limited-access drinking. It is possible that single-bottle access to EtOH may drive compulsive intake and that providing an alternative is sufficient to disrupt escalated drinking. Future lickometer assessments will see if presentation of a second bottle disrupts the pattern of licking during the four-hour DID access period. In addition, work should address how other environmental enrichments may disrupt single-bottle EtOH drinking and whether this effect is specific to H101R mutants.

To clarify the present findings, future work must establish how α2(H101R)-containing $GABA_A$ receptors respond to ethanol. In recombinant receptors, the α2(H101R) mutation produces a rightward shift of the GABA dose-response curve (Benson et al.,1998), which might suggest a reduced sensitivity to GABA *in vivo*. However, at least in central and lateral/basolateral amygdala, the amplitudes of extracellularly evoked inhibitory postsynaptic currents are unchanged by the α2(H101R) mutation, which is consistent with no change in GABAergic functions in the absence of benzodiazepines (Marowsky et al. 2004). Until we have a more complete understanding of how the α2(H101R) mutation alters the properties of $GABA_A$ receptors throughout the brain, we cannot formally exclude the possibility that increased binge-like drinking by H101R mutants may reflect altered GABA sensitivity and/or potentiation of GABA-induced currents by ethanol.

Animals rendered selectively insensitive to ALLO and THDOC at α2-containing $GABA_A$ receptors reduced their drinking in a protocol that models dependence-inducing ethanol intake. The Q241M mutation impedes neurosteroid positive modulator binding to the membrane-bound modulatory site of the α2-subunit (Hosie et al., 2006). Because both low-concentration potentiation and activation by high concentrations of neurosteroids require this modulatory site, these mutants should be insensitive to all neurosteroid action at α2-containing $GABA_A$ receptors. Indeed, *in vitro* dose-effect curves do show that this single amino acid substitution can block ALLO-potentiation of GABA currents (Hosie et al., 2009). A number of studies demonstrate that ALLO or its synthetic analog, ganaxolone, can increase responding for alcohol and can escalate alcohol intake (Janak and Gill 2003; Nie and Janak 2003; Ramaker et al., 2014). Yet, studies also provide evidence for reduced drinking with regional increases in ALLO or following ALLO or ganaxolone treatment (Besheer et al., 2010; Cook et al., 2014; Ramaker et al., 2015); conflicting evidence may reflect dose- and time-dependent effects of ALLO and/or an interaction between ALLO and history of alcohol consumption (Janak et al., 1998; Ford et al., 2005; Ramaker et al., 2011). In the present investigation, Q241M mutants showed reduced alcohol intake beginning with their first day of access, indicating that neurosteroids may need to act on α2-containing $GABA_A$ receptors for alcohol to have its rewarding effects. One hypothesis is that ALLO or THDOC initially binds to the membrane-bound $GABA_A$ receptor modulatory site to induce a conformational change in the receptor. This may subsequently render an extracellular site more accessible to ethanol, leading to receptor positive modulation. Additional studies need to establish whether or not α2(Q241M)-containing receptors are sensitive to ethanol-potentiation of GABA-induced currents. The possibility that ethanol reward value may be altered in Q241M mutants must be addressed directly in studies comparing alcohol-reinforced responding by mutants to responding by wild-type controls.

During a chronic intermittent access to alcohol procedure, wild-type, H101R, and S270H/L277A mice all consumed ~20 g/kg/24 hr for six weeks. Despite consistently drinking substantial amounts of alcohol, S270H/L277A mutants did not show wild-type-like deficits in social behavior during withdrawal from alcohol. These mutants harbor two mutations, one to block potentiation by ethanol and the other is a gain-of-function mutation that normalizes GABA sensitivity (Homanics et al., 2005; Borghese et al., 2006b). To identify the specific substitution that affects behavior in withdrawal, it would be necessary to pair the S270H mutation with an alternative gain-of-function mutation; if these mutants were to behave like the present

S270H/L277A mice, then withdrawal-like symptoms may specifically involve serine at location 270 in the α2-subunit protein sequence.

In agreement with earlier work, the present findings demonstrate similar binge-like ethanol intake by mutant male S270H/L277A and wild-type mice (Blednov et al. 2011). However, drinking by S270H/L277A mutants may exceed that of wild-types when animals receive intermittent access to a high concentration of ethanol (i.e. 20% EtOH (w/v); this difference is not evident when animals receive 15% EtOH (v/v; Blednov et al. 2011). S270H/L277A mutants may be less sensitive to some of the aversive effects of alcohol as supported by their escalated chronic ethanol consumption, insensitivity to conditioned taste aversion (Blednov et al. 2011) and intact social approach behavior in withdrawal from chronic alcohol. Future studies need to clarify whether these mice are selectively insensitive to the aversive effects of ethanol or if they also show deficits in their sensitivity to reward.

High-risk alcohol dependence–associated *GABRA2* allelic variants do not affect primary protein sequence in humans, and therefore, mutant mice with amino acid substitutions *do not* serve as humanized preclinical models to assess alcohol dependence risk. The present study does, however, provide insight regarding how alcohol or endogenous ligands may interact with the $GABA_A$ receptor α2-subunit protein to either increase or reduce drinking. Because previous studies using *Gabra2* null mutants did not reveal any differences in ethanol intake (Dixon et al., 2012), we chose to use mice harboring targeted amino acid substitutions to address the role of precise $GABA_A$ receptor modulatory sites in consumption. Although the action of alcohol on these mutated receptors is not fully characterized, we speculate that α2-containing $GABA_A$ receptor sensitivity to benzodiazepines, neurosteroids, or another presently unidentified endogenous ligand may influence specific patterns of drinking. To determine if the present preclinical findings translate to alcohol-dependent patients, clinical studies should investigate individuals with the high-risk *GABRA2* haplotype for their sensitivity to benzodiazepines, allopregnanolone and THDOC. Interestingly, recent clinical findings suggest that high AUD-risk *GABRA2* SNPs may occur in spans of sequence that regulate $GABA_A$ receptor gene expression during a specific perinatal period (Lieberman et al., 2015). Altered expression of receptor subunits during development may change $GABA_A$ receptor composition and sensitivity to endogenous modulators. Future research addressing the potential regulatory role of AUD-associated *GABRA2* SNPs may guide the development of pharmacogenetic tools to aid in the diagnosis and treatment of alcohol use disorders.

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Conflict of Interest The authors declare no conflict of interest.

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Table 1

Experimental group *n*s

	WT	H101R	Q241M	S270H/ L277A
DID	*n* =10	9	9	10
DID with lickometer	*n* =5	5	9	8
2-bottle choice DID	*n* =9	8	-	-
Intermittent Access (IA)	*n* = 11	10	10	10
IA social approach	*n* = 10	9	10	10
EtOH-naïve social approach	*n* = 9	11	9	11
Continuous Access	*n* = 9	8	9	9
Ascending concentrations of EtOH (3-20%)	*n* = 8	9	9	10
Ascending concentrations of sucrose or quinine	*n* =8	4	5	7-8[&]

[&] One mouse euthanized following sucrose testing