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MUTATIONS IN THE *GABRB1* GENE PROMOTE ALCOHOL CONSUMPTION THROUGH INCREASED TONIC INHIBITION

Quentin M. Anstee[1,2,3,*], **Susanne Knapp**[2,3,*], **Edward P. Maguire**[11], **Alastair M. Hosie**[5,%], **Philip Thomas**[5], **Martin Mortensen**[5], **Rohan Bhome**[5], **Alonso Martinez**[3,^], **Sophie E. Walker**[10], **Claire I. Dixon**[10], **Kush Ruparelia**[4], **Sara Montagnese**[4,+], **Yu-Ting Kuo**[8,†], **Amy Herlihy**[8], **Jimmy D Bell**[8], **Iain Robinson**[9], **Irene Guerrini**[6], **Andrew McQuillin**[6], **Elizabeth M.C. Fisher**[7], **Mark A. Ungless**[8], **Hugh M.D. Gurling**[6], **Marsha Y. Morgan**[4], **Steve D.M. Brown**[2], **David N. Stephens**[10], **Delia Belelli**[11], **Jeremy J. Lambert**[11], **Trevor G. Smart**[5], and **Howard C. Thomas**[2,3]

[1]Institute of Cellular Medicine, The Medical School, Newcastle University, 4th Floor William Leech Building, Framlington Place, Newcastle Upon Tyne, NE2 4HH, UK.

[2]MRC Mammalian Genetics Unit, Harwell, Oxford, OX11 0RD, UK.

[3]Department of Gastroenterology & Hepatology, Imperial College, St Mary's Hospital Campus, Praed Street, London W2 1NY, UK.

[4]UCL Institute for Liver & Digestive Health,, Royal Free Campus, University College London Medical School, London NW3 2PF, UK.

[5]Department of Neuroscience, Physiology & Pharmacology, University College London, Gower Street, London WC1E 6BT, UK.

[6]Molecular Psychiatry Laboratory, Department of Mental Health Sciences, Royal Free and University College London Medical School, 46 Cleveland Street, Windeyer Institute of Medical Sciences, London W1T 4JF, UK.

[7]Department of Neurodegenerative Disease, Institute of Neurology, University College London, Queen Square, London, WC1N 3BG, UK.

[8]MRC Clinical Sciences Centre, Imperial College London, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK

Corresponding Author/Reprint Requests Dr Quentin M. Anstee BSc, MB BS, PhD, MRCP(UK). Senior Lecturer & Honorary Consultant Hepatologist, Institute of Cellular Medicine, The Medical School, M4.090, 4th Floor, William Leech Building, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, Great Britain. Telephone: + 44 (0) 191 208 7012. Fax: + 44 (0) 191 208 0723 quentin.anstee@ncl.ac.uk.

[*]QMA and SK are joint first authors.

[^]Current address: Cancer Genetic Group, Infection & Cancer, Dept. Microbiology & Parasitology, School of Medicine, University of Antioquia, Medellin – Colombia.

[†]Current address: Department of Medical Imaging, Chi Mei Medical Center, Tainan City, Taiwan.

[+]Current address: Department of Medicine, via Giustiniani, 2, 35128 Padova, Italy

[%]Deceased

Contributions HCT, SDMB, EMCF conceived the research. The ENU screen was conducted by AM, SK, QMA and HCT. *In vivo* phenotyping and preference testing was conducted and overseen by SK and QMA. Endocrine studies were performed by IR. *In vitro* electrophysiology studies were conducted by AMH, PT, MM, RB and TGS. *In vivo* operant conditioning, ethanol kinetics, and ataxia studies were performed by SEW, CID and DNS. *Ex vivo* brain slice electrophysiology studies were conducted by EPM, DB and JJL. Additional phenotyping, data/sample acquisition and scientific support was performed by Y-TH, AH, MAU, JDB, MYM, SM, KR, HMDG, AM, IG. Data analysis and interpretation was performed by QMA, SK, TGS, DNS, EPM, DB, JJL and HCT. The manuscript was written and revised by QMA, SK, DB, JJL, DNS, TGS and HCT. All authors reviewed and approved the submitted manuscript. DNS, DB, SB, JJL, TGS & HCT are joint senior authors.

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[9]Division of Molecular Neuroendocrinology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

[10]School of Psychology, University of Sussex, Falmer, Brighton, BN1 9QG, UK.

[11]Division of Neuroscience, Medical Research Institute, University of Dundee, Ninewells Hospital, Dundee, DD1 9SY, UK.

Abstract

Alcohol-dependence is a common, complex and debilitating disorder with genetic and environmental influences. Here we show that alcohol consumption increases following mutations to the γ-aminobutyric acid$_A$ receptor (GABA$_A$R) β1 subunit gene (*Gabrb1*). Using *N*-ethyl-*N*-nitrosourea mutagenesis on an alcohol-averse background (F1 BALB/cAnN × C3H/HeH), we develop a mouse model exhibiting strong heritable preference for ethanol resulting from a dominant mutation (L285R) in *Gabrb1*. The mutation causes spontaneous GABA ion channel opening and increases GABA sensitivity of recombinant GABA$_A$Rs, coupled to increased tonic currents in the nucleus accumbens, a region long-associated with alcohol reward. Mutant mice work harder to obtain ethanol, and are more sensitive to alcohol intoxication. Another spontaneous mutation (P228H) in *Gabrb1* also causes high ethanol consumption accompanied by spontaneous GABA ion channel opening and increased accumbal tonic current. Our results provide a new and important link between GABA$_A$R function and increased alcohol consumption that could underlie some forms of alcohol abuse.

INTRODUCTION

Our understanding of the genetic and molecular basis of alcohol dependence is incomplete. Alcohol abuse has long been associated with facilitation of neurotransmission mediated by the brain's major inhibitory transmitter, GABA, acting via GABA$_A$ receptors (GABA$_A$Rs). Recently, a locus within human chromosome 4, containing GABA$_A$R subunit genes encoding α2, α4, β1 and γ1 subunits has been associated with alcohol dependence in humans [1, 2, 3, 4, 5, 6, 7]. In particular, haplotypic variations in the GABRA2 gene encoding the α2 subunit have been repeatedly linked with alcohol dependence[2, 8, 9, 10]. However, the neurobiological basis by which genetic variation translates into alcohol abuse is largely unknown.

Ionotropic GABA$_A$Rs are pentameric ligand-gated ion channels, drawn from a family of 19 proteins, which underpins the expression of ~20-30 neuronal GABA$_A$R isoforms[11]. These receptors have distinct physiological and pharmacological properties, are heterogeneously expressed in the mammalian CNS and as a consequence can differentially influence behavioral phenotypes[12, 13]. Synaptic GABA$_A$Rs mediate phasic inhibition, whereas extrasynaptic GABA$_A$Rs are activated by ambient concentrations of GABA and mediate a tonic form of inhibition. Recent evidence has suggested roles for both forms of GABAergic transmission in the neurobiology of addiction[14, 15, 16, 17].

With regard to ethanol, both consumption and preference are reduced following disruption of GABA-mediated tonic inhibition in δ subunit knock-out ($\delta^{-/-}$) mice [18] and a similar impact on ethanol drinking was achieved by RNAi-induced suppression of either α4 (a subunit partner of the δ subunit) or δ subunit expression in the rodent nucleus accumbens (NAc)[17, 18]. The reduced ethanol self-administration appeared to be a consequence of the altered reinforcing properties of the drug[17, 18]. These actions on ethanol drinking and self-administration appear to be specific to the activity of α4βδ receptors and not a general effect on manipulating GABAergic activity in the NAc, as no differences in ethanol self-

administration have been found in $\alpha2^{-/-}$ or $\alpha5^{-/-}$ mice[19, 20], when compared to wild-type (WT) counterparts.

It has been suggested that ethanol may exert a direct action on δ-$GABA_A$Rs to enhance their function, which might account for the rodent self-administration data. However, whether ethanol exerts such direct effects is controversial[21, 22, 23, 24]. An alternative interpretation of the behavioral data posits that the activity of NAc extrasynaptic $GABA_A$Rs influences the activity of neural circuits underlying certain addictive behaviors, such as the desire for alcohol. We were able to explore this possibility by exploiting the availability of two novel mutant mouse lines in which single point mutations in $GABA_A$R β1 subunits have occurred. The first line was identified through a phenotype-driven *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen[25, 26] for alcohol-preferring mice, whilst the second was a spontaneous mutation identified using a genotype-driven approach[27]. Both mutant mouse lines display increased alcohol consumption and self-administration, and are characterized by GABA channels that can open spontaneously, promoting an increased tonic inhibition in NAc medium spiny neurons (MSNs). These findings strongly suggest that the large tonic conductance of MSNs contributes to the increased preference and intake of ethanol by *Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$ mice. These data reveal a novel link between $GABA_A$R function and increased alcohol consumption that could lead to a better understanding of some forms of alcohol abuse.

RESULTS

Alcohol preferring mouse strains

We identified two dominant mutations in *Gabrb1* that induced a phenotypic switch from alcohol aversion to a sustained, strongly-heritable alcohol preference. One mutation was generated through phenotype-driven random ENU mutagenesis[25, 26] (*Gabrb1*^{L285R}), whilst the other was a spontaneous mutation (*Gabrb1*^{P228H}) identified through gene-driven screening of a DNA/sperm archive[27]. Both lines showed highly-penetrant ethanol preference throughout over eight and five backcross generations respectively.

An ENU-induced *Gabrb1* mutation confers alcohol preference—Alcohol-averse male BALB/cAnN mice were exposed to ENU[25] and crossed to WT C3H/HeH females. G1 progeny (n=1047) were screened in a two-bottle choice test for preference for 10% (v/v) ethanol with mice showing ethanol preference backcrossed to C3H/HeH to test heritability. The ENU-induced mutation was localized to a region on mouse chromosome 5 (71.45-73.05 Mb), syntenic with a region on human chromosome 4, containing 11 genes including *Gabra4 and Gabrb1* (Supplementary Fig S1). Sequencing identified just one mutation in the β1 subunit (*Gabrb1* exon 8), a leucine-to-arginine exchange (L285R) in the highly-conserved third transmembrane domain (M3), near the M2-M3 linker (Supplementary Fig S2A), an important area for GABA receptor activation and ion channel gating. The mutation was absent in both parental strains.

A spontaneous *Gabrb1* mutation confers alcohol preference—To determine if other *Gabrb1* mutations modified alcohol drinking, we screened a DNA library containing ~10,000 unique samples from ENU mutagenized male mice (F1 C57BL/6JxC3H/HeH) and identified one sample carrying a non-synonymous proline-to-histidine mutation (P228H) within M1 of the β1 subunit. This proline is highly conserved in $GABA_A$Rs from various species (Supplementary Fig S2B). While absent in both background strains, the mutation occurred in the non-ENU mutagenized C3H DNA strand and therefore had arisen spontaneously. Significantly, as with *Gabrb1*$^{+/L285R}$ above, *Gabrb1*$^{+/P228H}$ mutants displayed a heritable ethanol preference (Supplementary Fig S3).

Gabrb1$^{+/L285R}$ mutant mice were significantly smaller than WT (P<0.0001; Supplementary Figure S4). A similar but less pronounced size difference was also observed in *Gabrb1*$^{+/P228H}$ mutant mice. Both mutant lines exhibited complete female infertility characterized by underdeveloped *corpus luteii*. Consequently, we could only study heterozygotes (*Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$) and their corresponding wild-type (WT) mice. Reduced body size and impaired fertility most likely reflect hypothalamic-pituitary axis endocrine deficiency and so analysis of anterior pituitary hormone content was performed. This demonstrated that carriage of either mutant *Gabrb1* allele (*Gabrb1*^{L285R} or *Gabrb1*^{P228H}) was associated with significant hypothalamic-pituitary axis dysfunction (Supplementary Tables S1 & S2). Mutant mice displayed a significant preference for ethanol (*Gabrb1*$^{+/L285R}$: Fig 1A, B; *Gabrb1*$^{+/P228H}$: Supplementary Fig S3), but not for similarly presented sucrose, saccharin, or quinine solutions, suggesting taste or calorific requirements were not driving alcohol preference (Supplementary Table S3). Furthermore, all mice consumed similar daily fluid volumes, despite *Gabrb1*$^{+/L285R}$ and, to a lesser extent, *Gabrb1*$^{+/P228H}$ mutant mice possessing lower body weights (Supplementary Fig S4). The co-segregation of both alcohol preference and body weight traits were observed throughout all eight (for *Gabrb1*$^{+/L285R}$) and five (*Gabrb1*$^{+/P228H}$) generations studied, being present in 123/130 (93%) of *Gabrb1*$^{+/L285R}$ mice effectively excluding the possibility of separate mutations causing these components.

Gabrb1^{L285R} and alcohol seeking behavior

The motivation for *Gabrb1*$^{+/L285R}$ mice to obtain alcohol was assessed using operant self-administration of fluid coupled with a sucrose-fading technique[19, 20]. Self-administration of ethanol over 1 hour caused ataxia, consistent with alcohol intoxication (Supplementary Fig S5A). To determine whether the features of intoxication observed in mutant mice during the 1 hour operant sessions were due to increased intake or a heightened sensitivity to alcohol, WT and mutant mice were given a standard bodyweight-adjusted dose of ethanol and tested for the presence of ataxia on the rotarod (ethanol 3g/kg) and loss of righting reflex (ethanol 3.5g/kg; Supplementary Fig S5). Although study groups were relatively small (n = 7 - 8), limiting statistical power, there was a strong tendency for the mutant mice to be more impaired than the WT mice (two-way ANOVA, main effect of genotype: $F(1,13) = 4.46$; $p = 0.05$), and to recover more slowly (two-way ANOVA, time point*genotype interaction: $F(1,13)=1.76$; $p = 0.08$). Genotype did not affect the rate of loss of righting reflex, but there was a trend towards a more rapid recovery in WT mice (806±140 vs. 1466±448 seconds (SEM); t-test, $t = 1.41$; $p < 0.1$). Since neither the peak, nor time course of blood or brain alcohol levels differed significantly between mutant and WT mice following ethanol administration (Supplementary Fig S6), these data suggest an increased sensitivity of *Gabrb1*$^{+/L285R}$ mice to the ataxic effects of ethanol (Supplementary Figs S5B, S5C and S6).

To ensure ataxia did not affect performance, we analyzed only the first 30 min of each operant self-administration session. Although rates of lever pressing declined with lower sucrose and higher ethanol concentrations (P<0.001, two-way ANOVA n = 8 / group), these were still higher with 7/5% and 10/10% v/v sucrose/ethanol (Fig 1C, D) in *Gabrb1*$^{+/L285R}$ mice compared to WT (P<0.01, two-way ANOVA n = 8 / group). Above 5% v/v ethanol, mutant mice worked harder to obtain ethanol, irrespective of the sucrose concentration, tending towards lower lever-pressing rates for unadulterated sucrose (P<0.1, two-way ANOVA n = 8 / group). Thus, the *Gabrb1*^{L285R} mutation is specific in affecting the motivation to consume alcohol.

Consistent with higher response rates, inter-response times (IRTs) were significantly shorter in the mutant mice (Supplementary Fig S7). In order to obtain information on patterns of responding within a session, we analysed the pattern of IRTs within sessions. Interestingly, WT mice showed increases in IRTs as the session progressed, consistent with them satiating

on alcohol. In contrast, the *Gabrb1*$^{+/L285R}$ mice maintained their shorter inter-response times (Supplementary Fig S7). This pattern suggests that, in comparison with the WTs, their desire for alcohol decreased more slowly as they consumed alcohol.

Following the tests of alcohol self-administration, the mice were tested over two sessions in extinction (i.e. lever pressing no longer resulted in fluid presentation). Their response rates declined when alcohol was no longer delivered following lever presses. The higher response rates maintained by *Gabrb1*$^{+/L285R}$ mice during extinction sessions (Supplementary Fig S8), were not significant, suggesting that there were no major differences in motivation to obtain ethanol under deprivation conditions. However, mice could not consume ethanol during extinction sessions and so any differences in rate of satiation to ethanol would not influence responding.

Mutant $GABA_AR$ β1 subunits increase NAc tonic inhibition

The NAc is an important brain region for understanding the neurobiology of reward and addiction. Implicating $GABA_ARs$, specific suppression of either the $GABA_AR$ α4 or δ subunits in the NAc decreases both ethanol consumption and preference in rats [16, 17]. We therefore determined how the β1 mutations affected $GABA_AR$ function in NAc slices. Whole-cell voltage-clamp (−60mV) of *Gabrb1*$^{+/L285R}$ NAc core medium spiny neurons (MSNs) revealed greater membrane current noise (root mean square (RMS)) and holding currents than for WT (Table 1). These effects were $GABA_AR$-mediated as the receptor antagonist bicuculline (30μM) induced an ~6-fold greater outward current and a reduction in RMS for *Gabrb1*$^{+/L285R}$ than for WT MSNs (Table 1, Fig 2A, C).

Accumbal MSNs exhibit a GABA-dependent tonic conductance mediated by extrasynaptic α4βδ receptors[28]. However, the inward current induced by the agonist THIP, at a δ-$GABA_AR$-selective concentration (1 μM) was similar for WT and *Gabrb1*$^{+/L285R}$ MSNs, implying the large tonic current for *Gabrb1*$^{+/L285R}$ was not caused by increased δ-$GABA_AR$ expression (Table 1). In contrast to bicuculline (a partial negative allosteric modulator[29, 30, 31]), the competitive antagonist gabazine (20 μM) only induced small outward currents in both WT and L285R MSNs (Table 1, Fig 2C, D, E), suggesting increased ambient levels of agonist (e.g. GABA, taurine) do not cause the larger tonic current.

The differential influence of $GABA_AR$ antagonists on Gabrb1$^{+/L285R}$ neurons may indicate that bicuculline, but not gabazine, shuts spontaneously-open mutant β1 $GABA_AR$ channels[29, 32]. Indeed, picrotoxin (100 μM), which is a non-competitive antagonist of $GABA_AR$-gated chloride channels, when co-applied with bicuculline, produced an additional outward current selectively in *Gabrb1*$^{+/L285R}$ neurons (Table 1; Fig 2C); whilst gabazine (20 μM), which shares a common binding site with bicuculline, prevented the outward current induced by bicuculline (30 μM), but not by picrotoxin (100 μM; Table 1, Fig 2D, E).

Gabrb1$^{+/P228H}$ MSNs also exhibited a greater membrane current noise and holding current than WT MSNs (Table 1). In *Gabrb1*$^{+/P228H}$ neurons, bicuculline (30 μM) and, to a lesser extent, gabazine (20 μM), induced larger outward currents relative to WT (Fig. 2B, C). In common with the β1L285R mutation, after gabazine, the co-application of picrotoxin (100μM) to *Gabrb1*$^{+/P228H}$, but not to WT MSNs, induced an additional outward current (Fig 2F, G). Furthermore, as for β1L285R, gabazine prevented the additional outward current produced by bicuculline in *Gabrb1*$^{+/P228H}$ MSNs (Table 1; Fig 2F). Thus, *Gabrb1*^{P228H} enhanced the tonic conductance, consistent with the ability of these mutant β1 $GABA_ARs$ to open spontaneously.

β1 subunit mutations influence NAc phasic inhibition

The β1 subunit mutations also affected $GABA_AR$-mediated phasic inhibition. For *Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$ MSNs the frequency of mIPSCs was reduced and their decay times prolonged. Additionally, the mIPSC amplitude was increased for *Gabrb1*$^{+/L285R}$ relative to WT MSNs (Fig 2H, 2I; Table 2),which was associated with an increased population of large amplitude mIPSCs, exhibiting slow decays. A scatter plot revealed a cluster of large amplitude (peak amplitude > 105 pA), slowly decaying (T70 > 19ms) events prevalent in *Gabrb1*$^{+/L285R}$ MSNs constituting only 0.7% of the total number of events for WT MSNs, but 8.8% (> 10-fold increase) of the *Gabrb1*$^{+/L285R}$ MSNs (Supplementary Fig. S9). Conversely, the proportion of events with a peak amplitude < 105 pA and T70 < 19ms decreased from 81.7% for WT to 54.2% for the *Gabrb1*$^{+/L285R}$ MSNs. This loss is accounted for not only by the greater percentage of events with a peak amplitude > 105 pA and T70 > 19ms but, additionally, by a higher proportion of events with an amplitude >105 pA (T70 ≤ 19 ms) and of those with a T70 > 19ms (but pA ≤ 105pA) (Supplementary Fig. S9). Such events may originate from a population of mutant postsynaptic β1-$GABA_ARs$, with increased open probabilities compared to WT β1-$GABA_ARs$.

Both the L285R and the P228H mutations were associated with a reduced mIPSC frequency compared to WT counterparts (Supplementary Table S2). Activation by THIP of presynaptic δ-$GABA_ARs$ located either on accumbal interneurons (the main source of MSN somatic input) or on neighboring MSNs, reduces GABA release onto MSNs (mIPSC frequency, Control: 1.9 ± 0.2 Hz; + THIP 1 μM: 1.1 ± 0.2 Hz, data presented as ± SEM, n = 5; $p<0.05$ paired t-test). Therefore, it is conceivable that pre-synaptically located spontaneously-open β1-$GABA_ARs$ either on interneurons, or neighboring MSNs would similarly influence GABA release, thereby reducing MSN mIPSC frequency.

Mutant β1 subunit expression and spontaneous channel opening

To explore how the $\beta1^{L285R}$ and $\beta1^{P228H}$ mutations affected $GABA_AR$ function, we used heterologous expression of WT and mutant recombinant $GABA_ARs$ in HEK293 cells. Immunocytochemistry was used to assess the expression levels of myc epitope-tagged WT and mutant β1 subunits. The expression levels of $\beta1^{L285R}$ along with α2 and γ2L subunits, including enhanced green fluorescent protein (eGFP), revealed no differences either for cell surface or overall total fluorescence (Supplementary Fig S10). Similarly, the cell surface membrane and intracellular expression levels for $\beta1^{P228H}$ were also unaltered compared to WT β1 subunits (Supplementary Fig S11).

To examine whether the β1 subunit mutations (L285R and P228H) altered $GABA_AR$ receptor physiology and pharmacology, whole-cell recording was performed on $\alpha2\beta1^{L285R}\gamma2$ and $\alpha2\beta1^{P228H}\gamma2$ receptors expressed in HEK293 cells. Relative to WT, both mutations reduced the maximum current density induced by saturating GABA concentrations (1mM; Fig. 3A).

As native $GABA_ARs$ contain two β subunits and because our *in vivo* studies necessarily used heterozygous mice, a proportion of native $GABA_ARs$ could comprise a mixture of WT and mutant β1 subunits. To examine the amplitudes of GABA-evoked currents under these conditions, we recreated a binomial mixture of $GABA_ARs$ in HEK cells by co-expressing α2 and β1 with either $\beta1^{L285R}$ or $\beta1^{P228H}$, and γ2 subunits in an equimolar ratio thereby reproducing native receptor isoforms likely to be present in heterozygotes. Assuming receptor subunit assembly proceeds according to binomial probabilities, we would expect a mixture of pentameric receptors (2α:2β:1γ) to include: WT, full mutant (both β subunits are mutated) and two forms of partial mutant receptors containing only one copy of the mutant β subunit. The maximum current densities induced by saturating concentrations of GABA (up

to 1 mM) were larger for receptors containing only one copy of the mutant β subunit than those observed with full mutant receptors, and this approached that of WT receptors (Supplementary Fig S12).

GABA concentration response curves revealed that GABA potency was increased (~2-3-fold) by $\beta1^{L285R}$ and $\beta1^{P228H}$ (Fig 3B, D; Supplementary Table S4). Significantly, the holding current (at −40 mV) was greater for many mutant receptor-expressing cells compared to WT (Supplementary Table S4), indicative of spontaneous GABA channel activity[33]. Indeed, picrotoxin (100 μM) induced outward currents in the absence of GABA for cells expressing $\beta1^{L285R}$, and to a lesser extent for $\beta1^{P228H}$ mutant receptors, reflecting spontaneous channel activity (Fig. 3C). The spontaneous current revealed by picrotoxin (I_{PTX}) accounted for ~3-15% of the total current (= $I_{GABA,max}$ + I_{PTX}) for these mutant receptors.

We also examined the level of spontaneous current for α4β1γ2 receptors, with WT or mutant β1 subunits, as an alternative isoform that may populate synaptic and/or extrasynaptic sites. Maximal current densities and holding currents exhibited greater variability for the β1 mutants compared to WT (Supplementary Table S4), and $\alpha4\beta1^{L285R}\gamma2$ exhibited a spontaneous current revealed by picrotoxin (Fig 3C).

To examine the gating of GABA ion channels underlying the spontaneous current, we used outside-out patches from HEK cells expressing α2β1γ2, $\alpha2\beta1^{L285R}\gamma2$ or $\alpha2\beta1^{P228H}\gamma2$ receptors (Fig. 3E). Spontaneous channel activity was evident with mutant receptors, but absent in WT, and abolished by the GABA antagonist, bicuculline (50 μM), acting as a negative allosteric modulator[29] in the absence of GABA. Activating WT α2β1γ2 GABA channels with 10 μM GABA induced single channel currents that were indistinguishable from the spontaneous openings observed with the mutant receptors (Fig. 3E) and with only minor differences in open and shut time durations (Supplementary Table S5). Overall, $\beta1^{L285R}$, but to a lesser extent, $\beta1^{P228H}$ caused spontaneous channel opening and increased receptor sensitivity to GABA when co-assembled with either α2 or α4, and γ2 subunits.

Given the importance of α4 and δ subunits for tonic current in NAc MSNs[16, 17, 28] we explored whether β1 mutants also conferred spontaneous channel activity on δ-$GABA_A$Rs. Using HEK293 cells, β1 subunits were expressed with either α2 (co-located on same chromosome as β1, and expressed in accumbal synapses[34]), or α4 (co-expressed in the accumbens with δ forming extrasynaptic receptors)[28]. The $\beta1^{L285R}$ mutation increased the maximum current density to saturating GABA (300 μM) for both $\alpha2\beta1^{L285R}\delta$ and $\alpha4\beta1^{L285R}\delta$ receptors, compared to WT equivalents (α2/4β1δ), whereas $\beta1^{P228H}$ did not (Supplementary Fig. S13). Both β1 mutants increased GABA sensitivity by 3-5 fold at α2β1δ-$GABA_A$Rs (Fig. 4A, Supplementary Table S4), similar to the increased sensitivity for mutant α2β1γ $GABA_A$Rs (Fig. 3B), but only $\beta1^{L285R}$ affected α4β1δ receptor sensitivity to GABA (Fig 4B).

In the absence of GABA, picrotoxin (100 μM) induced outward currents only for $\alpha2/4\beta1^{L285R}\delta$ $GABA_A$Rs (Fig. 4C, D) with $\alpha2/4\beta1^{P228H}\delta$ showing similar levels of activity to WT. Given that $\beta1^{L285R}$ conferred substantial spontaneous activity on δ-$GABA_A$Rs, compared to $\beta1^{P228H}$, the increased tonic currents recorded in MSNs of the NAc and caused by the β1 mutants, are most likely due to the presence of $\alpha2/4\beta1^{L285R}\gamma2$, $\alpha2/4\beta1^{P228H}\gamma2$ and $\alpha2/4\beta1^{L285R}\delta$ isoforms.

Alcohol and $GABA_A$Rs

Although alcohol may directly modulate some $GABA_A$Rs, this remains controversial[21, 22, 35, 36]. Here, ethanol (30 - 200 mM) co-applied with low concentrations

(EC_{10}) of GABA, did not modulate α2/α4β1γ2 $GABA_A$Rs containing either WT, or mutant β1 subunits in HEK cells (Supplementary Table S4). Alcohol intoxication is associated with increased production of the endogenous neurosteroid allopregnanolone, an allosteric potentiator of $GABA_A$Rs[35]. However, WT and β1 mutant $GABA_A$Rs remained equally sensitive to potentiation by allopregnanolone (1 μM) and benzodiazepines (0.5 μM diazepam), and to inhibition by Zn^{2+} (100 μM) or bicuculline (50 μM); Supplementary Fig S14 and S15. Similarly, neither β1 mutant when co-expressed with α2δ, or α4δ subunits demonstrated sensitivity to 30 or 100mM ethanol (Fig. 4E). Potentiation of αβδ $GABA_A$R function by neurosteroids (THDOC) was also unaffected by the mutation (Supplementary Fig S13). A previous study in the dorsal striatum clearly demonstrated that acute ethanol decreased the amplitude of the electrically-evoked IPSCs recorded from MSNs of young rats, but with no effect on the paired pulse ratio, suggesting a postsynaptic locus[37]. However, in our recordings from mature mouse NAc MSNs, ethanol (50 mM) had no significant effect on the spontaneous IPSCs (paired recordings n = 5 neurons from 5 mice *per* genotype; Supplementary Figure S16). Note that although there was an apparent trend for ethanol to increase the frequency of sIPSCs in the *Gabrb1*$^{+/L285R}$ MSNs, it was not significant in this sample. In further agreement with the recombinant receptor experiments, ethanol (50 mM) had no effect on the holding current of WT or *Gabrb1*$^{+/L285R}$ MSNs (paired recordings n=5 neurons from 5 mice *per* genotype; Supplementary Fig S16). Thus, it is evident that the L285R and P228H mutations do not affect the co-assembly of β1 with α2/4, δ and γ2 subunits, or their pharmacological properties.

DISCUSSION

ENU mutagenesis[25] of alcohol-averse BALB/cAnN mice generated a stable line carrying a non-synonymous dominant mutation in *Gabrb1* (L285R) with a strong phenotypic preference for alcohol. Given that the probability of a second ENU-induced mutation within a 5Mb region is estimated to be $p < 0.002$ (using both a Maximum Likelihood Estimation method, adopting a Poisson model; and a Markov chain Monte Carlo method, with a Poisson Gamma model)[38, 39], the probability of a second functional mutation within the 1.6Mb genomic region of interest is extremely remote, and would continue to halve with each of the subsequent eight backcross generations that have been studied. This possibility has been further excluded by the absence of other exonic mutations following comparative sequencing across the candidate region. Importantly, the identification of a second, independent line with a spontaneous, non-synonymous single base-pair mutation (P228H) in the same gene that also consistently exhibits ethanol preference and similar phenotypic traits over the 5 backcross generations studied, provides further confirmation that the phenotype is caused by mutating *Gabrb1*.

Mutant and WT mice showed no differences in preference for saccharin or quinine solutions, discounting taste as a driver for high alcohol consumption by the mutants. That *Gabrb1*$^{+/L285R}$ mice worked harder than WT to obtain alcohol compared to sucrose solutions indicates that motivation for alcohol was also not driven by calorific requirement or taste. Importantly, mutants undertook this extra work despite possessing lower body weights than their WT counterparts. The mutant mice also showed greater sensitivity to the sedative/ataxic effects of ethanol, which together with increased consumption, contributed to an increased incidence of intoxication.

Previous studies in man demonstrated significant allelic association between the risk of alcohol dependence and *GABRA2* and *GABRB1* polymorphisms[4, 5, 40, 41, 42]. Although deleting *Gabra2* in mice did not alter ethanol self-administration[19], and human polymorphisms and mouse mutations may lack common effects on receptor function, our

studies are supportive of the view that the $GABA_AR$ β1 subunit is a modifier of alcohol consumption.

The NAc is associated with reward and plays an important role in addiction. Our recordings from MSNs within the accumbal core revealed that the β1 subunit mutations greatly impact upon both synaptic and tonic inhibitory transmission. For *Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$, mIPSCs were prolonged and less frequent. Given our previous demonstration that MSNs express synaptic α2-$GABA_ARs$[34], the changed mIPSC kinetics suggest that β1 subunits may co-assemble with α2 and γ2 subunits at inhibitory synapses. The $GABA_AR$-mediated tonic conductance was greatly increased in the NAc of *Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$ mice. The differential effects of $GABA_AR$ antagonists suggest that this perturbation results from spontaneous activity of mutant β1-$GABA_ARs$, though increased receptor sensitivity to GABA and, for *Gabrb1*$^{+/P228H}$, an increase in ambient GABA, may also contribute.

Our recombinant receptor studies indicate that mutant β1 subunits efficiently co-assemble with α and γ or δ subunits and are functionally expressed at the cell surface, with minimal effect on their pharmacological properties. Nevertheless, both $\beta1^{P228H}$ and $\beta1^{L285R}$ mutations increased receptor sensitivity to GABA and caused their channels to open spontaneously in the absence of GABA, particularly for L285R. Modeling the likely subunit combinations of synaptic α2- and extrasynaptic δ-containing $GABA_ARs$ found in accumbal MSNs, we recreated recombinant equivalents of extrasynaptic (α2/4β1δ) and synaptic (α2/4β1γ2)-type receptors. Mutating the β1 subunit initiated spontaneous GABA channel opening, with $\beta1^{L285R}$ exhibiting a greater degree of spontaneous activity, implying that both $\alpha\beta1^{L285R}\gamma$ and $\alpha\beta1^{L285R}\delta$ receptors are the most significant contributers to the increased spontaneous (tonic) current in the NAc of the *Gabrb1*$^{+/L285R}$ mouse. In comparison, $\beta1^{P228H}$ $GABA_ARs$ were less spontaneously-active, but such activity was nevertheless clearly evident for $\alpha2\beta1^{P228H}\gamma2$.

Recombinant $GABA_ARs$ (α2/α4β1γ2, α2/α4β1δ) incorporating either WT or mutant β1 subunits, were consistently insensitive to ethanol. Similarly, neither tonic, nor phasic inhibition of WT and β1 mutant MSNs was affected by ethanol. Although low ethanol concentrations (~10-30 mM) have been reported to enhance neuronal and recombinant δ-GABA R[21, 23] function, other studies have failed to corroborate such effects[22, 24, 43, 44, 45].

Thus, increased accumbal tonic current is associated with β1 subunit mutations leading to increased ethanol consumption, but not as a consequence of direct effects of ethanol on $GABA_ARs$. Notably, both ethanol preference and consumption are reduced in $\delta^{-/-}$ mice[18], a genetic manipulation that reduces accumbal tonic currents[28]. The importance of the tonic current for ethanol reward is emphasized by RNAi knock-down of either α4 or δ subunits in the accumbens, which reduced ethanol consumption and preference without affecting water or sucrose intake[16, 17]. Collectively, this strongly suggests that the large tonic conductance of MSNs contributes to the increased preference and intake of alcohol by *Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$ mice.

Our study identifies $GABA_ARs$ containing the β1 subunit as a key element in modulating alcohol consumption and suggests that spontaneous GABA channel opening and increased tonic inhibition in the accumbens are critically important factors in this debilitating behavioral phenotype that is so costly to individuals and society.

MATERIALS AND METHODS

Mice

Mice were housed under standard conditions with a commercial diet (SDS, UK) and drinking water *ad libitum*. Using alcohol-averse background strains, 12-week old male BALB/cAnN mice were exposed to ENU[25] and crossed to WT C3H/HeH females. G1 progeny (n=1047) were screened in a two-bottle choice test for preference for 10% (v/v) ethanol at age 7-12 weeks. Mice showing ethanol preference (>2.5 standard deviations above a control non-mutagenized BALB/cAnN × C3H cohort) were backcrossed to 8-12 week old C3H/HeH females to test heritability. Our study was approved by the local ethical review panels of the MRC Mammalian Genetics Unit (MRC Harwell), the University of Dundee and the University of Sussex and complies with the UK Animal (Scientific Procedures) Act 1986.

Phenotyping

Male and female 7-12 week old adult mice were singly housed with free choice of water or ethanol (3% or 10% v/v) during two 10-day test periods. Consumption was determined by weighing the drinking bottles. The amount consumed was corrected for leakage and evaporation and expressed as grams ethanol consumed daily per kg bodyweight of the mice measured at the beginning of each period. Preference was calculated as the ratio of ethanol over total amount of liquid imbibed. Taste preference was determined similarly using 15 and 120mM sucrose (caloric value), 0.25mM and 0.4mM saccharin (no caloric value) and 0.05mM quinine. For self-administration of ethanol studies, animals (n=8 group) were trained using a sucrose-fading procedure to self–administer up to 10% v/v ethanol[19, 20]. Intoxication was scored as: normal, mild ataxia, ambulatory impairment, upon removal of the animals from the operant boxes at the end of daily sessions.

Mapping of ENU Mutation

A genome scan was performed on 13 animals (2 G2, 11 G3) displaying high ethanol preference using 86 microsatellite markers distributed throughout the genome to differentiate DNA of BALB/cAnN or C3H/HeH origin. A further 169 animals were used to identify informative recombinants. Fine mapping with additional microsatellite markers in the candidate region was undertaken to narrow the location of the mutation.

Candidate Gene Sequencing

Exons and the exon/intron borders of twelve candidate genes were sequenced from both directions in mutant and wild-type mice. Oligonucleotides were designed using Ensembl v36 predictions (http://ensembl.org). Data were analyzed with BioEdit software. Primer sequences can be found in Supplementary Table S6.

Electrophysiology and analysis of brain slice preparations

Coronal slices (300 μm) containing the NAc were prepared from male *Gabrb1*$^{+/L285R}$, *Gabrb1*$^{+/P228H}$ and WT littermates (age 2-5 months). As previously described[34], slices were cut in oxygenated ice-cold maintenance solution containing (mM): 140 K gluconate, 15 Na gluconate, 4 NaCl, 10 HEPES, 0.2 EGTA (pH 7.2; 310-320 mOsm), before storage for at least 1hr at room temperature (20-23°C) in oxygenated, extracellular solution (ECS) containing (mM): 126 NaCl, 2.95 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 D-glucose and 2 $MgCl_2$ (pH 7.4; 300-310 mOsm). MSNs were identified with an Olympus BX51 microscope equipped with DIC/infrared optics. Whole-cell voltage-clamp recordings (−60 mV) were performed at 35°C, using the ECS containing 1 μM strychnine, 2 mM kynurenic acid and 0.5 μM tetrodotoxin (TTX). Patch electrodes (3-4 MΩ) were filled with (mM): 135

CsCl, 10 HEPES, 10 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 2 Mg-ATP, 5 QX-314 (pH 7.2-7.3 with CsOH, 300-308mOsm).

Recordings were discarded if series resistance changed >20%. Currents were filtered at 2 kHz (8-pole, low pass Bessel filter) and recorded for offline analysis (DTR1205 recorder). Bicuculline methobromide, gabazine, THIP and strychnine hydrochloride were prepared as aqueous stock solutions for dilution in ECS. Picrotoxin was prepared in ECS. All drugs were obtained from Sigma-Aldrich (Poole, UK) or Tocris Bioscience (Bristol, UK). THIP and ethanol were allowed to infiltrate the slice for at least 10 min before data acquisition. Recordings were digitized (NIDQMX, National Instruments) and sampled at 10kHz before analysis (WinEDR/WinWCP). The mIPSCs were threshold detected (-4 pA, duration 3 ms) and visually inspected. A minimum of 50-100 mIPSCs were used for analysis, including: peak amplitude, rise time (10-90% - ≤ 1ms), and decay times. The decay phase of mIPSCs was best fit (98-10% of the peak amplitude) with a bi-exponential [$y(t) = A_{fast}.e^{(-t/\tau fast)} + A_{slow}.e^{(-t/\tau slow)}$] function, where t is time, A is the amplitude, and τ is the decay time constant. A weighted decay time constant (τ_w) was calculated from: $\tau_w = \tau_1 P_1 + \tau_2 P_2$ where τ_1 and τ_2 are decay time constants and P_1 and P_2 the relative proportions of the decay described by each component. The mIPSC frequency was determined in 20s bins for 2 min, detected by the rate of rise (30-50 pA ms^{-1}), excluding spurious noise.

The mean current and associated RMS (root mean square) were calculated over 102.4 ms epochs for 1 min, using a 10 kHz sampling rate. Epochs containing mIPSCs or unstable baseline were excluded. To ensure changes in the holding current reflected a drug effect, two 1 min control holding current periods (C1 and C2) were sampled and a 1 min section after drug equilibration (D). The mean holding current for C1 and C2 were pooled and the standard deviation calculated. The drug effect was accepted if the absolute change in the holding current (D-C2) was greater than twice the standard deviation of the controls (C1, C2). For electrophysiological analysis of brain slice recordings all data reported represent the mean ± SEM of observations derived from a minimum of 3 animals and each determination is derived from an individual slice.

Site-directed mutagenesis

All point mutations in the $GABA_AR$ β subunit were introduced using Quikchange (Stratagene). Sequences of the full coding region of mutated murine cDNAs were determined by automated fluorescent sequencing. Plasmid DNAs for transfection were purified using the Hi-Speed Plasmid Midi Kit (Qiagen).

Cell culture and electrophysiology

HEK293 cells (ATCC, Middlesex, UK) were cultured in Dulbecco's modified Eagle's medium with 10%v/v fetal calf serum, 2mM glutamine, 100 units/ml penicillin G and 100 mg/ml streptomycin at 37°C in 95% air/5% CO_2. Cells were transiently transfected by calcium phosphate co-precipitation using 1 μg of each plasmid DNA encoding for α2, α4, β1, δ or γ2 and 0.5 μg pEnhanced Green Fluorescent Protein (GFP; Clontech) and used for electrophysiology after 20 hours.

Whole-cell currents were recorded at room temperature (20-22 °C) from single HEK293 cells voltage clamped at −40mV, using an Axopatch 200B amplifier (Molecular Devices, USA). Patch electrodes (3-5 MΩ) contained (mM): 144 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 11 EGTA and 2 adenosine triphosphate, pH 7.2. Cells were superfused with Krebs solution containing (mM): 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES and 11 glucose, pH 7.4. Recordings were filtered at 5 kHz (6-pole Bessel, 36dB/octave). Drugs and Krebs solution were applied using a modified U-tube. GABA, bicuculline, picrotoxin and ethanol

were dissolved directly into Krebs solution (adjusted to pH7.4). Allopregnanolone and diazepam were diluted in Krebs from a 10 mM stock in DMSO. For whole cell studies of ethanol potentiation, the GABA current evoked by a low GABA concentration (EC_{10}) was initially determined then 30-200 mM ethanol was applied for 30-60 s prior to the co-application of EC_{10} GABA and ethanol. Allopregnanolone potentiation was determined by co-application with EC_{10} GABA and the neurosteroid. Dose-response relationship data were fitted with a non-linear least squares fitting routine using Origin 6.1 (Microcal). Data points represent the mean ± SEM of at least three experiments. The relative proportion of spontaneous $GABA_A$ receptor activation was ascertained by dividing the amplitude of currents induced by saturating concentrations of PTX (I_{PTX}) by the summed current amplitudes induced by saturating concentrations of PTX and GABA ($I_{GABA} + I_{PTX}$). Single channel currents were recorded at room temperature from excised outside-out membrane patches maintained at −70 mV (sampling rate 50 kHz; low pass-filtered at 10 kHz during recording, and at 2-5 kHz during analysis). Patches showing multiple simultaneous channel openings (channel stacking) exceeding 2% of all detected openings were discarded.

Single channel data were analysed using WinEDR (v.3.3.8; Strathclyde electrophysiology software, J Dempster) and QuB (2.0.0.13, Buffalo, NY). Amplitude histograms were created by fitting Gaussian components to the amplitude distributions using a non-linear least-squares routine. Open probabilities (Po) were defined as the ratio between the Gaussian areas of open and shut time components. Individual open and shut time durations were idealised using either WinEDR's 50% threshold detection method or by QuB's segmental k-means (SKM) fitting routine. Open and shut dwell-time histograms were fitted with a mixture of exponentials using a Levenberg-Marquardt nonlinear least-squares method from which the areas representing the individual exponential components and their relative time constants were obtained. Mean dwell time durations were calculated from the individual open and shut times weighted by their areas[33,46, 47].

Confocal microscopy and immunocytochemistry

$GABA_AR$ myc receptor α2, $\beta1^{myc}$ or $\beta1^{myc,L285R}$ or $\beta1^{myc,P228H}$, and γ2S or γ2L subunits were expressed with eGFP in HEK293 cells and incubated at room temperature with 9E10 antibody (to the extracellular myc epitope; Santa Cruz Biotechnology) followed by TRITC- or Cy5-conjugated secondary antibody. The myc epitope in the β1 subunit is electrophysiologically silent[48]. Transfected HEK293 cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 15 min before being quenched with 50mM NH_4Cl in PBS for 10 min. After washing in PBS, cells were incubated for 45 min at room temperature with 9E10 antibody. Cells were washed in PBS containing 10% v/v fetal calf serum (FCS) and 0.4% w/v bovine serum albumin (BSA) before incubation for 45 min with a TRITC- or Cy5-conjugated secondary antibody. Cells were washed and then mounted in glycerol before imaging using a Zeiss Axiophot confocal microscope (LSM510 Meta). The detector gain, and amplifier offset and gain were set at the same levels for all FITC, TRITC and Cy5 images of wild-type and mutant receptors to compare expression intensities. The scanning slice depth was set to 2.1 μm.

Statistical analysis

Analyses were performed using SPSS v14 and SigmaStat software (SPSS, USA). Data are presented as mean ± SEM or 95% confidence intervals unless otherwise stated. Student's t-tests were used for two group comparisons (paired or unpaired as appropriate). Multifactorial linear model analysis was used to investigate the effect of genotype on alcohol preference correcting for generation, gender and weight. Two-way ANOVA, with the between-subject factor *genotype* and within-subject factor *session,* was used to evaluate potential genotype and reinforcer type effect and any interactions, and lever pressing in

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extinction. Categorized indices were compared using a Fisher's Exact Test. The non-parametric Kolmogorov-Smirnoff (KS) test was used to compare cumulative probability distributions of IPSC parameters. Statistical significance was routinely set at $p < 0.05$, and at $p < 0.01$ for the KS test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Whiting PJ, McKernan RM, Wafford KA. Structure and pharmacology of vertebrate GABAA receptor subtypes. Int Rev Neurobiol. 1995; 38:95–138. [PubMed: 8537206]
2. Edenberg HJ, Dick DM, Xuei X, Tian H, Almasy L, Bauer LO, et al. Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and with brain oscillations. Am J Hum Genet. 2004; 74(4):705–714. [PubMed: 15024690]
3. Enoch MA. The role of GABA(A) receptors in the development of alcoholism. Pharmacol Biochem Behav. 2008; 90(1):95–104. [PubMed: 18440057]
4. Parsian A, Zhang ZH. Human chromosomes 11p15 and 4p12 and alcohol dependence: possible association with the GABRB1 gene. Am J Med Genet. 1999; 88(5):533–538. [PubMed: 10490712]
5. Porjesz B, Almasy L, Edenberg HJ, Wang K, Chorlian DB, Foroud T, et al. Linkage disequilibrium between the beta frequency of the human EEG and a GABAA receptor gene locus. Proc Natl Acad Sci U S A. 2002; 99(6):3729–3733. [PubMed: 11891318]
6. Reck BH, Mukhopadhyay N, Tsai HJ, Weeks DE. Analysis of alcohol dependence phenotype in the COGA families using covariates to detect linkage. BMC Genet. 2005; 6(Suppl 1):S143. [PubMed: 16451603]
7. Agrawal A, Edenberg HJ, Foroud T, Bierut LJ, Dunne G, Hinrichs AL, et al. Association of GABRA2 with drug dependence in the collaborative study of the genetics of alcoholism sample. Behav Genet. 2006; 36(5):640–650. [PubMed: 16622805]
8. Bauer LO, Covault J, Harel O, Das S, Gelernter J, Anton R, et al. Variation in GABRA2 predicts drinking behavior in project MATCH subjects. Alcohol Clin Exp Res. 2007; 31(11):1780–1787. [PubMed: 17949392]
9. Covault J, Gelernter J, Hesselbrock V, Nellissery M, Kranzler HR. Allelic and haplotypic association of GABRA2 with alcohol dependence. Am J Med Genet B Neuropsychiatr Genet. 2004; 129B(1):104–109. [PubMed: 15274050]
10. Dick DM, Bierut L, Hinrichs A, Fox L, Bucholz KK, Kramer J, et al. The role of GABRA2 in risk for conduct disorder and alcohol and drug dependence across developmental stages. Behav Genet. 2006; 36(4):577–590. [PubMed: 16557364]
11. Olsen RW, Sieghart W. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev. 2008; 60(3):243–260. [PubMed: 18790874]
12. Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience. 2000; 101(4):815–850. [PubMed: 11113332]
13. Rudolph U, Mohler H. Analysis of GABAA receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Annu Rev Pharmacol Toxicol. 2004; 44:475–498. [PubMed: 14744255]

14. Boehm SL 2nd, Ponomarev I, Jennings AW, Whiting PJ, Rosahl TW, Garrett EM, et al. gamma-Aminobutyric acid A receptor subunit mutant mice: new perspectives on alcohol actions. Biochem Pharmacol. 2004; 68(8):1581–1602. [PubMed: 15451402]
15. Engin E, Liu J, Rudolph U. alpha2-containing GABA(A) receptors: a target for the development of novel treatment strategies for CNS disorders. Pharmacol Ther. 2012; 136(2):142–152. [PubMed: 22921455]
16. Nie H, Rewal M, Gill TM, Ron D, Janak PH. Extrasynaptic delta-containing GABAA receptors in the nucleus accumbens dorsomedial shell contribute to alcohol intake. Proc Natl Acad Sci U S A. 2011; 108(11):4459–4464. [PubMed: 21368141]
17. Rewal M, Jurd R, Gill TM, He DY, Ron D, Janak PH. Alpha4-containing GABAA receptors in the nucleus accumbens mediate moderate intake of alcohol. J Neurosci. 2009; 29(2):543–549. [PubMed: 19144854]
18. Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL, et al. GABA(A)-receptor delta subunit knockout mice have multiple defects in behavioral responses to ethanol. Alcohol Clin Exp Res. 2001; 25(12):1708–1718. [PubMed: 11781502]
19. Dixon CI, Walker SE, King SL, Stephens DN. Deletion of the gabra2 gene results in hypersensitivity to the acute effects of ethanol but does not alter ethanol self administration. PLoS One. 2012; 7(10):e47135. [PubMed: 23115637]
20. Stephens DN, Pistovcakova J, Worthing L, Atack JR, Dawson GR. Role of GABAA alpha5-containing receptors in ethanol reward: the effects of targeted gene deletion, and a selective inverse agonist. Eur J Pharmacol. 2005; 526(1-3):240–250. [PubMed: 16253225]
21. Wallner M, Hanchar HJ, Olsen RW. Ethanol enhances alpha 4 beta 3 delta and alpha 6 beta 3 delta gamma-aminobutyric acid type A receptors at low concentrations known to affect humans. Proc Natl Acad Sci U S A. 2003; 100(25):15218–15223. [PubMed: 14625373]
22. Borghese CM, Storustovu S, Ebert B, Herd MB, Belelli D, Lambert JJ, et al. The delta subunit of gamma-aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. J Pharmacol Exp Ther. 2006; 316(3):1360–1368. [PubMed: 16272217]
23. Santhakumar V, Wallner M, Otis TS. Ethanol acts directly on extrasynaptic subtypes of GABAA receptors to increase tonic inhibition. Alcohol. 2007; 41(3):211–221. [PubMed: 17591544]
24. Baur R, Kaur KH, Sigel E. Structure of alpha6 beta3 delta GABA(A) receptors and their lack of ethanol sensitivity. J Neurochem. 2009; 111(5):1172–1181. [PubMed: 19765192]
25. Nolan PM, Peters J, Strivens M, Rogers D, Hagan J, Spurr N, et al. A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. Nat Genet. 2000; 25(4):440–443. [PubMed: 10932191]
26. Justice MJ, Noveroske JK, Weber JS, Zheng B, Bradley A. Mouse ENU mutagenesis. Hum Mol Genet. 1999; 8(10):1955–1963. [PubMed: 10469849]
27. Coghill EL, Hugill A, Parkinson N, Davison C, Glenister P, Clements S, et al. A gene-driven approach to the identification of ENU mutants in the mouse. Nat Genet. 2002; 30(3):255–256. [PubMed: 11850622]
28. Maguire, EP.; Herd, MB.; Corteen, NL.; Swinny, JD.; Macpherson, T.; King, SL., et al. Society for Neuroscience: Neuroscience 2012. Society for Neuroscience; New Orleans, USA: 2012. Extrasynaptic α4βδ GABAARs mediate a tonic conductance in nucleus accumbens medium spiny neurons. p. 669.619/CC618
29. McCartney MR, Deeb TZ, Henderson TN, Hales TG. Tonically active GABAA receptors in hippocampal pyramidal neurons exhibit constitutive GABA-independent gating. Mol Pharmacol. 2007; 71(2):539–548. [PubMed: 17090706]
30. Luddens H, Korpi ER. Biological function of GABAA/benzodiazepine receptor heterogeneity. J Psychiatr Res. 1995; 29(2):77–94. [PubMed: 7545236]
31. Ueno S, Bracamontes J, Zorumski C, Weiss DS, Steinbach JH. Bicuculline and gabazine are allosteric inhibitors of channel opening of the GABAA receptor. J Neurosci. 1997; 17(2):625–634. [PubMed: 8987785]
32. Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, Orser BA. Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by

gamma-aminobutyric acid(A) receptors in hippocampal neurons. Mol Pharmacol. 2001; 59(4): 814–824. [PubMed: 11259626]

33. Mortensen M, Kristiansen U, Ebert B, Frolund B, Krogsgaard-Larsen P, Smart TG. Activation of single heteromeric GABA(A) receptor ion channels by full and partial agonists. J Physiol. 2004; 557(Pt 2):389–413. [PubMed: 14990676]
34. Dixon CI, Morris HV, Breen G, Desrivieres S, Jugurnauth S, Steiner RC, et al. Cocaine effects on mouse incentive-learning and human addiction are linked to alpha2 subunit-containing GABAA receptors. Proc Natl Acad Sci U S A. 2010; 107(5):2289–2294. [PubMed: 20133874]
35. Kumar S, Fleming RL, Morrow AL. Ethanol regulation of gamma-aminobutyric acid A receptors: genomic and nongenomic mechanisms. Pharmacol Ther. 2004; 101(3):211–226. [PubMed: 15031000]
36. Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, et al. Hormonally regulated alpha(4)beta(2)delta GABA(A) receptors are a target for alcohol. Nat Neurosci. 2002; 5(8):721–722. [PubMed: 12118257]
37. Blomeley CP, Cains S, Smith R, Bracci E. Ethanol affects striatal interneurons directly and projection neurons through a reduction in cholinergic tone. Neuropsychopharmacology. 2011; 36(5):1033–1046. [PubMed: 21289603]
38. Keays DA, Clark TG, Flint J. Estimating the number of coding mutations in genotypicand phenotypic-driven N-ethyl-N-nitrosourea (ENU) screens. Mamm Genome. 2006; 17(3):230–238. [PubMed: 16518690]
39. Quwailid MM, Hugill A, Dear N, Vizor L, Wells S, Horner E, et al. A gene-driven ENU-based approach to generating an allelic series in any gene. Mamm Genome. 2004; 15(8):585–591. [PubMed: 15457338]
40. Sun F, Cheng R, Flanders WD, Yang Q, Khoury MJ. Whole genome association studies for genes affecting alcohol dependence. Genet Epidemiol. 1999; 17(Suppl 1):S337–342. [PubMed: 10597459]
41. Song J, Koller DL, Foroud T, Carr K, Zhao J, Rice J, et al. Association of GABA(A) receptors and alcohol dependence and the effects of genetic imprinting. Am J Med Genet B Neuropsychiatr Genet. 2003; 117B(1):39–45. [PubMed: 12555233]
42. Edenberg HJ, Bierut LJ, Boyce P, Cao M, Cawley S, Chiles R, et al. Description of the data from the Collaborative Study on the Genetics of Alcoholism (COGA) and single-nucleotide polymorphism genotyping for Genetic Analysis Workshop 14. BMC Genet. 2005; 6(Suppl 1):S2. [PubMed: 16451628]
43. Botta P, Radcliffe RA, Carta M, Mameli M, Daly E, Floyd KL, et al. Modulation of GABAA receptors in cerebellar granule neurons by ethanol: a review of genetic and electrophysiological studies. Alcohol. 2007; 41(3):187–199. [PubMed: 17521847]
44. Yamashita M, Marszalec W, Yeh JZ, Narahashi T. Effects of ethanol on tonic GABA currents in cerebellar granule cells and mammalian cells recombinantly expressing GABA(A) receptors. J Pharmacol Exp Ther. 2006; 319(1):431–438. [PubMed: 16844844]
45. Kaur KH, Baur R, Sigel E. Unanticipated structural and functional properties of delta-subunit-containing GABAA receptors. J Biol Chem. 2009; 284(12):7889–7896. [PubMed: 19141615]
46. Mortensen M, Ebert B, Wafford K, Smart TG. Distinct activities of GABA agonists at synaptic- and extrasynaptic-type GABAA receptors. J Physiol. 2010; 588(Pt 8):1251–1268. [PubMed: 20176630]
47. Mortensen M, Smart TG. Single-channel recording of ligand-gated ion channels. Nature protocols. 2007; 2(11):2826–2841.
48. Connolly CN, Krishek BJ, McDonald BJ, Smart TG, Moss SJ. Assembly and cell surface expression of heteromeric and homomeric gamma-aminobutyric acid type A receptors. J Biol Chem. 1996; 271(1):89–96. [PubMed: 8550630]

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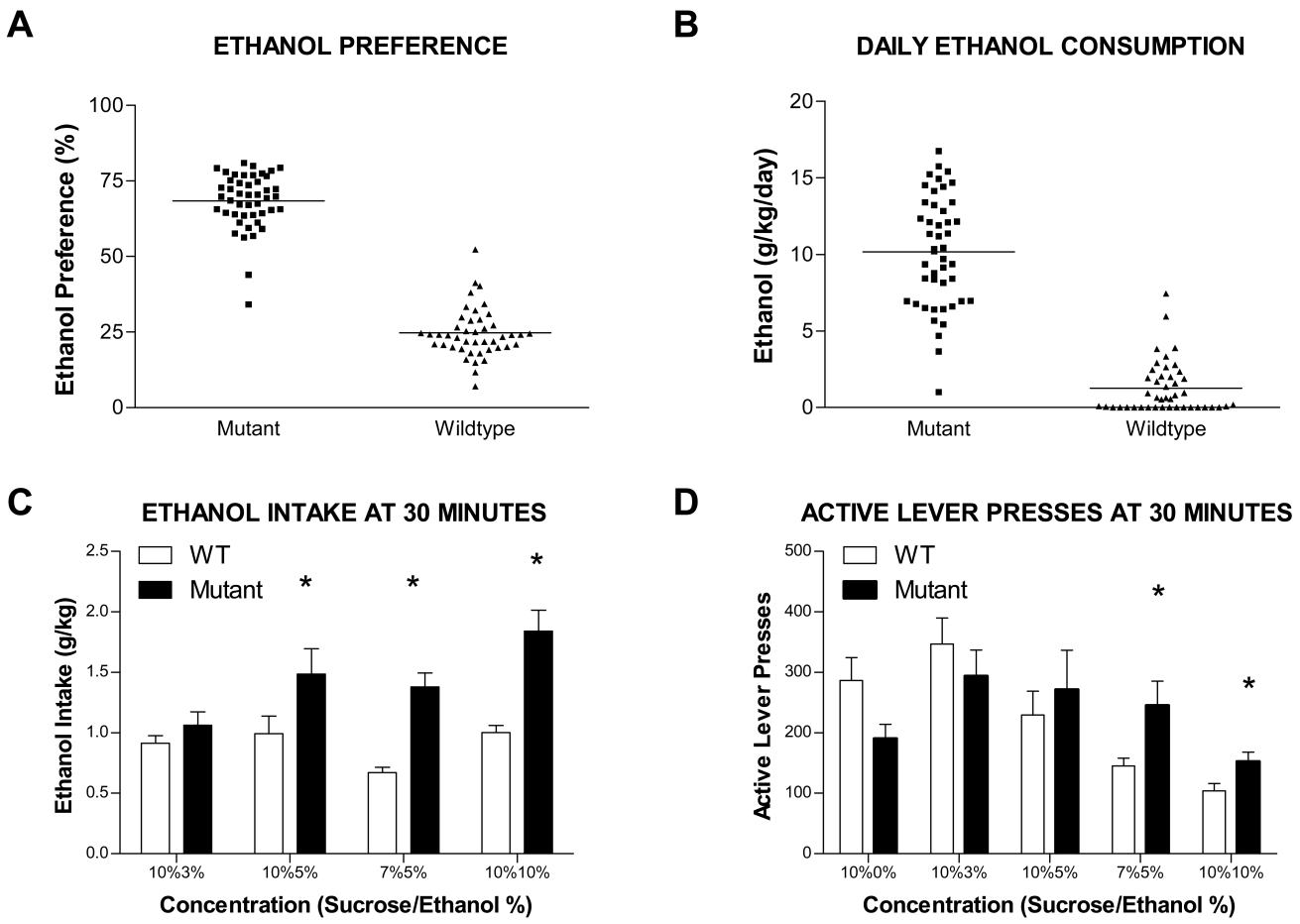

Figure 1. Alcohol Consumption and Behavioral Phenotype of Gabrb1$^{+/L285}$

(A) Ethanol (10% v/v) preference of male *Gabrb1*$^{+/L285}$ (Mean = 68.37%, 95% CI 65.61-71.13, n = 46) and WT littermates (24.67%, 22.20 −27.13, n = 44; t-test P<0.0001). **(B)** Daily ethanol consumption (g/kg body weight): Male *Gabrb1*$^{+/L285}$ (10.16, 95% CI 9.08-11.25, n = 46); WT littermates (1.24, 0.71-1.75; t-test P<0.0001, n = 44). **(C)** Amounts of ethanol earned (g/kg body weight ± SEM). * P<0.05 (n=8/group, ANOVA plus Bonferroni post-hoc test). **(D)** Numbers of lever presses ± SEM over 30 min by WT and mutant mice on a fixed ratio 4 (FR4) schedule for sucrose-ethanol reinforcers. Note increased number of lever presses for mutants at reinforcer mixtures of 7% sucrose / 5% ethanol, and 10% sucrose / 10% ethanol (n=8/group). * P<0.05, ANOVA plus Bonferroni post-hoc test.

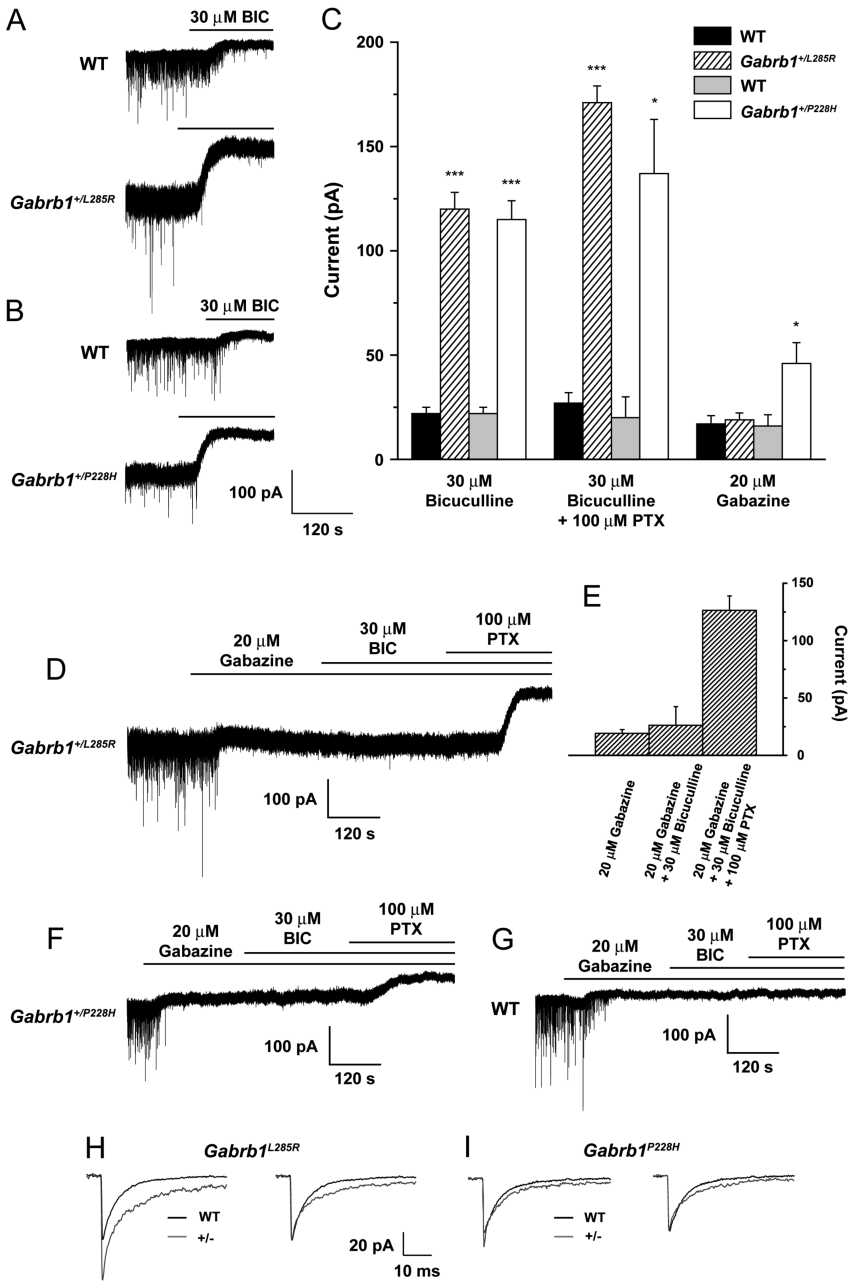

Figure 2. β1 Mutations Influence Tonic and Phasic Inhibition of Medium Spiny Neurons

Whole-cell recordings from accumbal MSNs of *Gabrb1*$^{+/L285R}$ **(A)**, *Gabrb1*$^{+/P228H}$ **(B)** and WT littermates. Note for both *Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$ MSNs the larger outward currents produced by bicuculline (30 μM). Scale bars apply to both A and B. **(C)** Outward currents induced by bicuculline (30 μM), gabazine (20 μM) and picrotoxin (PTX, 100 μM) from WT, *Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$ MSNs (means ± SEM; n = 3-12). **(D)** Whole-cell recording from a *Gabrb1*$^{+/L285R}$ MSN. Gabazine (20 μM) abolishes the mIPSCs, but produces only a relatively small outward current. However, gabazine prevents the further outward current usually produced by bicuculline for this mutant, but not that produced by

picrotoxin. **(E)** Mean outward current induced by $GABA_AR$ antagonists for *Gabrb1*$^{+/L285R}$ MSNs (means ± SEM; n = 4). Whole-cell recordings from a *Gabrb1*$^{+/P228H}$ **(F)** and a WT MSNs **(G)**. Gabazine (20 μM) abolishes mIPSCs, but additionally produces only a relatively small outward current for both the *Gabrb1*$^{+/P228H}$ and WT MSNs. However, for the *Gabrb1*$^{+/P228H}$ MSN gabazine prevents a further outward current by subsequent bicuculline, but this antagonist does not prevent the outward current produced by picrotoxin. By contrast, for WT MSNs after gabazine both bicuculline and picrotoxin are inert. Superimposed averaged mIPSCs are shown for WT (black) and *Gabrb1*$^{+/L285R}$ ***(H)***, or *Gabrb1*$^{+/P228H}$ **(I)** MSNs (grey). Right-hand traces of each pair are normalized to the mean peak amplitude of the appropriate WT mIPSC. Note the prolonged mIPSCs for *Gabrb1*$^{+/L285R}$ and *Gabrb1*$^{+/P228H}$ over WT. Scale bars apply to both H and I. *P<0.05, ** P<0.01, *** P<0.001 (unpaired Student's t-test for mutants relative to WTs).

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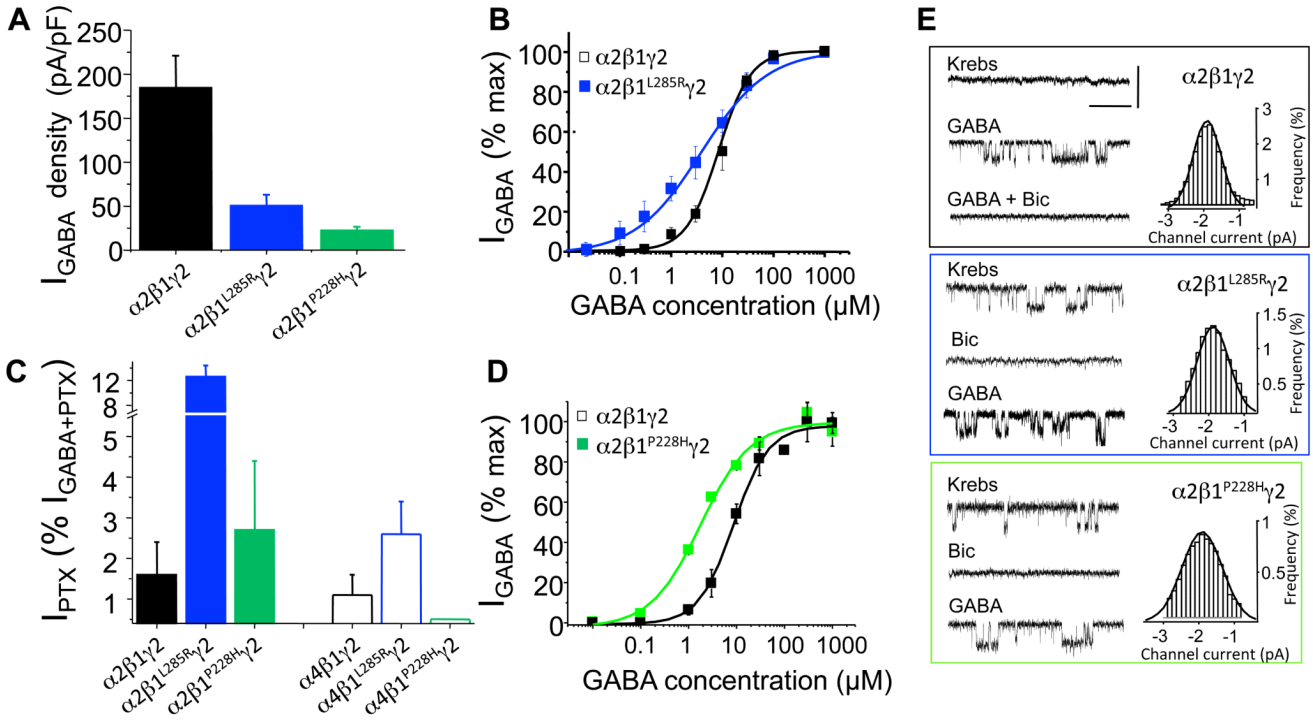

Figure 3. Effects of $\beta1^{L285R}$ and $\beta1^{P228H}$ on Recombinant $GABA_A$Rs

(A) Mean maximal GABA current densities to saturating GABA concentrations for WT and mutant, α2β1γ2 receptors. **(B)** GABA concentration-response curves for WT and $\alpha2\beta1^{L285R}\gamma2$ receptors. Responses are normalized to the maximum response from each cell. **(C)** Mean maximal outward currents produced by picrotoxin (PTX; 100μM) for WT and mutant receptors. Values are percentages of total current (= $I_{PTX}/(I_{PTX} + I_{GABA\ Max})$. **(D)** GABA concentration-response curves for WT and $\alpha2\beta1^{P228H}\gamma2$L receptors, analysed as in (B). **(E)** Single channel currents (sampled at 50 kHz) from outside-out patches (−70mV) of HEK293 cells expressing α2β1γ2, $\alpha2\beta1^{L285R}\gamma2$, and $\alpha2\beta1^{P228H}\gamma2$ receptors. Spontaneous channel activity is evident in the absence of GABA (traces labelled 'Krebs'). Both spontaneous and GABA-activated channel openings are inhibited by bicuculline (Bic: 50μM). Scale bars: 2 pA, 100 ms. Single channel amplitude histograms (right panels) are based on 10-30 s epochs for α2β1γ2 (activated by 10μM GABA) and for spontaneously-active $\alpha2\beta1^{L285R}\gamma2$ and $\alpha2\beta1^{P228H}\gamma2$. All single channel currents have mean amplitudes of ~ −1.9 pA (~27 pS). Error bars in panels A-D represent the mean ± SEM (n = 7-15 for panel A: n = 4-6 for panels B & D: n = 6-11 for panel C).

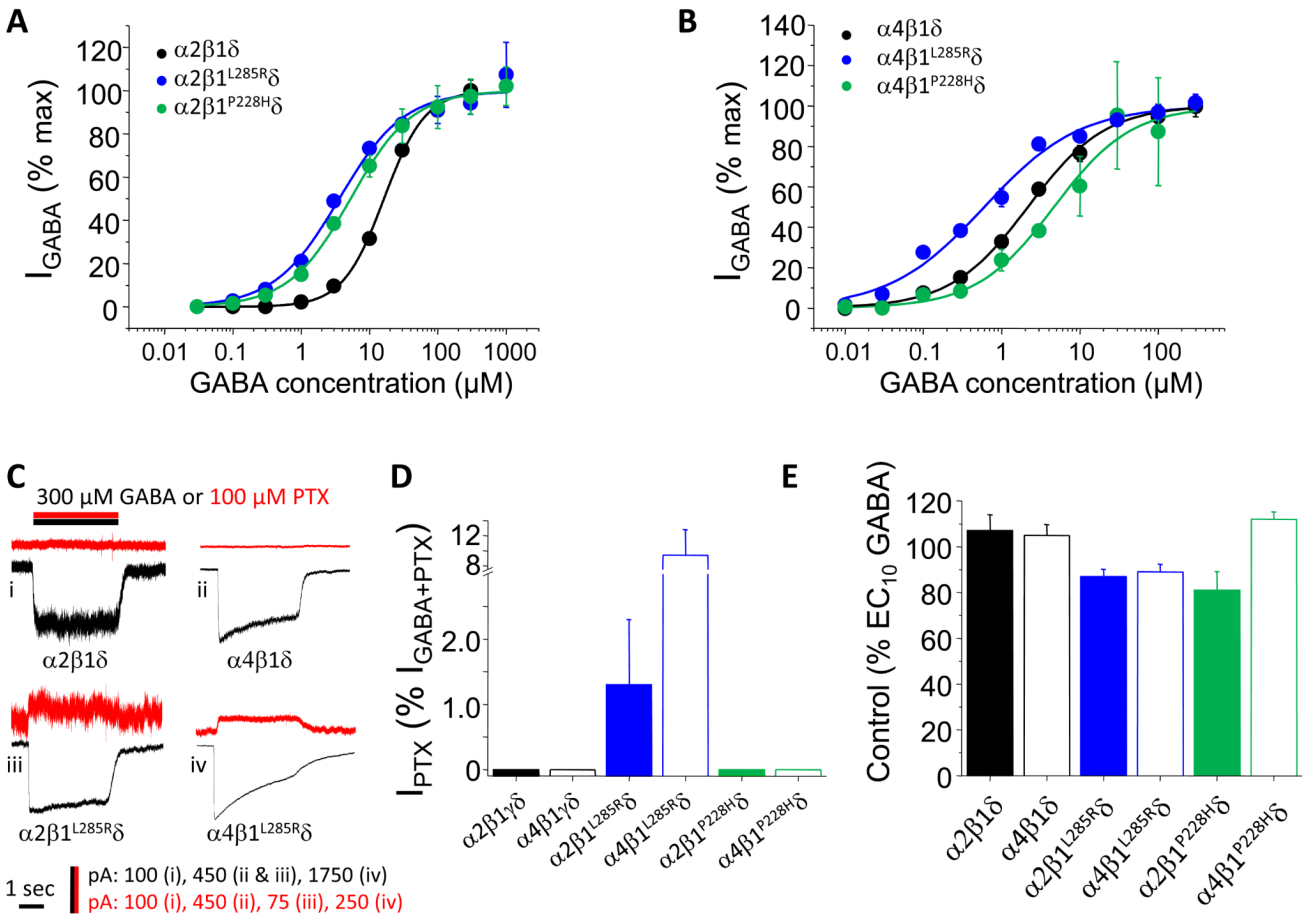

Figure 4. Effects of $\beta1^{L285R}$ and $\beta1^{P228H}$ on δ-containing recombinant $GABA_A$Rs

(A & B) GABA concentration-response curves for WT and mutant α2β1δ and α4β1δ receptors, analysed as in Figure 3B. **(C)** Representative membrane currents for GABA (black) or picrotoxin (red) recorded for WT and mutant (L285R) α2β1δ and α4β1δ receptors (V_H = −40mV). **(D)** Mean maximal outward currents after PTX (100 μM; expressed as in Figure 3C) applied in the absence of GABA for WT and mutant α2β1δ (solid bar) or α4β1δ (open bar) receptors. **(E)** Mean GABA responses (EC_{10}) in the presence of 30mM EtOH for WT and mutant receptors (Control EC_{10} responses =100%). All error bars in panels A, B, D & E represent the mean ± SEM (n = 5-12 for panels A & B: n = 7-12 for panel D: n = 3-13 for panel E).

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Table 1

NAc Tonic Conductance.

	WT	*Gabrb1* $^{+/L285R}$	WT	*Gabrb1* $^{+/P228H}$
I_{HOLD} (pA)	212 ± 24 n = 12	284 ± 14 ** n = 19	156 ± 12 n = 15	296 ± 20 *** n = 12
RMS (pA) (Control)	4.3 ± 0.2 n = 12	7.9 ± 0.2 *** n = 19	3.9 ± 0.1 n = 15	7.0 ± 0.2 *** n = 12
RMS (pA) (+ Bic)	2.7 ± 0.2 n = 6	4.4 ± 0. 2 *** n = 9	2.6 ± 0.1 n = 11	3.5 ± 0. 1 *** n = 8
Δ RMS (pA) (+ Bic)	1.1 ± 0.2 n = 6	3.7 ± 0.3 *** n = 9	1.1 ± 0.1 n = 11	3.7 ± 0.2 *** n = 8
$I_{BIC.}$ (pA)	22 ± 3 n = 6	120 ± 8 *** n = 9	22 ± 3 n = 11	115 ± 9 *** n = 8
$I_{GBZ.}$ (pA)	17 ± 4 n = 5	19 ± 3 ††† n = 4	16 ± 5 n = 4	46 ± 10 $^{*\,\dagger\dagger\,\ddagger}$ n = 3
I(pA) Bic + Ptx	27 ± 5 n = 4	171 ± 8 *** n = 4	20 ± 10 n = 3	137 ± 26 * n = 3
I(pA) Ptx after Bic	4 ± 2 n = 4	41 ± 8 ** n = 4	−5 ± 3 n = 3	19 ± 6 * n = 3
RMS (pA) (Bic + Ptx)	2.9 ± 0.2 n = 4	2.9 ± 0.2 n = 4	2.6 ± 0.1 n = 3	2.8 ± 0.1 n = 3
I_{THIP} (pA)	60 ± 15 n = 7	41 ± 9 n = 4	N.D.	N.D.

ND = Not determined; Bic = bicuculline; Ptx = picrotoxin; Gbz = gabazine

Statistical significance of mutant relative to its WT:

*P<0.05;

**P<0.01;

***P<0.001.

Statistical significance of I_{GBZ} relative to I_{BIC}:

††P<0.01;

†††P<0.001.

Statistical significance of *Gabrb1*$^{+/P228H}$ relative to *Gabrb1*$^{+/L285R}$:

‡P<0.05.

(unpaired Student's *t* test used in all cases)

Data presented as ± SEM

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Table 2
Brain Slice Electrophysiology: Synaptic transmission.

	WT n = 12	*Gabrb1*$^{+/L285R}$ n = 13	WT n = 6	*Gabrb1*$^{+/P228H}$ n = 7
Peak Amp. (pA)	62 ± 4	88 ± 6 **	53 ± 2	64 ± 5
Rise time (ms)	0.5 ± 0.02	0.5 ± 0.02	0.6± 0.02	0.5 ± 0.02
T_w (ms)	9.5 ± 0.6	14.4 ± 1.3 **	7.8 ± 0.5	10.9 ± 0.9 *
Frequency (Hz)	2.1 ± 0.5	0.6 ± 0.1 **	2.9 ± 0.5	0.8 ± 0.02 ***

Statistical significance of mutant relative to the appropriate WT:

* P<0.05;

** P<0.01;

*** P<0.001

(unpaired Student's *t* test).

Data presented as ± SEM