GABRA2 POINT-MUTATION: INCREASED BINGE-LIKE AND BLUNTED DEPENDENCE-INDUCING ALCOHOL DRINKING BY MICE

A thesis submitted by

Emily L. Newman

in partial fulfillment of the requirements for the degree of

Master of Science

in

Psychology

Tufts University

February 2016

Committee Members:

Klaus A. Miczek, Ph.D.[1,2]; Joseph F. DeBold, Ph.D.[1]; Uwe Rudolph, M.D.[3,4]

[1] Dept. of Psychology, Tufts University; [2] Dept. of Neuroscience, Tufts University;

[3] Laboratory of Genetic Neuropharmacology, McLean Hospital;

[4]Dept. of Psychiatry, Harvard Medical School

ABSTRACT

Alcohol use disorders have been associated with SNPs in *GABRA2*, the gene encoding the $GABA_A$ receptor α2-subunit in humans. Deficient GABAergic functioning is linked to impulse control disorders including 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. We assessed mice harboring specific amino acid point-substitutions in the α2-subunit protein sequence. Mutants rendered insensitive to GABAergic modulation by benzodiazepines (H101R), allopregnanolone or THDOC (Q241M), or to high concentrations of ethanol (S270H/L277A) at α2-containing $GABA_A$ receptors were assessed for binge-like, moderate or dependence-inducing drinking.

Mice with benzodiazepine-insensitive α2-containing $GABA_A$ receptors escalated their binge-like drinking, achieving blood ethanol concentrations of 127±19 mg/dl in 4h. 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. Mice harboring the Q241M mutation showed blunted moderate and dependence-inducing intake. This amino acid is necessary for neurosteroid positive modulation and activation of $GABA_A$ receptors; we postulate that neurosteroid action on α2-containing receptor may be necessary for escalated chronic ethanol intake. Like wild-type controls, S270H/L277A mutant mice consumed excessive amounts of alcohol but, unlike controls, they did not show withdrawal-induced deficits in social behavior. These findings suggest a role of amino acid residues: 1.) H101 for species-typical binge-like drinking, 2.) Q241 for escalated chronic, dependence-inducing drinking, and 3.) S270 and/or L277 for the development of alcohol withdrawal-associated social deficits which may increase the chance of relapse to drug taking.

ACKNOWLEDGEMENTS

First, I would like to thank my committee members for their invaluable guidance, expertise and patience along the way. Our discussions have taught me about animal behavior, research methodologies, pharmacology, and behavioral genetics. I would also like to thank our Senior Research Technician, T. John Sopko for his technical expertise and lickometer enthusiasm. Also, thank you to my contemporaries, Lara Hwa, Elizabeth Holly, and Xiao Han for their support and advice in the last two years. Also, thank you to all of the dedicated Tufts undergraduate assistants who helped with these experiments, including Georgia Gunner, Polly Huynh, Tiffany Wang, Yang Chen, Darrel Gachette, Johnny Auld and Henry Butler. Last, I would like to acknowledge the support for this work which was funded by the National Institute on Alcohol Abuse and Alcoholism (R01AA013983 awarded to KAM) and by the National Institute of Mental Health (R01MH080006 awarded to UR).

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ALCOHOL USE DISORDERS

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). Presently, the definition of AUD encompasses alcohol abuse and alcohol dependence; as such, it is characterized by an uncontrollable pattern of harmful drinking that impedes normal, adaptive functioning (World Health Organization 2014). The outward behavioral consequences of alcohol use disorders can include, but are not limited to unsatisfactory work performance and social functioning, criminal acts involving interpersonal violence or property damage that may result in repeated arrests, and high-risk sexual activity. Alcohol use disorders can also have severe psychological repercussions by either causing or exacerbating symptoms of depressive, anxiety, or sleep disorders or by inducing psychosis upon acute intoxication or during withdrawal. AUDs can also have a grave impact on systemic physiology and functioning; in particular, long-term excessive alcohol use can lead to irreversible and potentially fatal organ damage (World Health Organization 2014). While the impact of AUDs is severe, only a minor fraction of individuals who consume alcohol will suffer from such a disorder; this suggests that individual differences may play a role in whether or not we consume in moderation or in pathological excess. To clarify the etiology of AUDs and to improve our ability to predict vulnerability to and successful treatment of alcohol abuse and dependence disorders, researchers focus their attention on facets of the environments we live in, our biological makeup, and, importantly, the interactions between these external and internal influences.

Guided by research on the association between allelic variants in the human genome and alcohol dependence, the present work employs a preclinical genetic mouse model 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. If mutations in non-coding regions for *GABRA2* produce functional changes in α2-containing $GABA_A$ receptor sensitivity to endogenous modulators, the present findings may provide an understanding of how these changes may increase the risk for different patterns of pathological drinking.

OLD DRUG, NEW SNPS: *GABRA2* ALLELIC VARIANTS AND ALCOHOL DEPENDENCE

To identify genes and allelic variants that may be associated with the development of pathological patterns of alcohol consumption, the National Institute on Alcohol Abuse and Alcoholism has funded the Collaborative Studies on Genetics of Alcoholism (COGA). COGA gathers extensive clinical, electrophysiological, and neuropsychological data from families with a high incidence of individuals meeting the alcohol dependence (AD) criteria. Cell lines from these subjects are maintained, allowing researchers to assess numerous genetic factors and potential endophenotypes associated with AD (niaaa.nih.gov).

One of the most consistent findings using COGA data is the link between alcohol dependence and single-nucleotide polymorphisms (SNPs) in *GABRA2*, the gene encoding the $GABA_A$ receptor α2-subunit protein (Reich et al. 1998, Covault et al. 2004, Edenberg et al. 2004, Lappalainen et al. 2005, Lind et al. 2008, Bierut et al.

2010, Olfson and Bierut 2012, 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, Dick et al. 2006, Fehr et al. 2006, Soyka et al. 2008, Enoch et al. 2009).

Clinical studies reveal that high-risk *GABRA2* haplotypes may be associated with an individual's sensitivity to the rewarding and sedative effects of alcohol which may, in turn, promote a pathological pattern of drug-seeking behavior. It also seems that the pattern of alcohol consumption may interact with genotype to alter sensitivity to alcohol. In particular, recent high levels of alcohol consumption may predict reduced ethanol-induced euphoria in carriers of a high-risk allele (Haughey et al. 2008, Kosobud et al. 2015). Likewise, among frequent social drinkers, individuals with an AD-associated *GABRA2* haplotype report reduced EtOH-induced euphoria and sedation compared to low-risk controls (Pierucci-Lagha et al. 2005, Roh et al. 2011). In another sample in which more than half of individuals reported no heavy drinking days within the past month, results revealed a link between the AD-associated haplotype and ***greater*** stimulatory response to alcohol (Arias et al. 2014). An interaction between alcohol consumption and *GABRA2* genotype may regulate the subjective effects of alcohol. Specifically, individuals who experience a greater degree of euphoria after an initial drink may quickly escalate their consumption in the form of a binge; furthermore, this escalation may be potentiated by a rapid desensitization to ethanol's pleasurable effects in carriers of an AD-associated *GABRA2* haplotype. Rodent models of binge-like and dependence-

inducing alcohol consumption provide powerful tools for addressing the potential relationships between genetics and the trajectory of alcohol use and abuse.

GABRA2 SNPS: SILENT MUTATIONS DON'T FALL ON DEAF EARS

$GABA_A$ receptors are heteropentameric structures assembled from different subunits deriving from the same gene family (Schofield et al. 1987). In the mammalian CNS, synaptic $GABA_A$ receptors are commonly comprised of 2α, 2β, and 1γ subunits which surround a ligand-gated chloride ion channel (for reviews Mody 2001, Moss and Smart 2001, Farrant and Nusser 2005, Olsen and Sieghart 2008). Heterogeneity in receptor composition can determine sensitivity to endogenous and exogenous receptor modulators including benzodiazepines, neurosteroids, ethanol and general anesthetics (for reviews, see Farrant and Nusser 2005, Olsen and Sieghart 2008, Rudolph and Knoflach 2011, Howard et al. 2014).

Of the AD-associated SNPs, only rs279858 is exonic (chr4p12, exon 5): yet, because it encodes a synonymous substitution, this SNP does not alter the amino acid sequence of the gene product. All other identified AD-associated *GABRA2* SNPs are intronic and therefore, like rs279858, they are silent and do not alter primary protein sequence (Edenberg et al. 2004). Even so, silent mutations may have an effect on phenotype by producing functional changes in transcription, splicing of pre-mRNA, and by affecting mRNA stability and translation.

So, although AD-associated SNPs in the human *GABRA2* gene do not alter amino acid sequence, they may impact the relative expression of specific ligand-sensitive $GABA_A$ receptors (Edenberg et al. 2004, Tian et al. 2005, Lieberman et al.

2015). To clarify a possible role for AD-associated allelic variants in the regulation of *GABRA2* gene expression, Lieberman and colleagues used induced pluripotent stem cells from individuals with either the high- or low-risk allele of the rs279858 tag-SNP in *GABRA2* and then looked at differentiated neural cells from these lines (2015). Cell lines with reduced expression of the *GABRG1*, *GABRA2*, *GABRA4*, and *GABRB1* genes had a greater-than-chance likelihood of deriving from individuals with the AD high-risk allele (Lieberman et al. 2015). This suggests that *GABRG1*, *GABRA2*, *GABRA4*, and *GABRB1* may comprise a cluster of genes with a shared mechanism of transcriptional control (e.g. Uusi-Oukari et al. 2000).

These findings provide the first evidence that high-risk SNPs in *GABRA2* may be associated with altered gene expression; however, a mechanism of action for these mutations requires further investigation. By introducing targeted mutations, researchers have identified amino acid residues in the $GABA_A$ receptor α2-subunit protein that confer receptor sensitivity to specific positive modulators. The present experiments were designed to test whether targeted mutations in *Gabra2* could functionally yield an AUD-like behavioral phenotype in mutant mice. Binge-like and dependence-inducing voluntary drinking were examined in mutant mice with α2-containing $GABA_A$ receptors insensitive to modulation by benzodiazepines (Low et al. 2000), by allopregnanolone and THDOC (*in vitro*: Hosie et al. 2006, Hosie et al. 2009), or by alcohol and select general anesthetics (Werner et al. 2011, Blednov et al. 2011).

GABA$_A$ RECEPTORS AND BENZODIAZEPINES

In the mid-1970s, a benzodiazepines (BZD) target of action was identified on the postsynaptic membrane through application of [^{3}H]-diazepam to synaptosomal fractions (Mohler and Okada 1977a, Mohler and Okada 1977b, Braestrup et al. 1977, Squires and Braestrup 1977). Concurrent research revealed that BZDs could potentiate GABA-induced responses, but not those produced by glycine (Choi et al. 1977, Macdonald and Barker 1978), suggesting BZDs and GABA act on a shared receptor. This idea was strengthened by GABA$_A$ receptor immunoprecipitation and co-localization with BZD-binding sites, providing early evidence that the BZD site of action was a component of the GABA$_A$ receptor (Schoch et al. 1985).

With the ability to establish primary protein structure in the late 1980s, the $\alpha_{1\text{-}3}$ and β-subunits of the GABA$_A$ receptor were identified. Co-expression of $\alpha_{1,\ 2\ \text{or}\ 3}$ and β_1 RNAs in *Xenopus* oocytes yielded ligand-gated ion channels which could produce GABA-induced currents that were blocked with bicuculline or picrotoxin and potentiated by pentobarbitol (Schofield et al. 1987, Levitan et al. 1988a). In addition, differences in the EC$_{50}$ of GABA for oocytes expressing one $\alpha_{1\text{-}3}$ subtype along with β_1 mRNA indicated that the α-subtype conferred differential affinity for GABA (Levitan et al. 1988a, 1988b). However, such αβ channels did not show consistent potentiation of GABA currents by benzodiazepines (Levitan et al. 1988a, 1988b, Pritchett et al. 1989b, Schofield et al. 1987, Malherbe et al. 1990). In the following year, the discovery of a γ_2-subunit led to the discovery that enhancement of GABA responses by BZDs could be observed upon co-expression of α_1, β_1, and γ_2 in HEK293 cells (Pritchett et al. 1989a). The same group illustrated that the α-subunit

subtype could confer drug affinity; specifically, the β-carboline, β-CCM, and the imidazopyridine, zolpidem, showed significantly greater receptor-affinity in cells transfected with viral vectors encoding the genes for α_1, $\beta_{1 \text{ or } 3}$, and γ_2 subunit subtypes (Type I receptors) as compared to cells transfected with those for α_2, α_3, or α_5, along with $\beta_{1 \text{ or } 3}$, and γ_2 (Type II receptors; Pritchett et al. 1989b, Pritchett and Seeburg 1990, Sigel et al. 1990). Conversely, holding the α- and γ_2-subunits constant and varying the β-subtype did not alter binding affinity, whereas infection with α, β and γ_1 plasmids reduced maximal affinity (Pritchett et al. 1989a, 1989b, Hadingham et al. 1993). These findings indicated that while γ_2, β_x and $\alpha_{1\text{-}3 \text{ or } 5}$ subunits were all necessary to form BZD-sensitive $GABA_A$ receptors, the α-subunit isoform determined sensitivity to BZDs.

The discovery of cerebellar cells that were insensitive to diazepam sparked an interest in native BZD-insensitive receptors (Bonetti et al. 1989, Malminiemi and Korpi 1989). This eventually led to the identification of BZD-insensitive α_4-containing receptors in the thalamus, hippocampus, and cortex (Khrestchatisky et al. 1989, Wisden et al. 1991, Benke et al. 1997) and α_6-containing receptors in granule cells of the cerebellum, (Luddens et al. 1990, Turner et al. 1991). Evaluation of HEK cells expressing recombinant α_4- or α_6-containing receptors revealed a unique pharmacological profile; while cells were insensitive to BZDs, they maintained sensitivity to the structurally similar compound, Ro15-4513, which can block alcohol (10 mM) potentiation of GABA-induced Cl^- influx and antagonize the anticonflict and intoxicating effects of acutely administered ethanol (1 g/kg). Interestingly, pretreatment with the BZD antagonist, flumazenil (i.e. Ro15-1788)

prevents the antagonism of alcohol's cellular and behavioral effect by Ro15-4513 (Suzdak et al. 1986a, Suzdak et al. 1986b).

In an elegant series of experiments conducted by Wieland et al., chimeric α_1/α_6 proteins were used to pinpoint the α_6 domain responsible for BZD-insensitivity. To construct the chimera, oligonucleotides were hybridized to α_1- and α_6-encoding cDNAs to generate restriction sites at matching locations (Wieland et al. 1992). Ligation of fragments therefore resulted in molecules with alternating lengths of sequence originating from α1 or α6 cDNAs. Diazepam-insensitive chimeric receptors were identified in membranes from HEK cells transfected with wild-type β2 and γ2 cDNAs in conjunction with the chimeric cDNA encoding α6(1-158)/α1(159-428). This implicated the α-subunit large extracellular domain (1-158) in determining BZD-sensitivity. Within this domain, substituting the α_1-subunit histidine residue (101) with arginine (i.e. the residue at the corresponding location in the α_6-subunit), was sufficient to produce BZD-insensitive α_1-containing $GABA_A$ receptors (Wieland et al. 1992). Because this histidine residue is conserved, researchers were also able to engineer BZD-insensitive α1-3 or 5-containing receptors which, importantly, showed near-normal GABA responding (Benson et al. 1998). Like native, BZD-insensitive α4- or α6-containing $GABA_A$ receptors, those incorporating α1(H101R)-, α2(H101R)-, α3(H126R)- or α5(H105R)-subunits showed enhancement of submaximal GABA responding by Ro15-4513 (Benson et al. 1998). In stark contrast, Ro15-4513 acts as an inverse agonist on native BZD-sensitive $GABA_A$ receptors (Hadingham et al. 1996, Knoflach et al. 1996, Benson et al. 1998).

Following the identification of a single amino acid residue that confers BZD-sensitivity, the next step was to introduce this genetic point-mutation into inbred mouse lines to generate animals with targeted point-substitutions. This line of research would provide invaluable insight to the effects of targeted mutation on receptor assembly, trafficking, targeting and clustering, and also on behavior throughout development. Mouse lines were developed with a histidine-to-arginine point-substitution in the α_1, α_2, α_3, or α_5 subunit amino acid sequence at locations 101, 101, 126, or 105, respectively. As shown in transfected HEK cells (Benson et al. 1998), dissociated Purkinje cells harvested from α_1(H101R) mutants and cultured hippocampal pyramidal cells from α_2(H101R) mice showed normal GABA-induced currents, but no potentiation of currents by diazepam (Rudolph et al. 1999, Low et al. 2000).

Behavioral evaluations of histidine-arginine point-mutants implicate α_1-containing $GABA_A$ receptors in the amnestic, anticonvulsant, anti-fear and sedative effects of BZDs, α_2-containing receptors in the analgesic and anxiolytic effects of BZDs, and α_5-containing receptors in the development of tolerance to BZDs and in learning and memory in the absence of BZDs (see **Table 1** for summary of findings and for references). In addition, both α_1- and α_2-containing $GABA_A$ receptors necessary for the pro-aggressive and rewarding effects of benzodiazepines while receptors with α_2- or α_5-subunits play a role in the myorelaxant effect of BZDs (see **Table 1** for summary of findings and references). These findings suggest that the α-subunit subtype not only determines the sensitivity to specific BZDs, but also plays a distinct role in the specific behavioral effects elicited by benzodiazepines, likely due

in part to regional expression within the brain. Furthermore, this body of work suggests that drugs targeting specific $GABA_A$ receptor subtypes could provide a therapeutic effect without an extensive side effect profile as seen with non-specific benzodiazepines.

BENZODIAZEPINE BINDING, ANXIETY AND ALCOHOL DEPENDENCE

Until the mid-20th century, alcohol was considered to have its behavioral effects primarily by disrupting the lipid bilayer constituting the neuronal membrane (for review Tabakoff and Hoffman 2013). During the second half of the century, however, studies on the behavioral and cellular effects of ethanol and benzodiazepines sparked the hypothesis that these compounds may act on a shared, membrane-bound protein. Following the identification of central BZD-binding receptors (Mohler and Okada 1977a, Squires and Braestrup 1977), researchers quantified the density of BZD-receptor binding, showing that rats selectively bred for a high-fearfulness phenotype expressed significantly reduced ^{3}H-diazepam binding in the hippocampus, hypothalamus and midbrain compared to low-fearfulness animals (Robertson et al. 1978). In accordance with these findings, more recent, clinical studies show that patients with panic or posttraumatic stress disorder have reduced BZD-binding, particularly in the frontal and temporal cortices (using PET or SPECT: Kaschka et al. 1995, Malizia et al. 1998, Bremner et al. 2000a, Bremner et al. 2000b, Cameron et al. 2007, Hasler et al. 2008), reduced basal levels of GABA in the frontal and cingulate cortices, and diminished GABA

responding to clonazepam in the occipital cortex (using MRS: Long et al. 2013, Goddard et al. 2004).

Robertson's findings in high-fearfulness rats led to similar work predicated on the hypothesis that alcohol consumption may relate to an individual's level of anxiety. Therefore, early studies aimed to clarify how chronic alcohol consumption could impact ^{3}H-flunitrazepam binding in the brains of C57BL/6J mice (Freund 1980). Mice consuming a liquid alcohol diet (~8% EtOH v/v) for seven months expressed reduced BZD-receptor binding as compared to animals on an isocaloric sucrose control diet (Freund 1980). Impressively, similar data were documented in a comparison between brain samples from deceased alcoholic and non-alcoholics. As seen in the dependent mice, brain samples harvested from alcoholic individuals showed reduced ^{3}H-flunitrazepam binding in the frontal cortex, hippocampus and basal ganglia as compared to non-alcoholic controls (Freund and Ballinger 1988, Laukkanen et al. 2013; but Korpi et al. 1992, Lewohl et al. 1997). In addition, reduced BZD-binding was identified in the thalamus, basal ganglia, orbitofrontal and parietal cortices of conscious and sober alcoholics as compared to controls (using PET or SPECT: Volkow et al. 1993, Gilman et al. 1996, Lingford-Hughes et al. 1998).

This impressive body of work provides ample evidence for a link between $GABA_A$ receptor sensitivity to BZDs and pathological alcohol use. To illuminate causal relationships between these physiological and behavioral observations, two lines of experimental work have been pursued: the first aims to identify underlying genetic factors that may lead to pathological alcohol use and the second examines how alcohol impacts physiology and behavior to promote escalation toward

dependence. Because an individual's genes, physiology and behavior are intricately connected, so are the findings that arise from these two approaches.

ALCOHOL DEPENDENCE: FROM GENES TO EXCESS?

As an extension to the hypothesis that reduced BZD-binding in alcohol dependent individuals may serve as a marker for increased anxiety, researchers speculated that pre-existing receptor deficiencies may contribute to the development of alcohol dependence. Therefore, the children of alcoholics have been tested for detectable, phenotypic indicators that may predict their risk of eventually developing an AUD (Cloninger et al. 1987). Indeed, the sons of alcoholic (SOA) fathers, who appear to be particularly at-risk for alcoholism, show reduced sensitivity to the effects of diazepam on eye-tracking tasks (Roy-Byrne et al. 1990). Compared to individuals without a family history, SOAs report higher scores of euphoria in response to a low dose of diazepam, but lower ratings of sedation upon receiving a high dose (Cowley et al. 1994, Cowley et al. 1996); this suggests that underlying genetic factors may impact BZD-sensitivity in a dose-dependent manner. As suggested by the work of Volkow and colleagues, these findings may reflect reduced basal cerebellar metabolism and a blunted metabolic response to BZDs in individuals with a family history of alcoholism (1995). However, evidence for a relationship between subjective responding to diazepam and family history of alcoholism is not universally consistent (de Wit 1991). While family history is associated with increased AUD risk, it is not an absolute predictor. Therefore, because a single gene does not account for AUD, the interpretation of results from

studies that select samples based only on family history are obfuscated by random variability due to genetic factors that are either unrelated to AUD or non-specifically associated.

To avoid this issue, individuals are sampled based on an AUD-associated haplotype. If a phenotypic measure significantly and specifically segregates with an AD-associated genetic factor, it conveys the same risk of AUD as the genetic factor. Such analyses identify increased resting EEG beta wave power as an AUD endophenotype predicting the same amount of risk as an AUD-associated *GABRA2* haplotype (Edenberg et al. 2004, Dick et al. 2006, Rangaswamy and Porjesz 2008, Malone et al. 2014). Beta wave characteristics are highly heritable and may be involved in the balance of inhibitory and excitatory activity, particularly in the frontal cortex (van Beijsterveldt et al. 1996, Begleiter and Porjesz 2006, Dick et al. 2006). Interestingly, BZD treatment can increase EEG beta power in a plasma concentration-dependent manner (Brunner et al. 1991, Friedman et al. 1992, Feshchenko et al. 1997, Lingford-Hughes et al. 2005) to produce oscillations reminiscent of those observed in untreated individuals with the high-risk *GABRA2* haplotype. Like family history-based analyses, these findings suggest a possible relationship between AUD susceptibility and sensitivity to some effects of benzodiazepines.

As in humans, rats and mice administered BZDs also show significant increases in the power of cortical EEG signals within the 13-28 Hz range (Mandema et al. 1991, Kopp et al. 2004). Interestingly, mutant mice with α2(H101R)-containing $GABA_A$ receptors rendered insensitive to BZDs do not show

augmentation of cortical EEG beta wave power by diazepam (Kopp et al. 2004). Conversely, α1(H101R) and α3(H126R) mutant mice show wild-type-like sensitivity, implying a role of α2-containing receptors in BZD modulation of non-REM and waking EEG activity, but not α1- or α3- containing receptors (Tobler et al. 2001, Kopp et al. 2003). Considering these findings in BZD-insensitive point-mutants and numerous studies indicating reduced BZD-binding in the brains of alcoholic individuals, it is tempting to speculate that SNPs in *GABRA2* may alter gene regulation to functionally reduce expression of BZD-sensitive α2-containing $GABA_A$ receptors, thereby decreasing resting EEG beta band power. However, endophenotypic analyses reveal quite the opposite: a strong association between increased EEG beta activity and AD-associated *GABRA2* haplotypes (Edenberg et al. 2004). These seemingly contradictory findings require further investigation.

Modulation of extrasynaptic $GABA_A$ receptors by low-dose alcohol (<50 mM) and endogenous neurosteroids

Soon after the identification of α_1(H101) as a requirement for $GABA_A$ receptor sensitive to diazepam (Wieland et al. 1992), Korpi and colleagues established that alcohol non-tolerant (ANT) rats possessed a naturally occurring arginine-to-glutamine α6(R100Q) substitution (Korpi et al. 1993, Korpi and Seeburg 1993). These ANT rats were selectively bred for their sensitivity to the motor-impairing effects of ethanol and, as expected; they consume less alcohol voluntarily

in a two-bottle choice protocol as compared to their alcohol-tolerant counterparts (Sarviharju and Korpi 1993).[1]

The α6(R100Q) mutation may increase cerebellar sensitivity to ethanol due to its direct modulatory action on $GABA_A$ receptors, thereby increasing the aversive, motor impairing effects of alcohol in ANT rats (Hanchar et al. 2005). Indeed, evidence suggests that low, clinically relevant concentrations of alcohol (≥3 mM) can enhance currents via recombinant BZD-insensitive α6β3δ and α4β3δ (Wallner et al. 2003, Wallner et al. 2006), but not α1α6β3δ receptors (Baur et al. 2010). Due to their high-affinity GABA-binding and extrasynaptic localization, these δ-containing receptors are activated by GABA spillover from the synaptic cleft, and thus, they play a crucial role in maintaining levels of tonic inhibition (see **Fig. 1;** Nusser et al. 1998, Pirker et al. 2000, Wei et al. 2003, Wallner et al. 2003, for review Mody 2001).

Expressed in *Xenopus* oocytes, recombinant α6(R100Q)β2δ receptors showed greater potentiation of GABA-induced currents by low concentrations of alcohol (≥3 mM) in comparison with wild-type α6β2δ receptors (Hanchar et al. 2005). Additionally, application of moderate concentrations of alcohol (50 mM) can increase amplitude of tonic currents and can reduce firing rate after depolarization (Jia et al. 2008). Yet, a lack of reproducibility calls for additional investigations into this potential mechanism of action, particularly for very low concentration ethanol

[1] ANT rats also show a similar response to $GABA_A$ receptor positive modulators as they do to alcohol, demonstrating extreme sensitivity to barbital and lorazepam (Hellevuo et al. 1989).

(>50 mM; Borghese et al. 2006, Yamashita et al. 2006, Botta et al. 2007, Borghese and Harris 2007, Casagrande et al. 2007, Baur et al. 2009).

Heterogeneity of $GABA_A$ receptors contributes to their varying sensitivities to endogenously active and exogenously administered compounds; this appears to be the case with some endogenous neurosteroids and may also apply to alcohol. Within the CNS, centrally- or peripherally-synthesized progesterone serves as the precursor for 5α-reduced dihydroprogesterone (DHP) and dihydrodeoxycorticosterone (DHDOC) which are synthesized into the neuroactive steroids, allopregnanolone (ALLO) and tetrahydrodeoxycorticosterone (THDOC) by 3α-hydroxysteroid dehydrogenase (3α-HSD; i.e. 3α-HSOR), respectively. The resulting 5α, 3α-reduced neurosteroids, THDOC and ALLO, can act as potent positive allosteric modulators of $GABA_A$ receptors (for review Belelli and Lambert 2005). In mice, 5α-reductase and 3α-HSD are co-localized in glutamatergic neurons in the cortex, hippocampus, basolateral amygdala (BLA), olfactory bulb, and thalamus and within GABAergic neurons in the striatum and cerebellum (Agis-Balboa et al. 2006). Similarly, humans express these enzymes and allopregnanolone within the hippocampus and neocortex (for reviews Stoffel-Wagner 2001, Stoffel-Wagner 2003). In terms of $GABA_A$ receptor responding, it appears that low concentrations of ALLO (3-100 nM) most effectively potentiate GABA-evoked responses in frog oocytes expressing $\alpha_{1,3\ or6}$, β_1, and γ_{2L} while maximum inhibition by GABA is potentiated to the greatest extent by ALLO in oocytes with recombinant $\alpha_1\beta_1$, $\alpha_6\beta_1\gamma_{2L}$, or $\alpha_4\beta_3\delta$ receptors (EC_{50} ALLO:~380, 220, 183 nM, respectively; Belelli et al. 2002).

At lower concentrations (<100 nM), allopregnanolone and THDOC potentiate GABA-elicited Cl^- influx while higher concentrations (≥100 nM) can activate $GABA_A$ receptors in the absence of GABA (for review Belelli and Lambert 2005). To characterize the relevant domains for these different mechanisms, Hosie and colleagues generated chimeric proteins with amino acid sequences from the neurosteroid-insensitive RDL GABA receptor derived from *Drosophila melanogaster* alternating with lengths of sequence originating from neurosteroid-sensitive, mammalian $GABA_A$ receptors. In this process, they discovered two sites on the $GABA_A$ receptor that interacted with ALLO and THDOC: a site located within the membrane-bound region of the α-subunit and a superficial binding site at the α/β subunit interface (Hosie et al. 2006, Hosie et al. 2009). Studies revealed that activity on the intracellular site is necessary for $GABA_A$ receptor modulation by low concentrations of ALLO, and this binding site appears to be distinct from the site of action for negative modulation by sulfated neurosteroids (Park-Chung et al. 1999, Akk et al. 2001, Akk et al. 2008). Conversely, high concentrations of ALLO or THDOC can activate receptors in a GABA-independent manner by acting on both the superficial site on the α/β subunit interface and on the membrane-bound α site (Wohlfarth et al. 2002, Hosie et al. 2006, Hosie et al. 2009, Carver and Reddy 2013). The α-subunit amino acid residues at locations 241 and 236 are essential for ALLO or THDOC modulation and GABA-independent activation of $GABA_A$ receptors, respectively (Hosie et al. 2006). Studies on *Gabrd* knock-out mice implicate δ-containing receptors in the anxiolytic-like effects of neurosteroids (Mihalek et al. 1999) and in their low-dose modulatory action (Stell et al. 2003). The δ-subunit may

play a role in neurosteroid efficacy, but since the α4(Q246L) mutation can inhibit potentiation of GABA responses by ALLO when expressed with δ or γ2, it is unlikely that the δ-subunit harbors a separate neurosteroid modulatory site (Hosie et al. 2009).

Synaptic $GABA_A$ receptors and anesthetic concentrations of alcohol (≥50 mM)

It is tempting to speculate that alcohol may affect receptors via a concentration-dependent mechanism as is the case with endogenous neurosteroids. In parallel with allopregnanolone, the extrasynaptic δ-containing receptors seem to be sensitive to the clinically relevant doses of alcohol; yet, there appears to be no receptor selectivity for high-concentrations of alcohol (Mihic et al. 1994). Because a definitive alcohol site of action has not yet been identified, techniques for targeting single amino acids are essential for conducting alcohol experiments on potential receptor-mediated mechanisms of action. Using a similar approach as Wieland et al., two separate labs identified amino acid residues involved in the potentiation of GABA-induced currents by high concentrations of ethanol (200 mM) and general anesthetics (Belelli et al. 1997, Mihic et al. 1997). When applied to glycine or $GABA_A$ receptors, ethanol and anesthetics potentiate GABA currents whereas these compounds act as inverse agonists of GABA ρ1 receptors. Due to their sequence similarities, GABA ρ1 and glycine receptor cDNAs could be used to generate chimeric cDNAs. Characterizing the resultant proteins revealed that a domain spanning transmembrane regions 2 and 3 (TM2, TM3) was required and sufficient for potentiation of GABA-elicited currents by ethanol and general anesthetics.

Within TM2 and TM3, residues that were conserved in glycine and $GABA_A$ but not ρ1 receptors were targeted via point-mutation. Examining the $GABA_A$ receptor revealed that β_1-subunit residues Ser265 in TM2 and Met286 and conserved $\alpha_{1,2}$-subunit residues, Ser270 and Ala291 in TM3 were necessary for alcohol and enflurane potentiation of GABA responses (Mihic et al. 1997). Only in the past several years, however, have researchers been able to identify a candidate ethanol binding pocket (Sauguet et al. 2013, Sauguet et al. 2014). As of yet, this work has only been conducted using non-mammalian ion channels that share similarities with $GABA_A$ receptors, but are not identical.

Gabra2 point-mutated mice have been generated with α2-containing $GABA_A$ receptors insensitive to potentiation of GABA-evoked currents by ethanol or select general anesthetics (Werner et al. 2011). These mice harbor two mutations; the S270H mutation renders them insensitive to ethanol and selected general anesthetics but also produces abnormal behavioral and developmental effects which are likely due to increased sensitivity to gating by GABA (Homanics et al. 2005). The L277A gain-of-function mutation reduces GABA sensitivity, bringing it closer to normal without interfering with the loss-of-function S270H mutation (Homanics et al. 2005, Borghese et al. 2006). A thorough investigation of male α2(S270H/L277A) mutants revealed that these mice show abnormal development of conditioned taste aversion and are insensitive to the motor stimulant effects of alcohol in an open field test (Blednov et al. 2011). In addition, these mice consume significantly less ethanol when given continuous access to ascending concentrations (EtOH 3-18% v/v) as compared to their WT littermates (Blednov et al. 2011). These results suggest that

the potentiation of GABA responses by alcohol at α2-containing $GABA_A$ receptors may be necessary for low to moderate alcohol consumption. However, there is little evidence aside from these findings to indicate that low concentrations of alcohol achieved by moderate levels of drinking have an effect on synaptic $GABA_A$ receptors. Therefore, in the present study, α2(S270H/L277A) mutants were assessed in a model of alcohol intake that has been shown to elicit an excessive, dependence-inducing pattern of drinking (Hwa et al. 2011).

ALCOHOL-INDUCED NEUROADAPTATIONS: ARE YOU WHAT YOU DRINK?

In addition to evaluating possible genetic causes, research has characterized alcohol-induced physiological changes that may relate to or promote dependence. These effects appear to be contingent on the duration of alcohol intake and abstinence and may be receptor subunit-selective. One study used [^{11}C]Ro15-4513, which has a higher affinity for α5-containing receptors, to identify diminished uptake in the hippocampus and nucleus accumbens of alcoholics after six weeks of abstinence as compared to healthy controls (Lingford-Hughes et al. 2012). Since α5-containing receptors are extrasynaptic in these brain regions, these data provide a possible explanation for reduced tonic inhibition in withdrawal. Likewise, postmortem evaluations of specific $GABA_A$ receptor subunits show reduced δ subunit and increased γ1 in the basal ganglia of alcoholics versus controls (Bhandage et al. 2014). This is a particularly interesting dissociation considering native receptors with both γ1 and δ have not been observed, so these neuroadaptations may signify compensatory changes. Specifically, downregulation

of extrasynaptic δ-containing receptors may cause upregulation of synaptic γ1-containing receptors (Bhandage et al. 2014). Reduced tonic inhibition may engender a hyperexcitable state to produce some of the symptoms of ethanol withdrawal syndrome (Liang et al. 2004, Liang et al. 2006).

Correlational human studies have also identified effects related to the duration since alcohol consumption. Following a week of abstinence, dependent patients show reduced in ^{123}I-iomazenil uptake; however, this effect was absent after four weeks of abstinence (Stanley et al. 2005). Chronic intake may cause downregulation of BZD-sensitive $GABA_A$ receptors while withdrawal may result in receptor upregulation. It is highly likely that neuroadaptations due to alcohol intake and subsequent abstinence are $GABA_A$ receptor subtype-specific. This possibility has been addressed using experimental animal models of voluntary and forced alcohol exposure.

Preclinical studies reveal that chronic alcohol administration or chronic binge-like intake can reduce α1- and α2-subunit mRNA expression in the cortex and the dorsal raphe nucleus (Morrow et al. 1990, Morrow et al. 1992, Mhatre and Ticku 1992, McClintick et al. 2015) and protein expression in the cortex and cerebellum of rats (Ravindran et al. 2007). Likewise, cultured mouse cortical neurons exposed to chronic alcohol (75 mM) treatment for five days showed reduced expression of α1, α2 and γ2 subunit mRNA (Rani and Ticku 2006). In withdrawal, there appears to be reduced cortical α1, α2 and α5 subunit mRNA expression *in situ* (Mhatre and Ticku 1992). However, 3-6 hours into withdrawal from high-concentration ethanol (100 mM), there is upregulation of α2 and α4 but downregulation of α1, α6, and γ2

subunit mRNA in cell cultures (Sanna et al. 2003, Follesa et al. 2003, 2004, 2005, Biggio et al. 2007). During ethanol self-administration, downregulation compensates for increased receptor positive modulation by alcohol. Upon alcohol withdrawal, neuroadaptive changes may produce a hyperexitable state, and therefore, subsequent upregulation of $GABA_A$ receptors could be a homeostatic response to counteract hyperexcitability (Sanna et al. 1993, Cagetti et al. 2003, Sanna et al. 2003). These findings indicated that low, euphoric and stimulating concentrations as well as high, anesthetic concentrations of alcohol and their withdrawal can impact both mRNA and protein expression levels in a subunit subtype-specific manner (for review Uusi-Oukari and Korpi 2010).

Some effects of alcohol may be mediated by a rise in allopregnanolone and resulting increases in modulation of $GABA_A$ receptors (Sanna et al. 2004). Clinical studies reveal increased plasma ALLO levels in adolescents recovering from severe alcohol intoxication (Torres and Ortega 2003, Torres and Ortega 2004). Similarly, in preclinical studies, alcohol administration (1-2 g/kg) can increase ALLO levels in the cortex, hippocampus, paraventricular nucleus of the hypothalamus (hPVN), and in the bed nucleus of the stria terminalis (BNST) of male rats; yet, it can reduce ALLO levels in the nucleus accumbens (NAcc) and central amygdala (CeA; Barbaccia et al. 1999, Cook et al. 2014a; but Porcu et al. 2010). In an elegant design, ethanol was administered acutely to rats that were either adrenalectomized (ADX) or intact. While cortical increases in ALLO required an intact adrenal gland, both ADX and intact rats showed increased ALLO in the hippocampus, hPVN and BNST along with reduced levels in the NAcc and CeA (Cook et al. 2014b).

Studies provide strong evidence to suggest that ethanol administration can affect neurosteroid levels in the brain. However, it is less apparent how ethanol-associated fluctuations in ALLO and THDOC may affect subsequent drinking. In alcohol-preferring rats, treatment with the synthetic neurosteroid, ganaxolone reduces ethanol responding (Besheer et al. 2010). In addition, when P450ccc, the rate-limiting enzyme that converts cholesterol into the ALLO and THDOC precursor, pregnenolone, is amplified in the VTA, rats reduced their ethanol self-administration (Cook et al. 2014c). Conversely, findings also suggest that allopregnanolone treatment can escalate binge-like intake and alcohol responding when administered either systemically or into the lateral ventricle of C57BL/6J mice (Sinnott et al. 2002, Wang et al. 2005, Ford et al. 2005, 2007, 2008, Ramaker et al. 2011). Also, in contrast with results reported by Besheer et al., a priming dose of allopregnanolone or ganaxolone has been shown to induce reinstatement of alcohol responding by rats or mice (Nie and Janak 2003, Ramaker et al. 2014).

These seemingly contradictory findings may reflect the involvement of distinct $GABA_A$ receptor subtypes according to the pattern of alcohol consumption. In addition, the pattern of ethanol intake or administration may have a specific effect on the synthesis of endogenous neurosteroids. Future drinking and the likelihood of escalation may depend on alterations in ALLO and THDOC synthesis elicited by this initial intake pattern.

EXPERIMENTAL AIMS AND HYPOTHESES

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, some studies have identified the α4βδ or α6βδ $GABA_A$ subtypes for potentiation of inhibitory currents by low concentrations of alcohol: yet, to date, these findings have been inconsistent. 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 protein site of action, such studies are informative in the absence of non-specific pharmacological tools and can reveal which protein domains are necessary for a non-specific drug to elicit a specific effect.

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 ALLO and THDOC (*in vitro:* Hosie et al. 2006, Hosie et al. 2009), or modulation by high concentrations of ethanol (Werner et al. 2011, Blednov 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. In addition, these findings may direct future clinical research by suggesting target sequences that could be differentially regulated or spliced due to AD-associated SNPs in the human *GABRA2* gene.

Aim1: Alcohol-dependent humans express fewer cortical BZD-binding sites as compared to non-dependent controls; this physiological difference may serve as a predictor of AD. To establish whether BZD-sensitive α2-containing $GABA_A$ receptors are necessary for the escalation of alcohol drinking, we propose an evaluation of BZD-insensitive *Gabra2* (H101R) mutants for binge-like and dependence-inducing drinking. We hypothesize that benzodiazepine-insensitive mutants will consume more alcohol as compared to wild-type C57BL/6J mice.

Aim 2: Ethanol can escalate plasma allopregnanolone levels in humans while finasteride can block self-administration in mice. To determine if sensitivity to neurosteroids at α2-containing $GABA_A$ receptors is required for escalated intake, mutant *Gabra2*(Q241M) mice will be evaluated for their short-access, binge-like drinking and in a protocol that can model dependence-inducing intake. The clinical and preclinical data seem to support the idea that escalated allopregnanolone levels following alcohol consumption may be responsible for some of the rewarding effects of alcohol. Therefore, we hypothesize that Q241M mutants will reduce their ethanol consumption.

Aim 3: Because sensitivity to ethanol potentiation of GABA responses at α2-containing $GABA_A$ receptors may be necessary for the rapid induction of neuroadaptations during escalated alcohol intake and subsequent withdrawal, ethanol-insensitive *Gabra2* (S270H/L277A) mutants will be assessed for dependence-inducing alcohol intake and for behavioral deficiencies associated with alcohol withdrawal. We hypothesize that S270H/L277A mutants will not escalate

their ethanol intake during intermittent access, and therefore, they will also not show subsequent behavioral deficiencies associated with withdrawal.

METHODS

I. ANIMALS

Mutant H101R mice were homozygous for a histidine to arginine point-substitution in *Gabra2* which confers selective 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, in theory, 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* (*in vitro*: Hosie et al. 2006, Hosie et al. 2009). 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 2** 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.

II. ALCOHOL INTAKE PROCEDURES

Binge-like ethanol intake: 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, 4-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).

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.

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 α2(H101R) mutants escalated their binge-like drinking in the one-bottle DID test, only α2(H101R) and wild-type mice were assessed in this two-bottle adaptation of the procedure.

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 2-, 4- or 24-hr 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. (2011).

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 (see **Fig. 2**).

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.

III. Social approach after alcohol withdrawal

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 in a repeatable test adapted for mice (Overstreet et al. 2002, Knapp et al. 2005, previously in mice Newman et al. 2015).

Male mutant and WT mice either received intermittent access to 20% EtOH or access to two bottles of tap water for 16 consecutive weeks (see **Fig. 2**). In week seven, mice were habituated to intraperitoneal injections and, prior to any assessment of social approach, mice were evaluated for side preference. Two ethanol-drinking mice (one WT, one H101R) and two ethanol-naïve mice (one H101R, one Q241M) were excluded because they continued to show a >60% side preference after two trials in the absence of a social stimulus mouse (see **Table 2** for group *n*s).

From week eight to sixteen, mice were tested for social approach behavior toward a novel OVX stimulus mouse during withdrawal from ethanol (see **Fig. 3**). Six to eight hours after the 20% EtOH bottle was replaced with water, the male experimental mouse was moved to the central chamber of a three-chamber apparatus. An OVX stimulus mouse was held in a wire mesh cage in either the right or left chamber. After 5-min., doors on either side of the central chamber were lifted, allowing the experimental male to move freely between the central chamber, the

chamber with the OVX stimulus mouse, and a third chamber with an empty stimulus cage for a 10-min. behavioral 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.

Experimental males in withdrawal from alcohol were compared to EtOH-naïve mutant and WT males that consumed water for 6-wks. To establish which genotypes demonstrated this phenotype, all mice received either 20% βCD or 0.9% saline prior to their first withdrawal test. These initial data were used to apply drug administration inclusion criteria (see Results).

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. In an initial test, , withdrawing and ethanol-naïve mice were administered vehicle 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. EtOH-naïve (Naïve) and EtOH-withdrawn (WD) mutant and WT mice received each dose of both compounds in a randomized order according to a mixed, factorial design. For withdrawing animals, alcohol intake (g/kg) returned to baseline 24-48-hr after drug treatment and social approach testing.

RESULTS

Escalated binge-like ethanol intake by H101R mutants

For the three versions of the drinking in the dark (DID) experiment (1-bottle, 1-bottle with lickometer, or 2-bottle choice), data were analyzed by protocol using two-way repeated measures analysis of variance (2-way RM ANOVA; genotype x day). For significant 2-way ANOVA results, Dunnett's test was used to compare treatment levels to a control condition (for DID, CA, and IA drinking: wild type x mutant; for social approach and locomotion in withdrawal: wild type x mutant, vehicle x drug dose; for ascending concentrations of alcohol: 10% x all other concentrations, wild type x mutant). In the original DID protocol, there was an effect of day ($F(3, 34)=13.32$, $p<0.001$) with escalated drinking during the binge and an effect of genotype ($F(3,102)=77.67$, $p<0.001$) with H101R mutants drinking the greatest amount of alcohol (**Fig. 4A**).

For DID that was adapted using the lickometer setup, two-way ANOVA detected an interaction between genotype and day ($F(9,66)=2.28$, $p=0.027$) driven by escalated drinking by all genotypes during the binge except for wild-type mice. This absence of significant escalation by wild-type mice on day four may contribute to the significant difference observed between wild-type and H101R mice during the binge (**Fig. 4B**).

According to the final adaptation of the DID protocol, mice received two-bottle choice access to water and alcohol according to the same schedule as the original DID protocol. Because only α2(H101R) mice were significantly different

from wild-type mice using the original protocol, we tested wild-type and α2(H101R) mutants in this protocol. Two-way RM ANOVA on genotype x day did not reveal any significant effects, suggesting that availability of a water bottle in addition to 20% EtOH will suppress alcohol binging. Another 2-way ANOVA was conducted to compare four-hour intake values between the original DID protocol and the two-bottle choice adaptation. This revealed a significant interaction between genotype and drinking protocol ($F(1, 32)=9.53$, $p=0.004$) reflecting significantly greater intake by α2(H101R) mutants that were assessed in the original DID protocol as compared to two-bottle choice DID **(Fig.5)**.

For a higher resolution analysis of alcohol self-administration, the four-day DID protocol was conducted in the lickometer setup. Nozzle contacts that closed the electrical circuit were assumed to be licks. Data are displayed as licks per ten-minute time bin throughout the four hour binge (**Fig. 6**). Two-way RM ANOVA on 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 10-min bin regardless of genotype . Interestingly, during the binge, wild-type mice reduced their drinking after two-hour access (**Fig. 6**), coinciding with the two-hour time point when ethanol bottles would have been removed on the preceding days. Conversely, none of the *Gabra2* mutants appeared to do this, which may indicate deficiencies in circadian rhythm or in expectancy coding.

Licking data were also portrayed in a log survival plot (**Fig. 7**) as the number of licks that occurred with a specified inter-lick interval to convey the number of high- versus low-frequency licks. Unfortunately, we found that the rate of licking

was so rapid that our mode of detection imposed an artificial cap on the detection of extremely high-frequency inter-lick intervals. Therefore, we cannot say with confidence that the wild-type mice actually engaged in more high-frequency bouts of licking (**Fig. 6**), or if the majority of high-frequency licks by mutants were faster than the limit of detection.

Lickometer data were useful in interpreting the discrepancy between low chronic drinking and wild-type-like binge drinking by the Q241M mutants. While the recorded g/kg intake for Q241M mutants was nearly identical to their wild-type counterparts, licking data revealed a remarkably low number of circuit completions. Paired with their low post-binge BECs (**Fig. 8**), these findings could suggest that Q241M mutants did not consume the entire volume of ethanol calculated for their 4-hour binge. The contrast between blunted chronic drinking by Q241M mutants and their wild-type–like binge "drinking" may be an artifact. A one-way ANOVA on BEC by genotype revealed a non-significant trend with $p=0.062$, driven by low BECs in Q241M mutants (M=73.93±12.3) and slightly higher than average BECs in H101R mutants (M=127.43±18.72).

Reduced chronic ethanol intake by Q241M mutants

For mice receiving continuous (CA) or intermittent access (IA) to alcohol, individual mean intake (g/kg) and percent ethanol intake for 18 ethanol-access days were analyzed by 2-way ANOVA to detect interactions between protocol and genotype (**Fig. 4C, 4D**). For g/kg intake and percent ethanol intake, separate two-way ANOVA revealed main effects of the drinking protocol (g/kg: $F(1, 68)=157.9$,

$p<0.001$; percent intake: $F(1, 68)=73.11$, $p<0.001$) and of genotype (g/kg: $F(3, 68)=24.16$, $p<0.001$; percent intake: $F(3,68)=6.26$, $p<0.001$). Intermittent alcohol access yielded significantly higher intake values than continuous access. Q241M mutants consumed less than wild-type mice while S270H/L277A mice consumed more.

For each chronic drinking protocol, 2-way RM ANOVA was run on individual weekly averages (g/kg) and percent daily alcohol intake to detect genotype-associated differences in the progression of drinking (genotype x week). For intermittent access g/kg intake data, 2-way RM ANOVA revealed a significant interaction between genotype and week ($F(15, 185)=2.13$, $p=0.01$). Wild-type and Q241M mice escalated their drinking following the third and fourth weeks, respectively whereas S270H/L277A mutants achieved their highest weekly averages in the second and third weeks (**Fig. 9A**). Throughout all six weeks, wild-type mice consumed significantly more than Q241M mutants. During the second week, S270H/L277A mice had higher intake values than wild-type mice. H101R mutants showed stable levels of drinking that did not differ from wild-type values. Two-way RM ANOVA also detected significant main effects of genotype ($F(3, 37)=8.33$, $p<0.001$) and of week ($F(5, 185)=5.15$, $p<0.001$). Q241M mice had significantly lower preference for alcohol as compared to wild-type. For all genotypes, percent preference increased from weeks four through six as compared to week one (**Fig. 10A).**

Two-way RM ANOVA on g/kg intake values from mice on the continuous access protocol revealed significant main effects of genotype ($F(3, 31)=9.94$,

$p<0.001$) and week ($F(5, 155)=5.72$, $p<0.001$). Similar to the intermittent access protocol, Q241M mutants consumed less than wild-type mice. However, in contrast with intermittent access, the continuous access protocol reduced drinking, with significantly lower intake values from weeks two onward as compared to week one (**Fig. 9B**). Continuous access percent ethanol intake values were also analyzed by two-way RM ANOVA, identifying main effects of genotype ($F(3, 31)=3.39$, $p=0.03$) and of 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, but no mutant line differed appreciably from wild-type. As with the g/kg intake, percent alcohol intake decreased though after the first week (**Fig. 9B**).

Disrupted social approach in withdrawal

Alcohol dependence was defined as a pattern of drinking that produces withdrawal symptoms in the form of disrupted social approach behavior. Because the aim of drug treatment was to diminish the signs of withdrawal, only mutants that displayed withdrawal-like phenotypes or different intermittent access alcohol intake were treated with midazolam or allopregnanolone.

Initially, two-tailed t-tests were used to determine if a specific genotype showed signs of withdrawal in the form of reduced time spent engaging with a social partner. In the case that data were not normally distributed, Mann-Whitney rank sum test was used. This analysis revealed that EtOH-naïve wild-type ($t(17)=3.11$, $p<0.01$) mice spent significantly more time in the social approach zone as compared to wild-type mice that had consumed alcohol on the intermittent

access protocol for six weeks and were tested for social behavior 6-8 hours into ethanol withdrawal. Mann-Whitney rank sum test detected a significant difference between EtOH-naïve H101R mice and their withdrawn counterparts (U=20, n_1=9, n_2=11, p=0.028). There was no difference in social approach time for EtOH-naïve and EtOH-withdrawn Q241M or S270H/L277A mutants (**Fig. 11C, D**, respectively). Since Q241M mice showed a trend toward reduced social approach compared to wild-types, regardless of drinking condition, they were tested for social approach with midazolam and ALLO treatments along with wild-type and H101R mice.

Because six-week intermittent access drinking differed by genotype and was predicted to impact behavior in withdrawal, social approach was analyzed by one-way RM ANOVA by genotype. Social approach after midazolam or allopregnanolone treatment was compared to behavior after vehicle administration according to a within subjects design. This analysis revealed a significant treatment effect in wild-type mice (F(4, 36)=3.77, p=0.012) with the 0.56 and 1.0 mg/kg doses of midazolam and the 10.0 mg/kg dose of allopregnanolone increasing social approach in withdrawal as compared to vehicle (**Fig. 11A**). For H101R mice, separate one-way RM analyses were run by drug since we anticipated that these generally anxiolytic compounds could have opposite effects in the BZD-insensitive mutants. There was a significant effect of midazolam (F(2, 16)=4.17, p=0.035) with the 1.0 mg/kg dose reducing social approach time, which was likely due to sedation (**Fig. 11B, 12**). 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 (χ^2(2)=8.22, p=0.016). As compared to vehicle, both the 3.0 and

10.0 mg/kg doses of allopregnanolone increased social approach in withdrawal from alcohol. For Q241M mice, one-way RM ANOVA revealed a significant effect ($F(4, 36)=3.23$, $p=0.023$) with the 1.0 mg/kg dose of midazolam increasing social approach. While these mice did not show signs of withdrawal, they did show a trend of reduced social approach as compared to wild-type mice. It appears as though this anxiogenic-like phenotype can be recovered to a degree with a moderate dose of midazolam, but not allopregnanolone (**Fig. 11C**).

Distance travelled during social approach testing in withdrawal was used as a metric of both withdrawal-induced locomotor impairments and for allopregnanolone- or midazolam-induced sedation. None of the genotypes showed motor impairment due to withdrawal as revealed by one-way ANOVA between EtOH-naïve and EtOH-withdrawn mice. 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. 12**). However, Friedman RM ANOVA did detect a significant effect of treatment in H101R mutants ($\chi^2(4)=22.93$, $p<0.001$) driven by reduced distance travelled following treatment with the highest dose of midazolam (1.0 mg/kg). This suggests that reduced social approach at this dose was associated with increased sedation. In Q241M mice, one-way RM ANOVA revealed a significant effect of drug administration ($F(4, 36)=11.61$, $p<0.001$) with the highest does of allopregnanolone (10.0 mg/kg) reducing locomotor behavior (**Fig. 12**).

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 ($F(9,96)=11.28$, $p<0.001$). 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. 13A**). When analyzed as percent ethanol intake, two-way RM ANOVA detected a significant effect of the ethanol 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. 13B**). 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); this analysis revealed a main effect of concentration with mice preferring the 100 mM solution ($F(2, 42)=44.04$, $p<0.001$; **Fig. 14A**). Two-way RM ANOVA on quinine percent intake also revealed a main effect of concentration ($F(1,20)=29.13$, $p<0.001$; **Fig. 14B).**

DISCUSSION

In the present study, mutant mice with BZD-insensitive α2-containing $GABA_A$ receptors escalated their binge-like alcohol intake, achieving blood ethanol concentrations of 90-240 mg/dl within four hours. In contrast, α2(H101R) mutants were indistinguishable from their wild-type counterparts in a protocol used to

model dependence-inducing drinking. In contrast, α2(Q241M) mutant mice rendered insensitive to neurosteroids at α2-containing $GABA_A$ receptors mutants consumed less than wild-types in the dependence-inducing and moderate drinking protocols. Finally, the α2(S270H/L277A) mutation, which rendered mice insensitive to ethanol potentiation of GABA-induced currents, did not impact the amount of alcohol consumed; yet, unlike wild-type mice, these mutants did not show disrupted social approach in withdrawal from chronic, excessive alcohol intake.

By using knock-in mice with specific amino acid substitutions in the α2-subunit primary protein sequence, the present study reveals: 1.) the H101R substitution escalates binge-like alcohol intake, 2.) the Q241M mutation suppresses escalation of chronic, 24-hour access drinking, and 3.) the S270H/L277A point substitutions have no effect on binge-like or chronic alcohol drinking but prevent social deficits in withdrawal. In addition, effects of genotype on ethanol intake were not associated with differences in consumption of bitter or sweet solutions.

Clinical studies have revealed a correlational relationship between reduced $GABA_A$ receptor BZD-binding sites and alcohol-dependence. By evaluating α2(H101R) mutant mice, we provide evidence to suggest that BZD-insensitivity can promote excessive binge-like alcohol consumption. In contrast, when α2(H101R) mutants received chronic intermittent, chronic continuous, or brief intermittent access to ethanol along with water, they did not escalate their intake (**Fig. 5**). This pattern of results could suggest that the α2(H101R) mutation promotes excessive drinking once a threshold of intoxication has been surpassed. Future lickometer studies will test this by priming α2(H101R) mutants with a low dose of ethanol (0.5

g/kg) or saline and assessing alcohol drinking. If a threshold model is at play, ethanol-primed α2(H101R) mutants would be expected to consume more than their saline-treated counterparts. Similarly, ethanol-primed animals may work harder to gain access to alcohol which could be assessed using an operant conditioning task on a progressive ratio schedule.

In addition to being BZD-insensitive, mutant α1(H101R)- and α2(H101R)- and recombinant α4- and α6-containing receptors share an unusual response to Ro15-4513, the BZD-derivative and "alcohol antagonist" introduced in the 1980s (Suzdak et al. 1986a). Specifically, Ro15-4513 can act on mutant and native BZD-insensitive receptors to *strongly potentiate* GABA-induced currents; in stark contrast, this compound acts as a *partial inverse agonist* on BZD-sensitive receptor subtypes (Wafford et al. 1992, Hadingham et al. 1996, Knoflach et al. 1996, Whittemore et al. 1996, Benson et al. 1998, Rudolph et al. 1999, Low et al. 2000, Crestani et al. 2002b). Although Ro15-4513 and flumazenil are often referred to as "BZD-site" ligands, both compounds can act on classic BZD-sensitive and BZD-insensitive α4/α6βδ and α4/α6βγ $GABA_A$ receptor subtypes, though with different efficacies (Mohler et al. 1981, Wisden et al. 1991, Whittemore et al. 1996, Iyer et al. 2011). Therefore, positive modulatory action on BZD-insensitive α4- or α6-containing receptors and inverse agonist action on BZD-sensitive receptors may produce what seem like mutually exclusive characteristics.

Considering the varied actions of *diazepam binding inhibitor* (*DBI*) gene products as either a negative or positive allosteric modulators, it is worth questioning if such endogenous peptides may act on $GABA_A$ receptors like Ro15-

4513. Indeed, in their initial 1978 report identifying *DBI* as a GABA receptor ligand, Guidotti, Toffano and Costa mention a high-affinity *DBI* binding site of action independent of the diazepam and GABA sites. This site of action may very well be on high-affinity, extrasynaptic $GABA_A$-receptors; yet, to my knowledge, this has not been investigated. Interestingly, chronic alcohol and withdrawal from alcohol are associated with upregulation in *DBI* (Katsura et al. 1995a, Katsura et al. 1995b). If these two modulators have a similar mechanism of action, then Ro15-4513 may have its anti-alcohol effects by acting on receptors absent of *DBI* to oppose or equalize its effects. Related work demonstrates that repeated dosing with Ro15-4513 produces locomotor sensitization in α2(H101R) mutants but not wild-type mice (Morris et al. 2008, Dixon et al. 2010). In α2-subunit-rich brain areas including the cortex, hippocampus, amygdala, striatum and motor nuclei, *DBI* may act on BZD-insensitive α2(H101R)-containing receptors as a partial agonist. Theoretically, Ro15-4513 administration could augment potentiation of these receptors to cause downregulation of the α2(H101R)-subunit, perhaps on medium spiny neurons within the NAcc, to facilitate locomotor sensitization (Dixon et al. 2010). Because the drinking in the dark procedure follows a schedule of repeated, brief periods of ethanol self-administration, this protocol may also produce sensitization in the α2(H101R) mutants. Such a sensitization effect could explain why these animals escalate their drinking on the fourth consecutive day of alcohol access during the binge. Repeated 24-hr access to alcohol during the continuous or intermittent access protocols may not fit the requirements for sensitization to occur, leading to wild-type-like long-term drinking by α2(H101R) mutants.

It is also possible that the H101R point-mutation may alter drinking behavior by hindering $GABA_A$ receptor assembly and subsequent receptor targeting. A study assessing the crucial span of amino acids necessary for assembly identified amino acids 80-100 in the α1-subunit protein as required for the α1 and γ2 subunits to assemble into functional receptors targeted to the membrane (Klausberger et al. 2001). When amino acids β3(77-98) were substituted for α1(80-100), there was an absence of α1β3γ2 cell surface expression. Conversely, surface expression of α1β3 was apparent and γ2 could be visualized in permeabilized cells, indicating that when α1γ2 assembly fails due to a mutation within amino acids 80-100, α1β3 assembly can still occur while the γ2 subunits likely remain within the endoplasmic reticulum (Klausberger et al. 2001). If the histidine to arginine mutation at amino acid 101 were to impede αγ subunit assembly, you may expect insensitivity to benzodiazepines to result from the formation of the $\alpha_2\beta$ configuration rather than α2(H101R)βγ (Levitan et al. 1988a, Levitan et al. 1988b, Pritchett et al. 1989b, Schofield et al. 1987, Malherbe et al. 1990). Due to the crucial site of α2βγ-comprised $GABA_A$ receptors at the AIS of pyramidal neurons, improper assembly would likely give way to dramatic behavioral and physiological abnormalities which were not observed. In addition, a recent study confirmed that brain slices harvested from α_5(H105R) mutant mice bound [^{3}H]L655,708, a compound with high affinity for α_5-subunit containing receptors with the αβγ $GABA_A$ receptor composition (Balic et al. 2009). Yet, we cannot eliminate the possibility that the α2(H101R) mutation may alter receptor assembly and targeting to impact receptor sensitivity.

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 allopregnanolone can increase responding for alcohol and can escalate alcohol intake (Janak et al. 1998, Sinnott et al. 2002, Ford et al. 2005, Wang et al. 2005, Ford et al. 2007, Ford et al. 2008, Ramaker et al. 2011, Nie and Janak 2003, Ramaker et al. 2014). Yet, many studies also provide evidence for reduced drinking by ALLO or THDOC treatment (Besheer et al. 2010, Ramaker et al. 2015). In the present investigation, α2(Q241M) mutants show 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 modulatory site to induce a conformational change in the receptor. This may subsequently render an extracellular site (perhaps S270?) more accessible to ethanol, leading to receptor positive modulation. In this case, low doses of neurosteroid would activate the modulatory site and lead to a potentiation of alcohol's effects; conversely, higher doses of neurosteroid would compete with

alcohol for the extracellular site resulting in blunted ethanol action. Such a bitonic dose-effect curve might explain the mixed findings within the literature. Additional studies need to address whether or not α2(Q241M)-containing receptors are sensitive to ethanol-potentiation of GABA-induced currents. If these receptors are indeed insensitive to alcohol, targeted amino acid substitutions, perhaps around residue S270, may yield a mechanistic explanation for the interaction between neurosteroids and alcohol.

During a chronic intermittent access to alcohol procedure, wild-type, α2(H101R), and α2(S270H/L277A) mice all consumed ~20 g/kg/24 hr for six weeks. In withdrawal from alcohol, wild-type mice and α2(H101R) mutants showed reduced social approach compared to their ethanol-naïve counterparts. Treatment with allopregnanolone could recover social approach behavior in both genotypes while only wild-type mice were sensitive to midazolam, thereby functionally confirming the H101R mutation. Despite consistently drinking substantial amounts of alcohol, α2(S270H/L277A) mutants did not show deficits in social behavior during withdrawal. These results suggest that ethanol potentiation of GABA responses at α2-containing $GABA_A$ receptors may be necessary for some of the effects of alcohol withdrawal. However, 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. 2006). 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 α2(S270H/L277A) mice, then

withdrawal-like symptoms may require enhancement of GABA currents by ethanol. If so, future studies should also investigate whether reduced withdrawal symptoms in S270H/L277A mutants might protect these animals from relapse to drug-taking behavior (i.e. reinstatement).

Endophenotypic analyses reveal that individuals with a high-risk AD-associated *GABRA2* haplotype have increased resting EEG beta band (13-28 Hz) activity (Edenberg et al. 2004). Benzodiazepines and allopregnanolone can have a similar effect on EEG power, possibly by acting on α2-containing $GABA_A$ receptors on the axon initial segment of hippocampal and cortical pyramidal neurons (for review on subcellular localization, see Fritschy and Brunig 2003). Assessments of α1(H101R), α2(H101R) and α3(H126R) mutant mice reveal a requirement for BZD-sensitive α2-, but not α1 or α3-containing receptors in enhancement of cortical EEG beta activity by diazepam (Tobler et al. 2001, Kopp et al. 2003, Kopp et al. 2004). In addition, reduced baseline hippocampal beta/theta band power was detected in α2 global knockout, but not α1 global knockout mutants (Heistek et al. 2013). It is possible that blunted baseline EEG beta activity in α2(H101R) mutants is due to reduced sensitivity to an endogenous modulator of the BZD-binding site. Studies have shown that BZD-sensitive α2-containing $GABA_A$ receptors are necessary for BZD-escalated intra-cranial self-stimulation. In contrast, we show that α2(H101R) mutants actually escalate their binge-like drinking, thereby suggesting that alcohol and BZDs may require different sites of action to produce their rewarding effects (Reynolds et al. 2012, Engin et al. 2014).

Because high-risk AD–associated *GABRA2* allelic variants do not affect primary protein sequence in humans, mutant mice with amino acid substitutions do not serve as humanized preclinical models to assess alcohol dependence risk. However, the present study does 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. 2008), 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. Specifically, a histidine residue at location 101 in the $GABA_A$ receptor α2-subunit protein, which is required for benzodiazepines to produce their anxiolytic, analgesic, and rewarding effects, is also involved in regulating binge-like, excessive alcohol drinking. Second, a glutamine residue at location 241 in the α2 protein sequence is necessary for neurosteroid modulation and activation of $GABA_A$ receptors and for excessive chronic, dependence-inducing drinking. Last, a shared site for selected general anesthetics and ethanol to potentiate GABA-induced currents (S270H) may be necessary for some symptoms of ethanol withdrawal. To determine if these 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 (for review

Roche et al. 2015). Such findings may guide the development of pharmacological tools to aid in the diagnosis and treatment of alcohol dependence (for review Kranzler and Edenberg 2010).

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Table 1

$GABA_A$ receptor α-subunit subtypes required for BZD-induced behavioral effects in histidine-to-arginine point-mutants

Effect of BZD	α_1	α_2	α_3	α_5	
Amnestic					
Passive avoidance	+	nt	nt	nt	Rudolph et al. 1999
Analgesic					
Formalin test	-	++	+	+	Knabl et al. 2008, 2009, Ralvenius et al. 2015
Neuropathic pain	-	++	+	+	Knabl et al. 2008, Paul et al. 2014, Ralvenius et al. 2015
Anticonvulsant					
Pentylenetetrazole	+	-	-	-	Rudolph et al. 1999, Low et al. 2000, Crestani et al. 2000, 2002a
Anti-fear					
Fear-potentiated startle	+	+*	-	nt	Smith et al. 2012, Newman et al. 2015
Anxiolytic					
Elevated X/plus maze	-	+	-	-	Rudolph et al. 1999, Low et al. 2000, Crestani et al. 2002a, Smith et al. 2012
Light/dark choice	-	+	-	-	Rudolph et al. 1999, Low et al. 2000, Crestani et al. 2002a
Conditioned emotional response	nt	+	nt	nt	Morris et al. 2006
Novelty-induced risk assessment	+	+	-	nt	Koester et al. 2013
3-chamber social approach	-	+	+	nt	Newman et al. 2015
Hyperphagia					
Behavioral satiety sequence	nt	-	nt	nt	Morris et al. 2009
Myorelaxant					
Horizontal wire	-	++	+	-	Rudolph et al. 1999, Crestani et al. 2001, Ralvenius et al. 2015
Pro-aggressive					
Resident-intruder	+	+	-	nt	Newman et al. 2015
Rewarding					
BZD+cocaine-induced hyperactivity	nt	+	nt	nt	Morris et al. 2008, Dixon et al. 2010
BZD-reduced ICSS	(+)	++	+	nt	Reynolds et al. 2012, Engin et al. 2014
Oral MDZ self-administration	+	+	-	nt	Tan et al. 2010, Engin et al. 2014

Table 1 cont.

GABA$_A$ receptor α-subunit subtypes required for BZD-induced behavioral effects in histidine-to-arginine point-mutants					
Effect of BZD	α_1	α_2	α_3	α_5	
Sedative					
Ethanol-potentiation of LORR	-	+	-	-	Rudolph et al. 1999, Tauber et al. 2003
Open field, circular chamber, or 2-chamber	+	-	-	-	Rudolph et al. 1999, Low et al. 2000, McKernan et al. 2000, Crestani et al. 2000, 2001, 2002a, Zeller et al. 2008, Smith et al. 2012
Tolerance to chronic BZD					
Tolerance to sedative effect	+[&]	-	-	+	van Rijnsoever et al. 2004
Effects of α-subunit point-mutations in the absence of BZDs					
Anxiety-like behavior					
Light/dark choice	nt	nt	nt	=	Prut et al. 2010
Locomotion					
Open field	nt	nt	nt	↑	Hauser et al. 2005, Prut et al. 2010
3-chamber social approach	=	=	=	nt	Newman et al. 2015
Learning					
Trace fear conditioning	nt	nt	nt	↑	Crestani et al. 2002b, Yee et al. 2004
Trace appetitive conditioning	nt	nt	nt	↑	Yee et al. 2004
Fear-potentiated startle	=	=	=	=	Crestani et al. 2002b, Smith et al. 2012, Newman et al. 2015
Contextual fear conditioning	nt	nt	nt	=	Crestani et al. 2002b, Prut et al. 2010
Pre-pulse inhibition	nt	nt	nt	↓	Hauser et al. 2005
Object location learning	nt	nt	nt	↓	Prut et al. 2010
Latent inhibition	nt	nt	nt	↓^	Gerdjikov et al. 2008
Social approach and aggression	=	=	=	nt	Newman et al. 2015

+ α subunit is required, ++ α subunit shows greater involvement than other α subunits that also appear to mediate the effect
(+) possible involvement of α subunit, some conflicting evidence
- α subunit is not required
nt indicates not tested
*BZD-specific effect; α2 required for reduced startle responding by diazepam and chlordiazepoxide, but not midazolam
& likely due to a lack of BZD-induced sedation in α1(H101R) mice
^ Only observed in males. No effect in
females.
BZD benzodiazepine; MDZ midazolam; ICSS intracranial self-stimulation; LORR loss of righting reflex

Table 2

	Experimental group *ns*			
	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

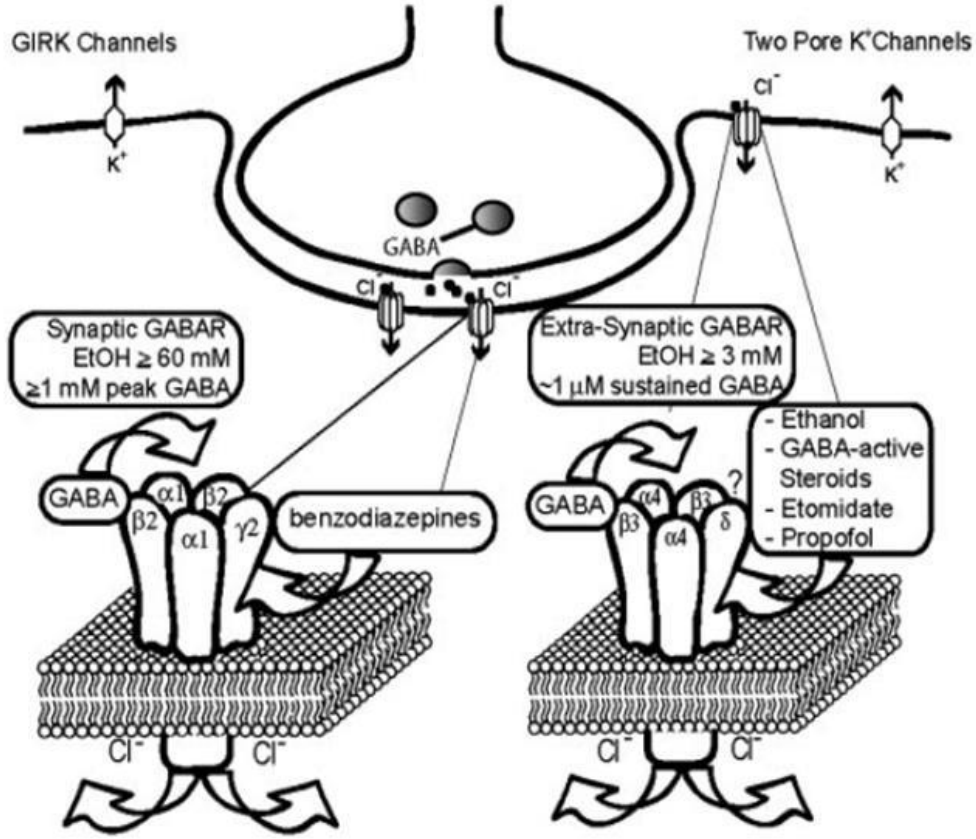

FIG. 1. ***SYNAPTIC VS. EXTRASYNAPTIC GABA$_A$ RECEPTORS***

Synaptic receptor (left) portrayed as the most common receptor assembly in the mammalian CNS (α1β2γ2). Clustering at the synapse allows for synchronized activation in response to saturating concentrations of GABA (>1 mM); show high efficacy but fairly low potency. Extrasynaptic receptor (right; composed of α4δ- or α6δ - and most likely β3-subunits); activated by persistent and usually non-saturating ambient GABA concentrations (0.5–1μM), and, even at saturating GABA concentrations, are characterized by low-current levels (high-potency, low efficacy receptors). **Figure and caption adapted from Wallner et al. 2003**

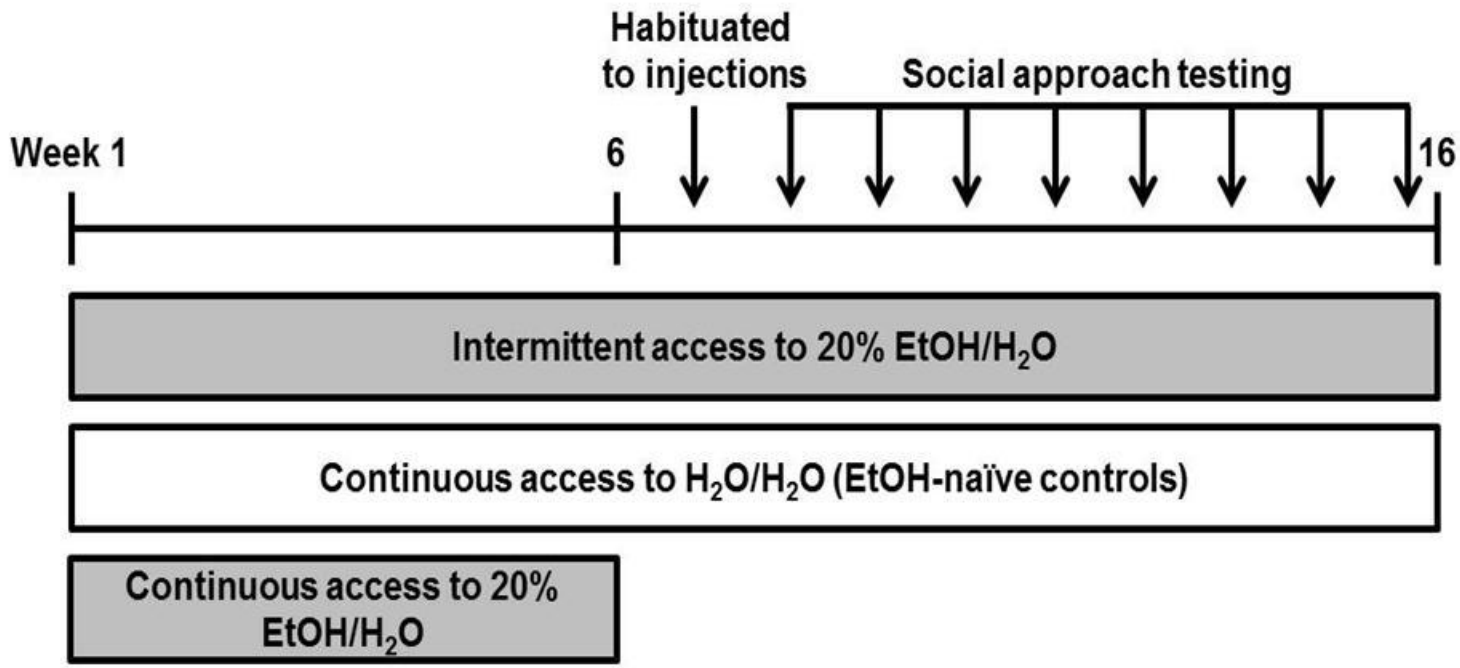

FIG 2. ***CHRONIC ALCOHOL DRINKING TIMELINE***

Each bar represents a separate cohort comprised of wild-type, H101R, Q241M and S270H/L277A $GABA_A$ receptor α2-subunit point-mutated males.

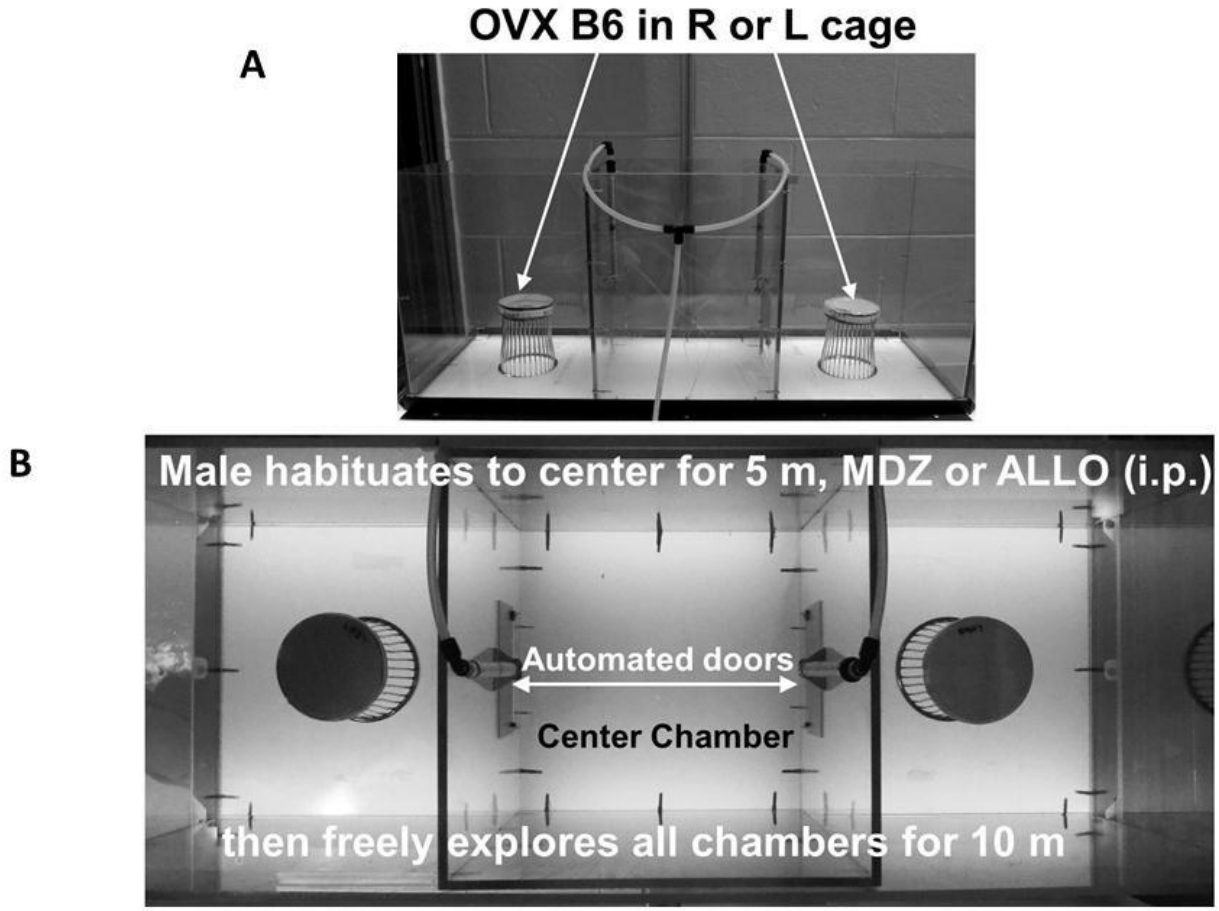

FIG 3. ***SOCIAL APPROACH 3-CHAMBER APPARATUS***

The two doors to the center chamber are operated by pressurizing two air piston systems, on e attached to each door. An OVX stimulus female is contained in the stimulus cage within the right or left chamber (A). The experimental male is administered drug and allowed to habituate to the center chamber for five minutes (B) after which the pressure is released to open the doors. The male freely explores the three chambers for a ten-minute test.

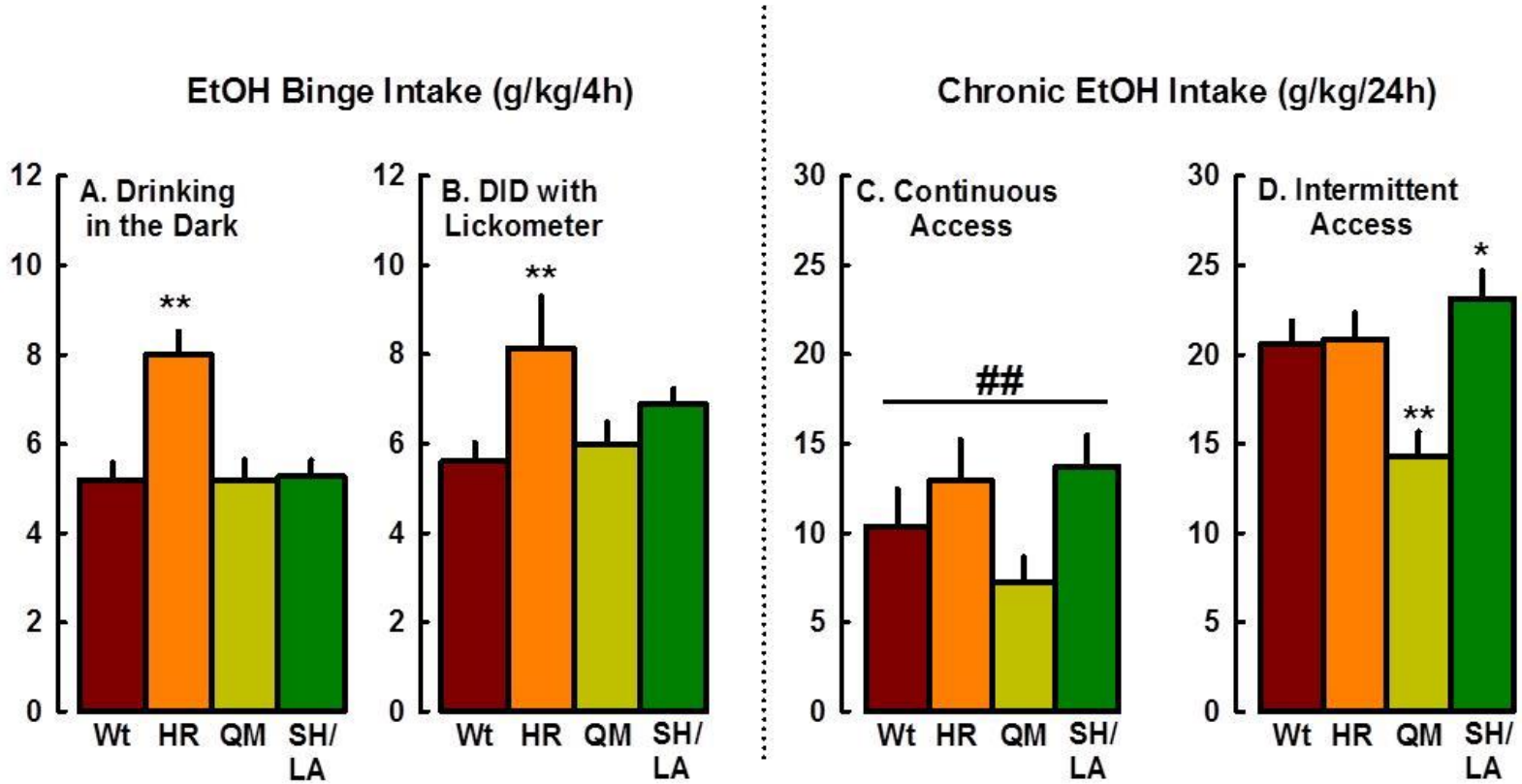

FIG 4. ***BINGE-LIKE AND CHRONIC ALCOHOL DRINKING SUMMARY***

Wild-type (Wt) and α2-subunit point-mutated males: H101R (HR), Q241M (QM), and S270H/L277A (SH/LA) in binge-like (A, B), moderate (C), or dependence-inducing (D) models of alcohol intake; Four-hour binge (20% EtOH (w/v))on the last day of the drinking in the dark protocol either without (A) or with the lickometer setup (B); Daily average intake (g/kg) for six weeks of continuous access to alcohol with 20% EtOH (w/v) and water available daily (C); Daily average intake (g/kg) for the initial six weeks of intermittent access to alcohol with 20% EtOH and water available M, W, F (D)

Data are shown as mean ± SEM; each bar represents a different group of animals

****p<0.01, *p<0.05 compared to wild-type, ##p<0.01 compared to continuous access**

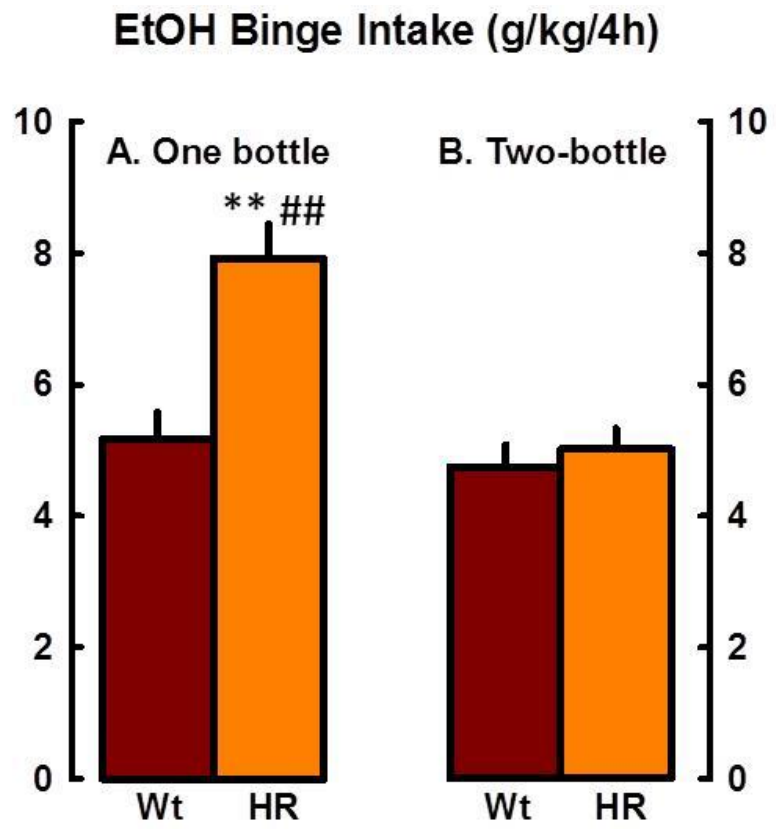

FIG 5. *BINGE-LIKE ALCOHOL INTAKE VERSUS 2-BOTTLE CHOICE DID*
Wild-type (WT) and *Gabra2* H101R (H-R) point-mutants; Four-hour binge (20% EtOH w/v) on the last day of the drinking in the dark protocol (A); Four-hour intake on the final day of the adapted, two-bottle choice drinking in the dark protocol (20% EtOH and H2O; B).

Data are shown as mean ± SEM; each bar represents a different group of animals
****p<0.01 compared to wild-type, ##p<0.01 compared to two-bottle choice drinking in the dark**

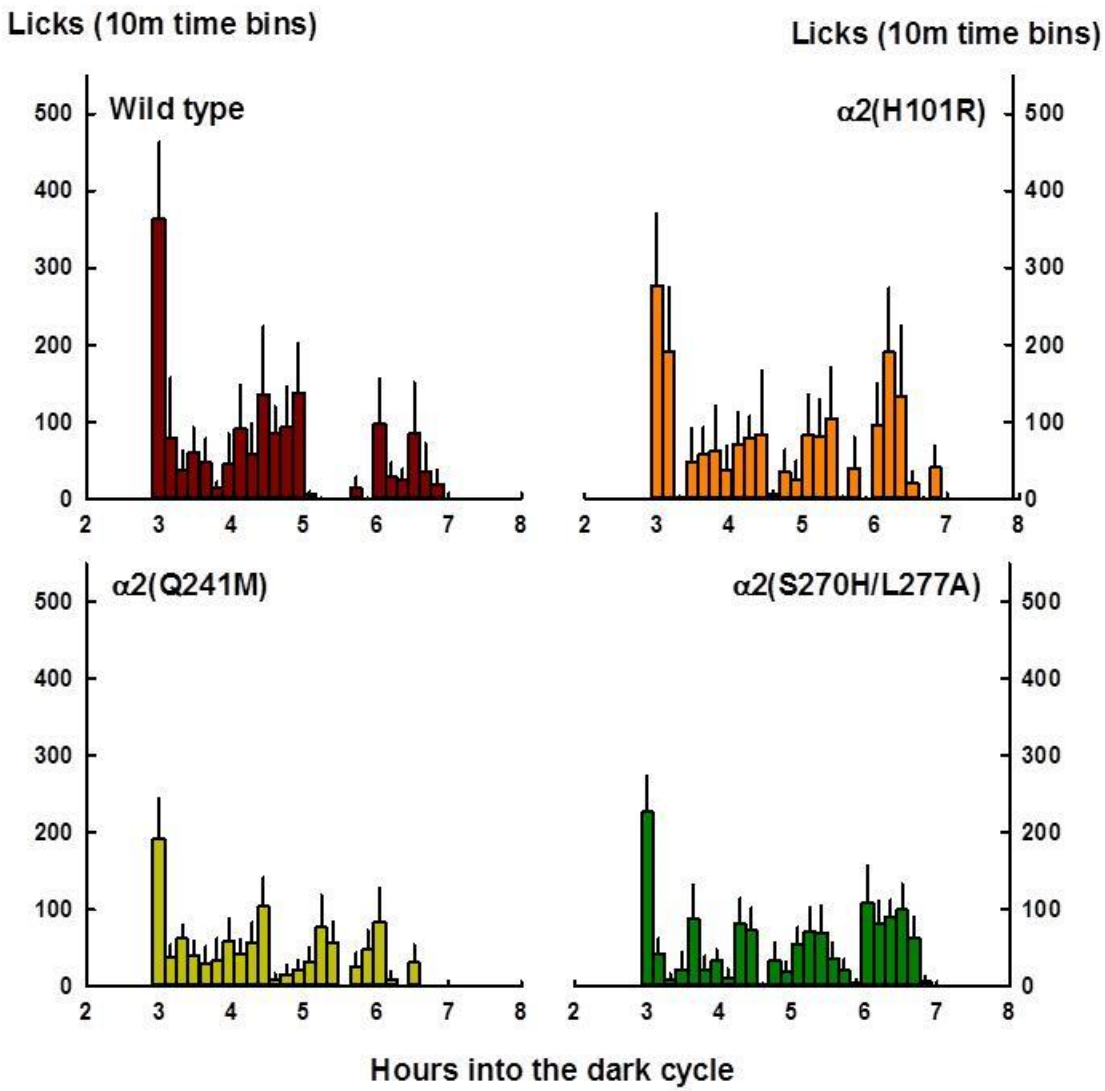

FIG 6. *THE BINGE, DECONSTRUCTED*

Wild-type and α2-subunit H101R, Q241M. and S270H/L277A point-mutated males; Contacts made during the four-hour binge on the final day of the DID protocol; represented as contacts per 10-minute time bin

Data are shown as mean ± SEM

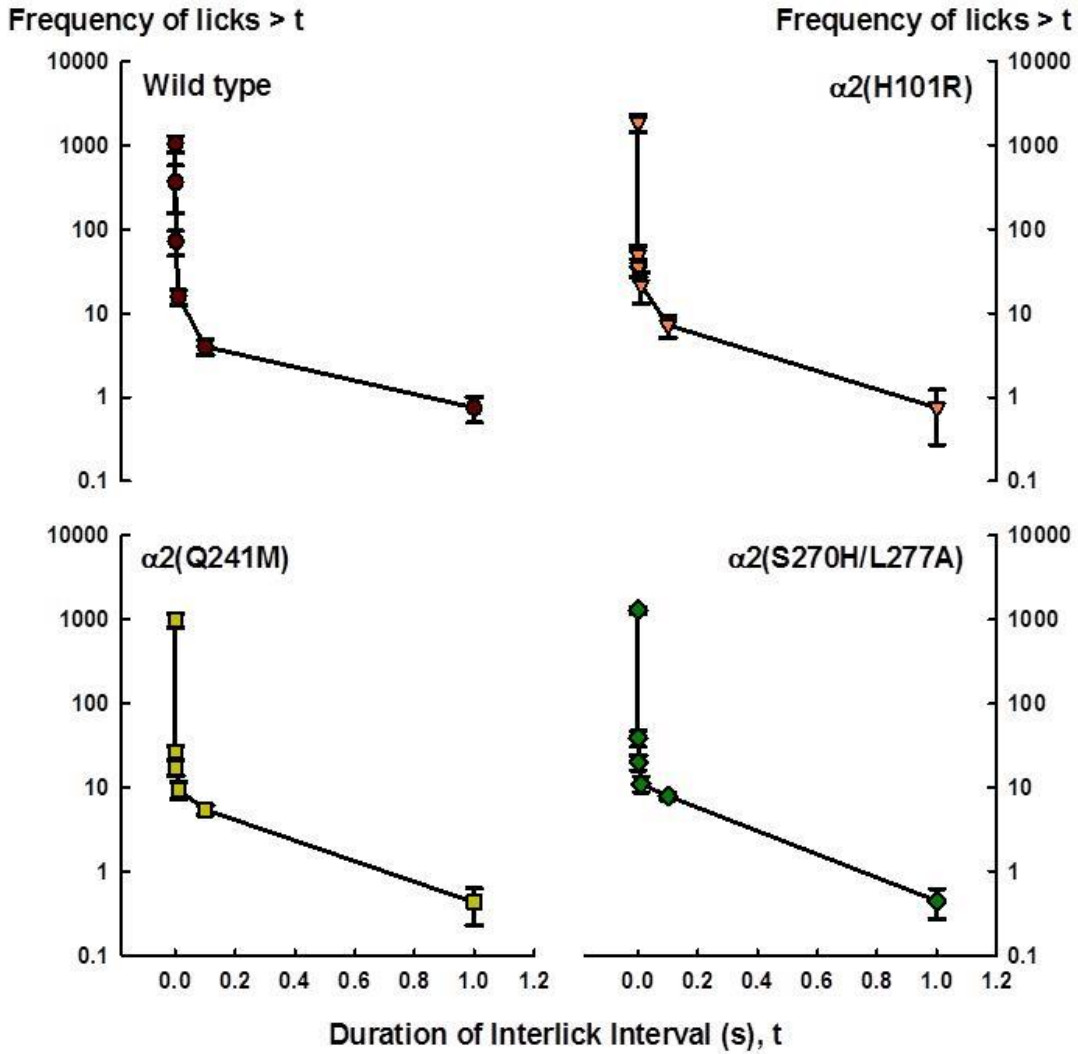

FIG 7. *RAPID LICKING DURING THE BINGE*

Wild-type and α2-subunit H101R, Q241M. and S270H/L277A point-mutated males; Number of licks according to duration between consecutive licks; a metric of low- versus- high-frequency licking bouts. All genotypes show a pattern of rapid licking behavior with 50% of licks occurring <0. 1 seconds apart.

Data are shown as mean ± SEM

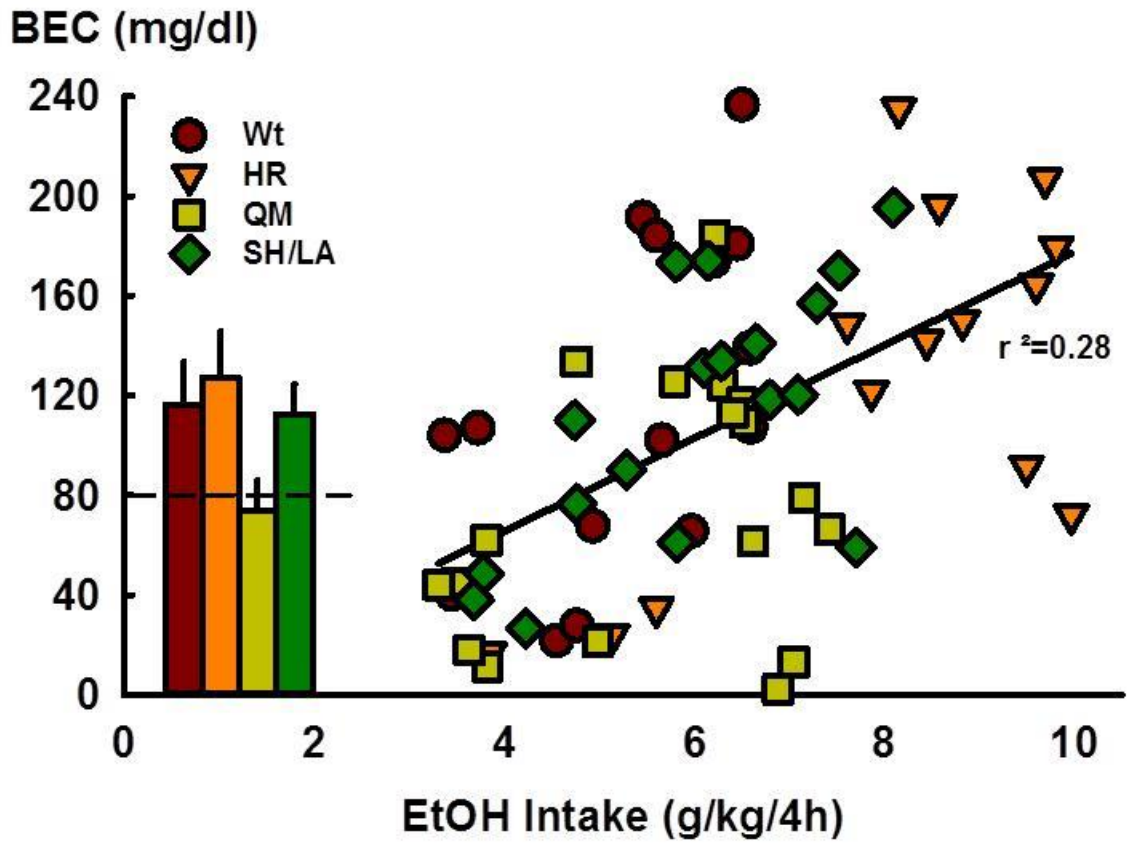

FIG 8. ***POST-BINGE BLOOD ETHANOL CONCENTRATIONS***

Wild-type and α2-subunit H101R, Q241M. and S270H/L277A point-mutated males; Blood was collected immediately after the four-hour binge from the submandibular vein and plasma was analyzed for BEC in mg/dl. BEC >80 mg/dl is considered impaired. Each point represents a single animal while the histogram portrays genotype mean BEC ± SEM.

Data are shown as mean ± SEM in the histogram, p =0.062 for mean BECs according to genotype
Scatter plot shows individual animal BEC

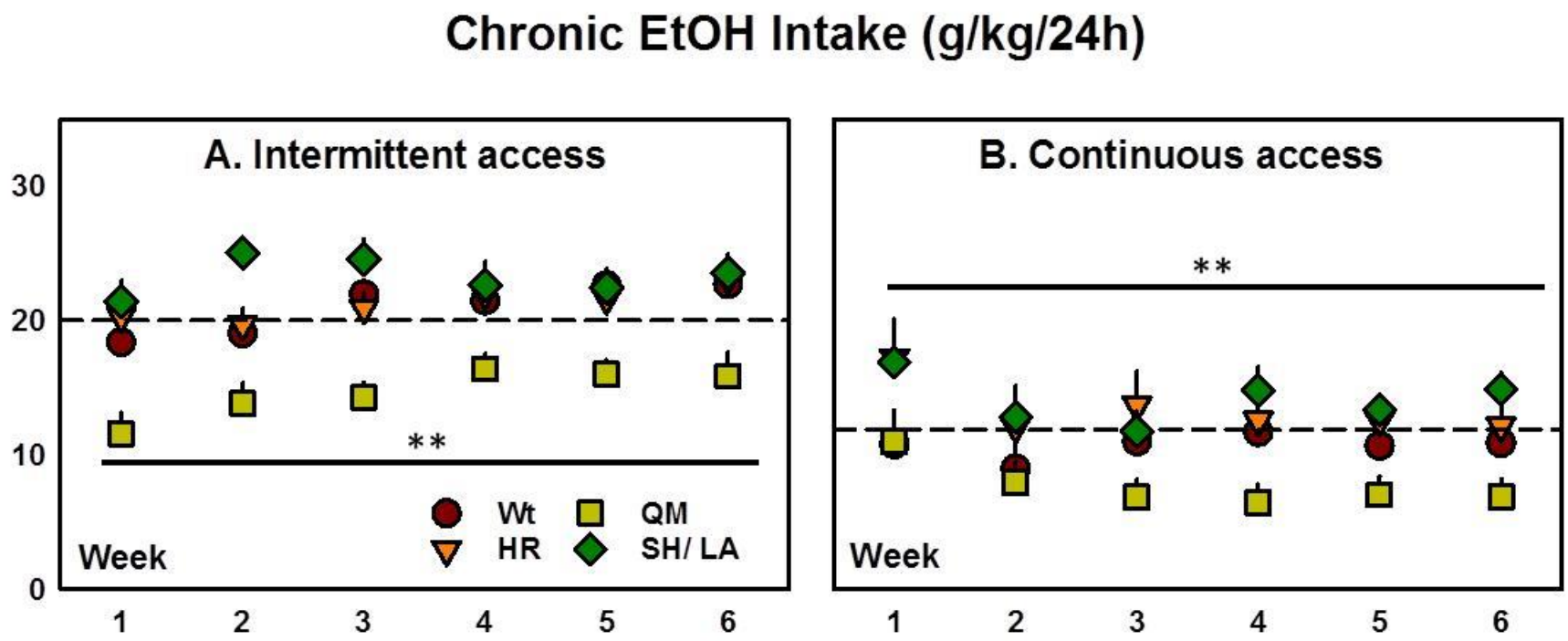

FIG 9. *CHRONIC MODERATE VS DEPENDENCE-INDUCING INTAKE*

Wild-type and α2-subunit H101R, Q241M. and S270H/L277A point-mutated males either had six weeks of continuous daily access to 20% EtOH (w/v) and water (A; moderate drinking); or six weeks of intermittent access to 20% EtOH (w./v) and water on M, W, F with only water all other days (B; dependence-inducing). Data are expressed as the daily average g/kg ethanol intake ± SEM.

Dashed lines are group means: IA: M=19.7, SEM±0.6, CA: M=11.0, SEM±0.652, $F(1,68)=157.9$ $p < 0.001$

****p<0.01 WT vs. Q241M**

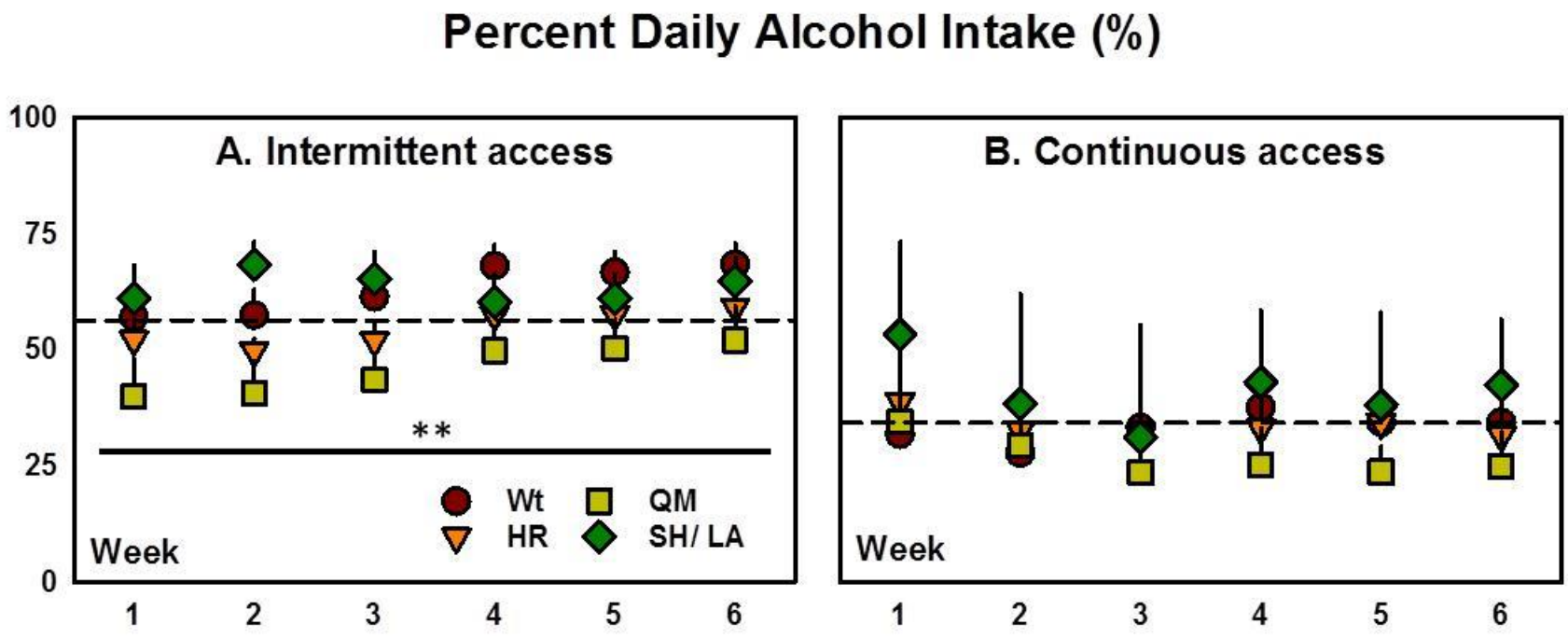

FIG 10. ***CHRONIC DRINKING PREFERENCE FOR ALCOHOL***
Wild-type and α2-subunit H101R, Q241M. and S270H/L277A point-mutated males either had six weeks of continuous daily access to 20% EtOH (w/v) and water (A; moderate drinking); or six weeks of intermittent access to 20% EtOH (w./v) and water on M, W, F with only water all other days (B; dependence-inducing). Data are expressed as the daily average g/kg ethanol intake ± SEM.

Dashed lines are group means: IA: *M*=56.32, SEM±01.75, CA: *M*=34.27, SEM±1.89, F(1,68)=73.107 $p < 0.001$
****p<0.01 WT vs. Q241M**

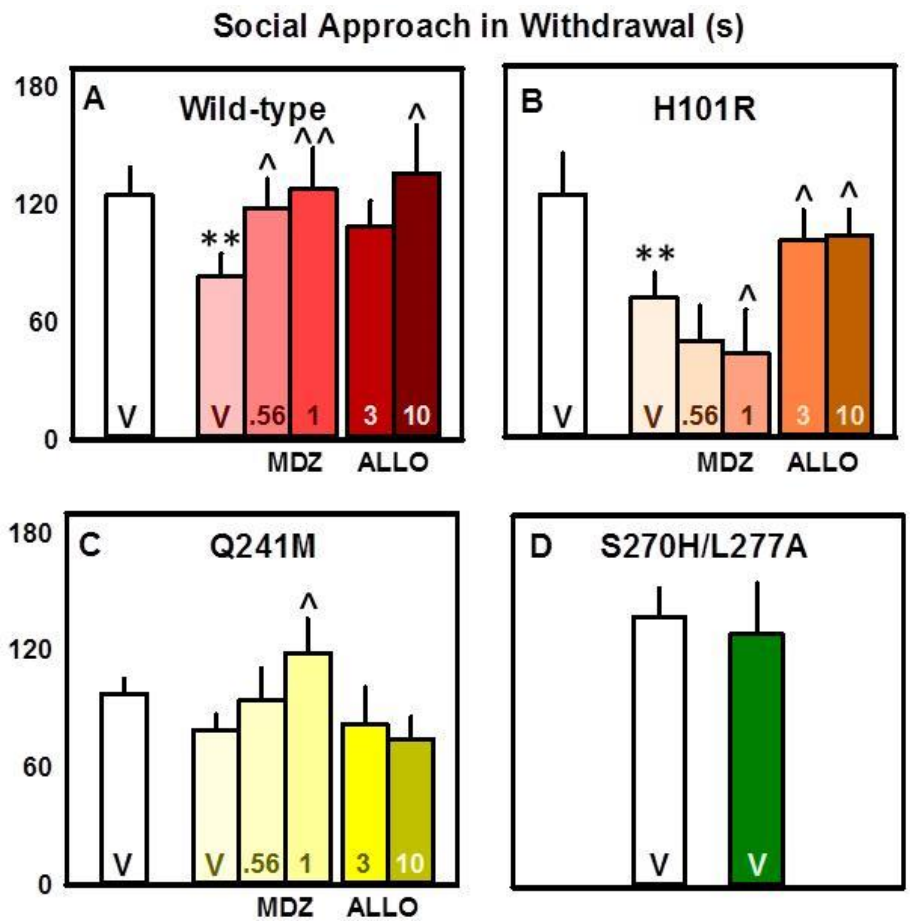

FIG 11. ***DISRUPTED SOCIAL APPROACH IN WITHDRAWAL RECOVERED WITH MIDAZOLAM OR ALLOPREGNANOLONE***
Wild-type (A), H101R (B), Q241M (C). and S270H/L277A (D) mice were maintained for six weeks on the IA protocol and were tested weekly for social approach, 6-8 hours after removal of ethanol bottles. S270H/L277A mutants were not tested under drug conditions because the did not meet the withdrawal criteria. White bars signify the matched, EtOH-naïve control mice tested after vehicle administration; data are portrayed in each panel from left to right: vehicle EtOH-naïve, vehicle EtOH withdrawal (EtOH WD); 0.56 mg/kg midazolam EtOH WD; 1.0 mg/kg midazolam EtOH WD; 3.0 allopregnanolone EtOH WD; 10.0 allopregnanolone EtOH WD

***p<0.05, **p<0.01 compared to EtOH-naïve vehicle; ^p<0.05, ^^p<0.01 compared to EtOH-withdrawn vehicle**

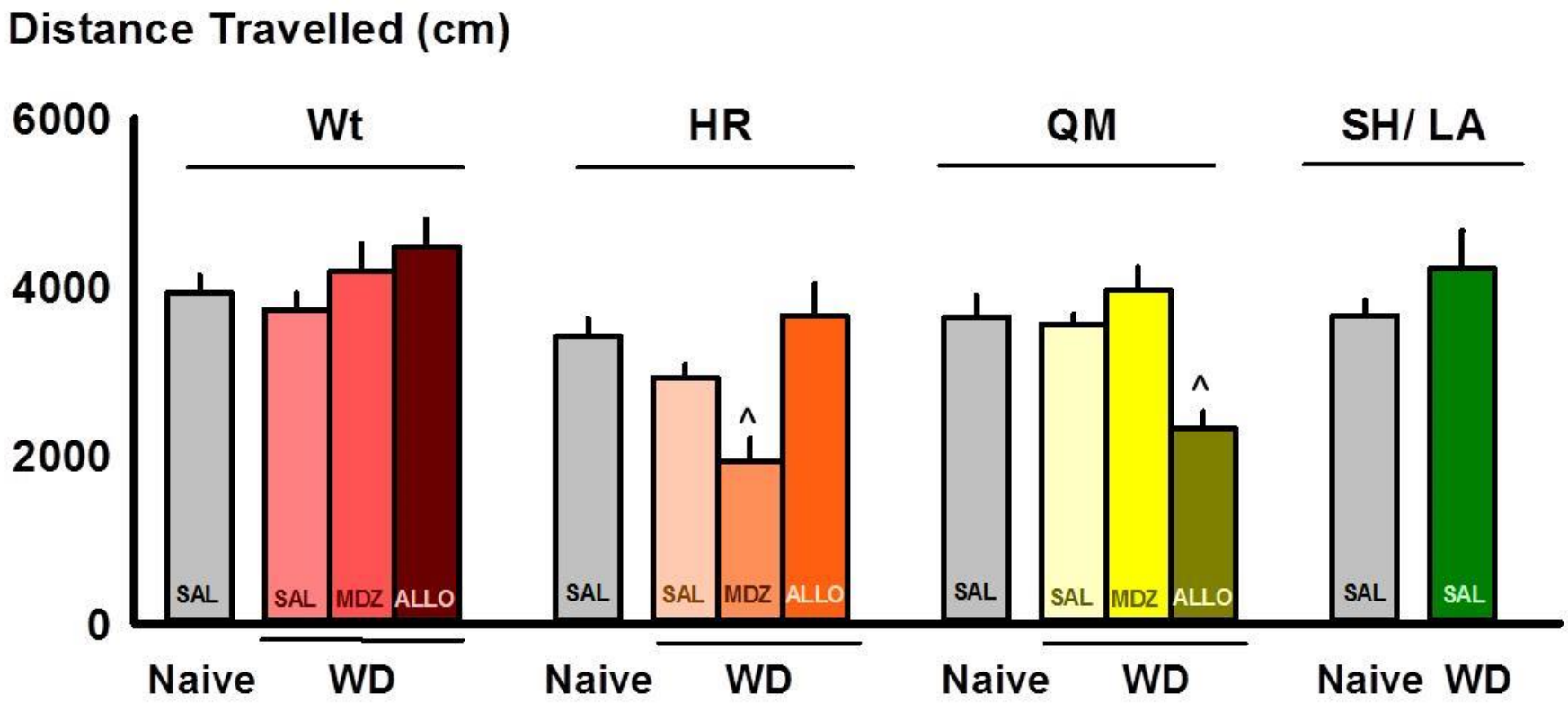

FIG 12. ***LOCOMOTOR BEHAVIOR IN WITHDRAWAL***

Wild-type (A), H101R (B), Q241M (C) and S270H/L277A (D) mice were maintained for six weeks on the IA protocol (WD) or given six-week access to only water (Naïve) Locomotor behavior , defined as distance travelled, was tracked during weekly, 10 minute social approach tests. Gray bars represent locomotor data from ethanol-naïve mice treated with vehicle (SAL). Only the highest drug doses are shown; MDZ, 1.0 mg/kg midazolam; ALLO, 10.0 mg/kg allopregnanolone

Data are shown as mean ± SEM

***p<0.05, **p<0.01 compared to EtOH-naïve vehicle, ^p<0.05, ^^p<0.01 compared to EtOH-withdrawn vehicle**

FIG 13. *PREFERENCE FOR 3-20% ETOH*

Mice received four consecutive days of ascending concentrations of alcohol (3, 6, 10, 20% w/v) with water. Data are shown as mean daily intake values in g/kg (**13A**) and in percent (**13B**).

#p<0.05, ##p<0.01 compared to 10% EtOH
****p<0.01 compared to wild-type**

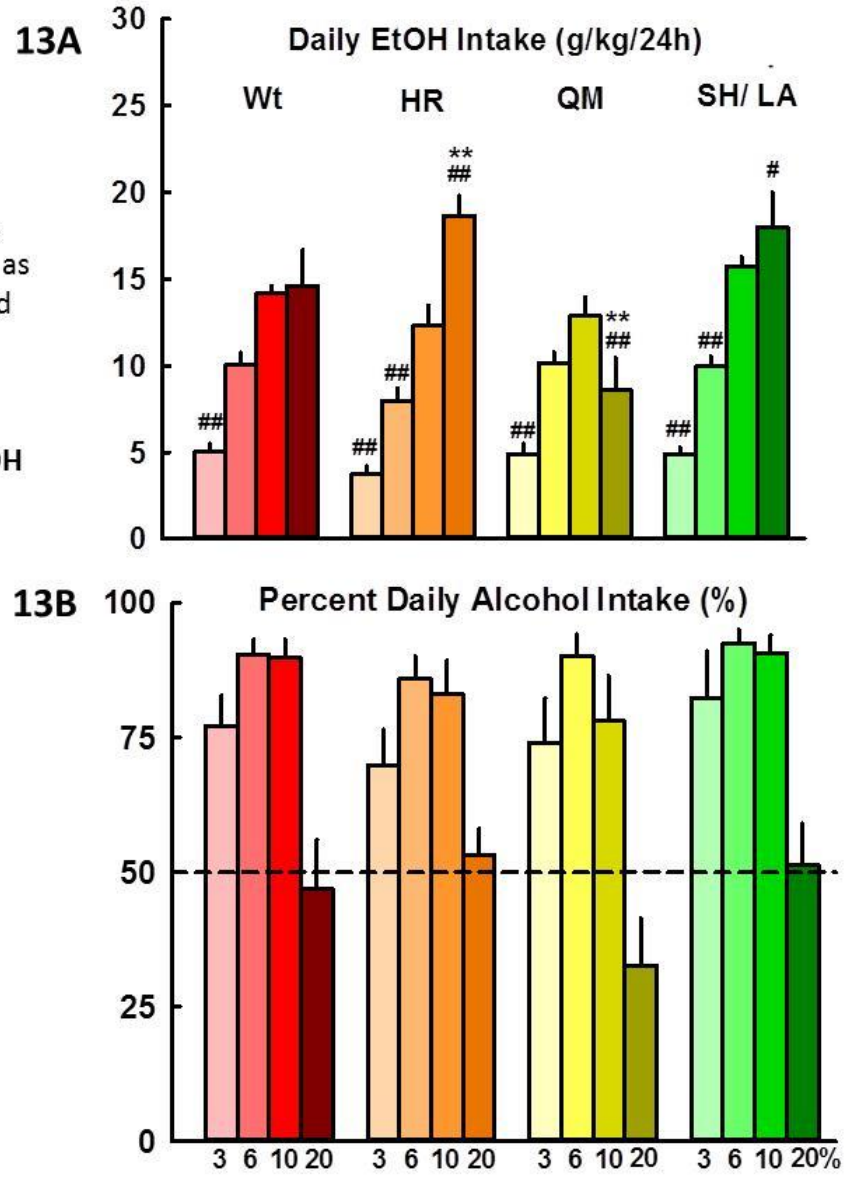

FIG 14A. % Sucrose Solution Intake (10, 30, 100 mM)

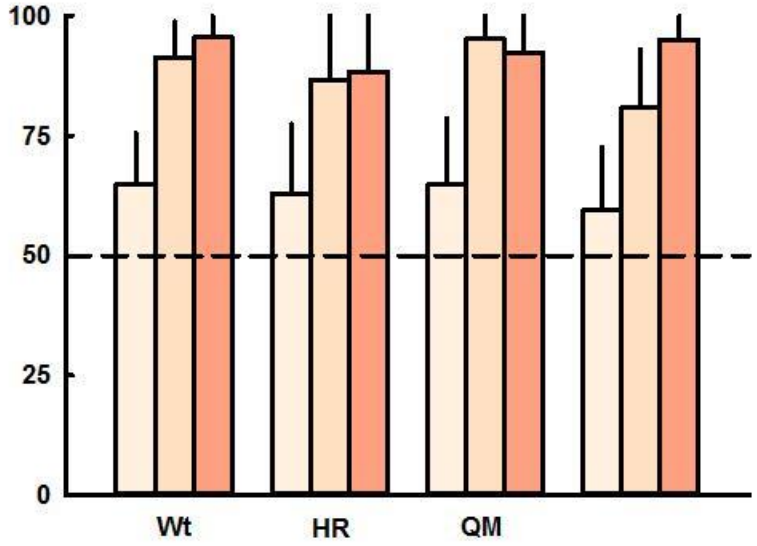

FIG 14B. % Quinine Solution Intake (0.1, 0.3 mM)

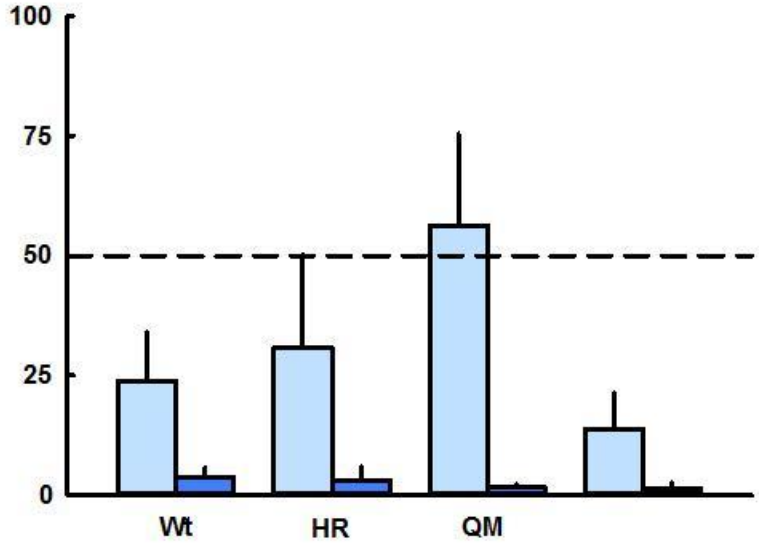

FIG 14. ***PERCENT SUCROSE OR QUININE SOLUTION INTAKE***

Wild-type and mutant mice received four days of access to each concentration of sucrose solution (10, 30, 100 mM) or quinine (0.1, 0.3 mM) with water.

Data are represented as mean± SEM