0026-895X/10/7805-918–924$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 78:918–924, 2010

Vol. 78, No. 5
62687/3633959
Printed in U.S.A.

Alcohol- and Alcohol Antagonist-Sensitive Human GABA$_A$ Receptors: Tracking δ Subunit Incorporation into Functional Receptors

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Received December 3, 2009; accepted August 10, 2010

ABSTRACT

GABA$_A$ receptors (GABA$_A$Rs) have long been a focus as targets for alcohol actions. Recent work suggests that tonic GABAergic inhibition mediated by extrasynaptic δ subunit-containing GABA$_A$Rs is uniquely sensitive to ethanol and enhanced at concentrations relevant for human alcohol consumption. Ethanol enhancement of recombinant $\alpha 4\beta 3\delta$ receptors is blocked by the behavioral alcohol antagonist 8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester (Ro15-4513), suggesting that EtOH/Ro15-4513-sensitive receptors mediate important behavioral alcohol actions. Here we confirm alcohol/alcohol antagonist sensitivity of $\alpha 4\beta 3\delta$ receptors using human clones expressed in a human cell line and test the hypothesis that discrepant findings concerning the high alcohol sensitivity of these receptors are due to difficulties incorporating δ subunits into functional receptors. To track δ subunit incorporation, we used a functional tag, a single amino acid change (H68A) in a benzodiazepine binding residue in which a histidine in the δ subunit is replaced by an alanine residue found at the homologous position in γ subunits. We demonstrate that the δH68A substitution confers diazepam sensitivity to otherwise diazepam-insensitive $\alpha 4\beta 3\delta$ receptors. The extent of enhancement of $\alpha 4\beta 3\delta$H68A receptors by 1 μM diazepam, 30 mM EtOH, and 1 μM β-carboline-3-carboxy ethyl ester (but not 1 μM Zn^{2+} block) is correlated in individual recordings, suggesting that δ subunit incorporation into recombinant GABA$_A$Rs varies from cell to cell and that this variation accounts for the variable pharmacological profile. These data are consistent with the notion that δ subunit-incorporation is often incomplete in recombinant systems yet is necessary for high ethanol sensitivity, one of the features of native δ subunit-containing GABA$_A$Rs.

Introduction

Classic synaptic GABAergic inhibition is characterized by the pulsatile release of the neurotransmitter GABA onto a molecularly distinct subset of GABA$_A$Rs that contain $\gamma 2$ subunits. In addition, there is a fundamentally different form of sustained (tonic) GABAergic inhibition mediated by circulating low levels of GABA that exerts a powerful depressant effect on neuronal excitability. Tonic inhibition is produced by extrasynaptic GABA$_A$R subtypes that exhibit high affinity for GABA and slow desensitization, with much of this tonic inhibition mediated by δ subunit-containing GABA$_A$Rs (Farrant and Nusser, 2005).

Although GABA$_A$Rs have for decades been implicated in EtOH actions (Liljequist and Engel, 1982; Suzdak et al., 1986), direct actions of relevant EtOH concentrations—defined as concentrations up to 30 mM, or slightly less than twice the legal driving limit—on classic synaptic GABA$_A$Rs have been elusive (Wallner et al., 2006a). A possible solution for this conundrum was provided by findings that extrasynaptic δ subunit-containing GABA$_A$Rs are enhanced by relevant low EtOH concentrations (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Hanchar et al., 2004). Support for this hypothesis comes from the observation that a single nucleotide polymorphism in the $\alpha 6$ gene (α6R100Q), initially identified in rats with increased alcohol-induced motor impairment (also known as alcohol nontolerant rats) (Uusi-Oukari and Korpi, 1989), further increases the EtOH sensitivity of $\alpha 6\beta 3\delta$ receptors in vivo and in vitro (Hanchar et al., 2005). In addition, ethanol enhancement of δ subunit-containing re-

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This work was supported by the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grant NS035985]; the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grant AA017891]; and by funds to the University of California to support research and a new drug development program for alcoholism and addiction.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.109.062687.

ABBREVIATIONS: GABA$_A$R, GABA$_A$ receptor; HEK, human embryonic kidney; BZ, benzodiazepine; β-CCE, β-carboline-3-carboxy ethyl ester; DZ, diazepam.

ceptors is competitively antagonized by the rat behavioral alcohol antagonist 8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester (Ro15-4513) (Hanchar et al., 2006; Wallner et al., 2006b). These results provided evidence that EtOH/Ro15-4513-sensitive $GABA_AR$ subtypes mediate behaviorally relevant alcohol effects in mammals and provide a detailed molecular explanation for the efficacy of the imidiazobenzodiazepine Ro15-4513 as an alcohol antagonist (Suzdak et al., 1986; Wallner et al., 2006b; Wallner and Olsen, 2008). It is noteworthy that the extrasynaptic $GABA_AR$ hypothesis has been extensively corroborated by recordings from δ subunit-containing native neurons (Wei et al., 2004; Hanchar et al., 2005; Fleming et al., 2007; Glykys et al., 2007; Liang et al., 2007; Santhakumar et al., 2007; Jia et al., 2008). Yet despite the abundant evidence that native extrasynaptic $GABA_ARs$ are ethanol-sensitive, the hypothesis has been challenged by groups unable to replicate the high ethanol sensitivity of δ subunit-containing $GABA_ARs$ reconstituted using recombinant receptor cDNAs in *Xenopus laevis* oocytes (Borghese et al., 2006; Baur et al., 2009) or in mammalian cells (Yamashita et al., 2006).

Functional $GABA_ARs$ are pentamers; prototypical synaptic $GABA_ARs$ in vertebrate brains are generally believed to be formed by two α, two β, and one γ subunit, and it is assumed that a single γ subunit in these pentameric receptors can be replaced by the δ or ε subunit (Olsen and Sieghart, 2008) to yield $GABA_ARs$ with distinctive pharmacological properties (Davies et al., 1997; Wallner et al., 2003). $GABA_ARs$ reconstituted only from α and β subunits (i.e., lacking γ, δ, or ε subunits) readily form functional receptors in recombinant expression systems, and such receptors are generally characterized by high sensitivity to blockade by Zn^{2+} (Smart et al., 1991; Thompson et al., 2002).

Expression of α and β subunits in recombinant systems leads to the formation of functional benzodiazepine-insensitive $GABA_ARs$, and benzodiazepine sensitivity is conferred by $\gamma 2$ subunit coexpression (Pritchett et al., 1989). It has been shown that the formation of "binary" $\alpha\beta$ receptors leads to pharmacologically heterogeneous receptor populations when "synaptic," γ subunit-containing $GABA_ARs$ are expressed in recombinant systems (Boileau et al., 2002; Baburin et al., 2008). To mitigate such problems with heterogeneous populations of $\alpha\beta$ and $\alpha\beta\gamma$ $GABA_ARs$ in recombinant expression systems, γ subunit cRNA or cDNAs are generally supplied in excess over α and β subunits in recombinant expression.

In this report we tested the hypothesis that, similar to what has been described with the $\gamma 2$ subunit, transfection of HEK 239 T cells with human and rat $\alpha 4$, $\beta 3$, and the δ subunit results in the formation of heterogeneous, pharmacologically distinct populations of functional $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ receptors. We show here that δ subunit coexpression led to functional rat and human $GABA_ARs$ that were enhanced by 30 mM EtOH, and that this EtOH enhancement was blocked by the behavioral BZ alcohol antagonist Ro15-4513. However, we found substantial variability in the amount of EtOH enhancement among individual recordings, a small fraction of cells showing no detectable enhancement by 30 mM EtOH. To determine whether this variability resulted from differences in the amount of δ subunit-incorporation, we exploited a "functional tag," a mutation in the δ subunit (δH68A) that conferred diazepam sensitivity to $\alpha 4\beta 3\delta$H68A receptors with no changes in EtOH or GABA sensitivity.

Using the δH68A mutation, we found that the magnitudes of EtOH, β-CCE [another allosteric modulator at the EtOH/Ro15-4513 site in $\alpha 4\beta 3\delta$ receptors (Hanchar et al., 2006; Wallner et al., 2006b)], and DZ enhancement covary in individual recordings. This is consistent with our hypothesis that incomplete δ subunit incorporation causes variability in EtOH responses in recombinant systems. It is noteworthy that we found that the extent of inhibition by 1 μM Zn^{2+} was not well correlated with DZ and EtOH enhancement, which suggests that loss of Zn^{2+} sensitivity is disconnected from allosteric modulation by alcohol.

Experimental data shown here confirm the unique alcohol sensitivity of human and rat δ subunit-containing receptors and the reversal of EtOH enhancement by the behavioral alcohol antagonist Ro15-4513 when these receptors are expressed in a human immortalized cell line, and are consistent with the notion that δ subunit incorporation, although difficult to achieve, is necessary to confer EtOH/Ro15-4513-sensitivity in rodent and human $GABA_ARs$.

Materials and Methods

Diazepam and β-CCE were gifts from Hoffman-La Roche (Nutley, NJ) and Ferrosan (Soeborg, Denmark), respectively. Most other standard chemicals, including EtOH, were obtained from Sigma (St. Louis, MO). Human $\alpha 4$, $\beta 3$, and δ cDNAs were either cloned by RT-PCR using human total brain mRNA (Invitrogen, Carlsbad, CA) as described previously (Wallner et al., 2003) or were from cDNA repositories. Clones were sequenced to ensure that the protein sequences conform to consensus human protein sequences found in the RefSeq public database (http://www.ncbi.nlm.nih.gov/RefSeq/). For functional expression in mammalian cells, human $GABA_AR$ cDNAs were subcloned into a eukaryotic expression vector containing a cytomegalovirus promoter as well as a T7 RNA polymerase promoter. Oocyte expression methods and the rat clones used are as described previously (Wallner et al., 2003). HEK-293 T cells (American Type Culture Collection, Manassas, VA) were transfected using a dextran transfection method as described previously (Meera et al., 1997). Cotransfections with δ subunit contained a 5-fold excess of δ and δH68A mutant over $\alpha 4$ and $\beta 3$ subunits, and a limiting amount of EGFP cDNA to identify successfully transfected cells by green fluorescent protein epifluorescence. Total amounts of plasmid DNA were 4 μg of $\alpha 4$, 4 μg of $\beta 3$, and 20 μg of δ or δH68A (δ cDNA omitted for $\alpha\beta$ receptors) together with 0.4 μg of enhanced green fluorescent protein-plasmid DNA for each 10-cm diameter plate. Whole-cell electrophysiological recordings were performed between 70 and 150 h after transfection. Recordings were made from individual cells plated on poly-D-lysine–coated cover slips at room temperature. Voltage was clamped using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) at a holding potential of −60 mV. The external solution was 142 mM NaCl, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 8 mM KCl, 10 mM glucose, and 10 mM HEPES, pH 7.4 (327–330 mOsM). The pipette internal solution consisted of 140 mM CsCl, 4 mM NaCl, 0.5 mM $CaCl_2$, 10 mM HEPES, 5 mM EGTA, 2 mM Mg^{2+} ATP, and 0.2 mM GTP. Drug solutions were applied using a multibarrel pipette driven by a stepper motor (SF-77B; Warner Instruments, Hamden, CT) with an onset exchange time of around 10 ms. Recording pipettes had a bath resistance of ~4 MΩ.

Data Analysis. Whole-cell currents were analyzed using Clampfit 9 (Molecular Devices). The normalized concentration-response data were least-squares-fitted [with the "Solver" function in Excel (Microsoft Corp., Redmond, WA)] using the Hill equation: $I/I_{max} = (1/(1 + (EC_{50}/[A])^{nH})$, where EC_{50} represents the concentration of the agonist ([A]) inducing 50% of the maximal current evoked by a saturating concentration of the agonist and n_H is the Hill coefficient. I is the peak current evoked by a given concentration of GABA. I_{max} is the maximal current at a saturating GABA concentration. Correlation analysis was performed with Igor (Wavemetrics, Lake Oswego, OR).

Results

Given the importance of alcohol actions for human health and the debate about whether δ subunit-containing $GABA_A$Rs are EtOH- and Ro15-4513-sensitive (Lovinger and Homanics, 2007), we decided to test human and rat α4, β3, and δ subunit $GABA_A$R clones coexpressed in a mammalian cell line (HEK 293T) for EtOH sensitivity and unique alcohol-related pharmacology (Suzdak et al., 1986; Wallner et al., 2003, 2006b; Hanchar et al., 2005). Human α4, β3, and δ cDNAs were subcloned into the same vectors that we previously used to express rat subunits in HEK 293T cells (Hanchar et al., 2006); in this process we replaced the original 5′-untranslated regions with a 182 base-pair 5′-untranslated region from the Shaker K^+ channel, a membrane protein with high levels of expression in recombinant systems.

Characterization of α4β3 and α4β3δ Receptors in HEK Cells. Figure 1 compares GABA dose-response curves of HEK cells transfected with human α4 and β3 subunit alone with cells cotransfected with α4 and β3 subunits and a 5-fold excess of δ subunit cDNA. GABA responses were evoked by perfusion of GABA from a threshold concentration of 100 nM to a saturating concentration of 30 μM. In both α4β3- and α4β3δ-transfected cells, currents are substantially activated by a GABA concentration of 300 nM (300 nM GABA is ~EC_{15} for α4β3-transfected cells and ~EC_{30} for α4β3δ-transfected cells). Responses from human and rat α4β3δ-transfected cells did not seem to be different in their GABA sensitivity ($p = 0.98$; Fig. 1C) or in their sensitivity to other modulators (discussed in the paragraphs below). On this basis and considering the fact that rat and human sequences show 90 to 97% amino acid identity, rat and human data were pooled unless otherwise indicated. Analysis of the summary data from pooled rat and human $GABA_A$Rs suggests that δ subunit coexpression leads to a small yet statistically significant ($p = 0.008$) increase in GABA sensitivity of α4β3δ receptors ($EC_{50} = 0.53 \pm 0.04$ μM, $n = 7$) compared with binary α4β3 $GABA_A$Rs ($EC_{50} = 1.3 \pm 0.1$ μM, $n = 8$).

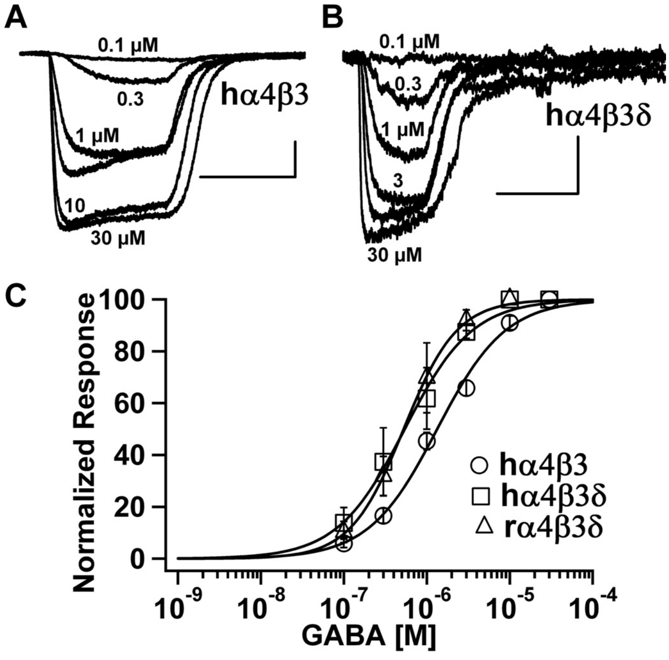

Fig. 1. Coexpression of the δ subunit increases GABA sensitivity. A and B show representative GABA dose-response traces recorded from HEK cells transfected with human α4β3 (A) and α4β3δ (B) receptors using GABA concentrations ranging from 0.1 to 30 μM (as indicated). The GABA EC_{50} values ± S.D. for human α4β3δ- and α4β3-transfected cells were 0.54 ± 0.05 μM ($n = 3$) and 1.3 ± 0.1 μM ($n = 8$), respectively. Because rat α4β3δ receptors [$EC_{50} = 0.53 \pm 0.02$ μM ($n = 4$)] are similar to human clones in their GABA sensitivity ($p = 0.98$) (C), the data for human and rat receptors were pooled. Statistical analysis (Student's t test) of EC_{50} values of individual recordings from α4β3 and α4β3δ subunit-transfected cells shows that δ subunit coexpression leads to a small, yet significant (**, $p < 0.01$), increase in GABA sensitivity (C). Horizontal time scale for both A and B is 5 s; the vertical scale in A is 200 pA and in B is 50 pA.

Recombinant α4β3δ $GABA_A$Rs Are Enhanced by 30 mM EtOH, an Effect Blocked by 300 nM Ro15-4513, a Behavioral Alcohol Antagonist. To study human and rat recombinant receptors expressed in HEK cells, we decided to use an alcohol concentration of 30 mM, which is close to the mean blood alcohol concentration reported for authority-apprehended intoxicated suspects (Khiabani et al., 2008) and less than twice the legal driving limit for adult drivers in the United States (17.4 mM).

GABA currents in human and rat α4β3δ- and α4β3-transfected cells were evoked by application of 300 nM GABA (labeled G in Fig. 2A) and tested for modulation by 30 mM EtOH alone (G+E), and for reversal of EtOH effects by ap-

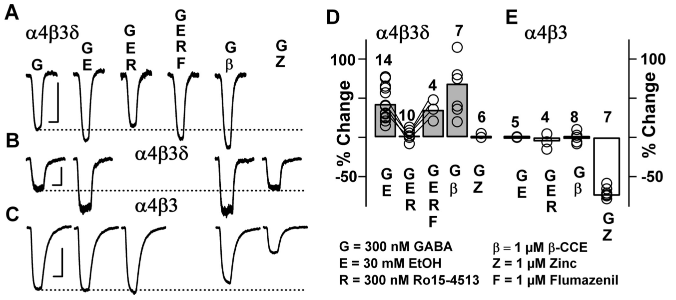

Fig. 2. Alcohol and alcohol antagonist-related pharmacology of human and rat α4β3δ $GABA_A$Rs. A to C show current traces recorded from three individual HEK cells transfected with human α4β3δ (A), rat α4β3δ (B), and human α4β3 (C) receptors. GABA currents were evoked by 1) 300 nM GABA alone (G, responses marked with a dotted line in A to C), with 300 nM GABA together with 2) 30 mM EtOH (G+E), 3) 30 mM EtOH + 300 nM Ro15-4513 (G+E+R), 4) 30 mM EtOH + 300 nM Ro15-4513 + 1 μM flumazenil (G+E+R+F), 5) 1 μM β-CCE (G+β), and 6) 1 μM Zn^{2+} (G+Z). Note that not all modulators (or combinations of modulators) were tested in all cases of currents recorded (blank spaces). D and E show summary data, with responses from individual cells shown as circles to demonstrate the considerable variability among individual recordings (numbers above data points are the number of experiments). GABA currents from individual α4β3δ-transfected cells that showed no enhancement by 30 mM EtOH and/or 1 μM β-CCE were, when tested, inhibited by 1 μM Zn^{2+}, indicative of α4β3 expression (E) and lack of δ subunit expression; therefore, such cells were excluded from the summary data in Fig. 2. Data obtained from human and rat receptors were similar: e.g., enhancement by 30 mM EtOH (percentage ± S.D.) with rat and human clones was 42 ± 21% for rat α4β3δ ($n = 8$) and 43 ± 23% for human α4β3δ ($n = 6$) ($p = 0.98$). Therefore, data from human and rat receptors were pooled in the summary data (Fig. 2D). Mean values for 300 nM GABA current enhancement with human and rat α4β3δ-transfected cells in percentage ± S.D. are as follows: 30 mM EtOH ($n = 14$), 43 ± 21%; 30 mM EtOH + 300 nM Ro15-4513, 2 ± 6% ($n = 10$). For α4β3-transfected cells, the modulation was as follows: 30 mM EtOH, −1 ± 3%, ($n = 5$); 1 μM β-CCE, 0.1 ± 5% ($n = 8$); 1 μM Zn^{2+}, −70 ± 6% ($n = 8$). Vertical scale is 100 pA for A and B and 200 pA for 2C; horizontal time scale is 5 s for all panels.

plication of 30 mM EtOH together with 300 nM Ro15-4513 (G+E+R). In four recordings, we also tested for Ro15-4513 alcohol antagonism reversal by 1 μM flumazenil (G+E+R+F); data points from individual experiments in which Ro15-4513 alcohol antagonism was tested for reversal by 1 μM flumazenil are connected by lines in Fig. 2D. Furthermore, we tested for enhancement of 300 nM GABA responses by the β-carboline β-CCE (Fig. 2A, G+β) and block by 1 μM Zn^{2+} (G+Z).

Mean enhancement of 300 nM GABA responses by 30 mM ethanol showed no statistically significant differences in human and rat $\alpha 4\beta 3\delta$-transfected cells ($p = 0.95$; human = 33 ± 9%, $n = 6$, rat = 34 ± 5%, $n = 8$, mean ± S.D.); therefore, data were pooled in summary Fig. 2D. Data obtained from individual $\alpha 4\beta 3\delta$-transfected cells showed considerable variability, as illustrated in the scatter plot, in which each point represents the percentage change in an individual $\alpha 4\beta 3\delta$-transfected cell (Fig. 2D). Despite this variability, 30 mM EtOH enhancement of 300 nM GABA currents was statistically highly significant (paired t test, $p < 0.001$ G versus G+E, $n = 14$). Enhancement by 30 mM EtOH was blocked by 300 nM Ro15-4513 ($p < 0.001$, G+E versus G+E+R, $n = 10$), and in four experiments (individual recordings connected by lines in Fig. 2D), it was tested whether the actions of Ro15-4513 were reversed by 1 μM flumazenil ($p < 0.01$, G+E+R versus G+E+R+F, $n = 4$). GABA currents from $\alpha 4\beta 3\delta$-transfected cells were enhanced by 1 μM β-CCE ($p < 0.01$, G versus G+B, $n = 7$) but not blocked by 1 μM Zn^{2+} ($p = 0.4$, G versus G+Z, $n = 6$). In contrast, recordings from cells transfected with $\alpha 4$ and $\beta 3$ subunits alone exhibited GABA responses that were enhanced by neither 30 mM EtOH ($p = 0.45$ versus G+E, $n = 5$) nor 1 μM β-CCE ($p = 0.96$, G versus G+B, $n = 8$), but were inhibited 70 ± 6% by 1 μM Zn^{2+} ($p < 0.001$, G versus G+Z, $n = 7$).

We noted that enhancement by 30 mM EtOH and by 1 μM β-CCE seemed to be correlated (compare amount of EtOH and β-CCE responses in individual traces in Fig. 2, A and B), an observation consistent with the notion that individual HEK cells have variable fractions of $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ receptors. On the other hand, the lack of inhibition by 1 μM Zn^{2+} implies that there was very little "contamination" by $\alpha\beta$ receptors in these experiments. In an attempt to resolve this apparent discrepancy, we developed an independent strategy to determine the extent to which δ subunits were present in the receptors generating GABA current within single HEK cells.

The δH68A Mutation Confers Diazepam Sensitivity to δ Subunit-Containing $GABA_AR$s. To find differences that might be responsible for lack of effects of classic BZs (such as DZ) on δ subunit-containing receptors, we explored regions of the δ subunit that are homologous to regions in the γ subunit that contribute to BZ binding pockets at $\alpha+/\gamma-$ subunit interfaces (Kucken et al., 2003). Our attention was drawn to a histidine residue in the δ subunit (δH68) that is an alanine residue (γ2A79) at the homologous position in the γ2 subunit (see Fig. 3A).

To test whether this residue influences the sensitivity of δ subunit-containing receptors to DZ, we converted histidine 68 to an alanine, cotransfected the δH68A mutant δ subunit with $\alpha 4$ and $\beta 3$ subunits, and tested for enhancement of 300 nM GABA responses by 1 μM DZ. Recordings in Fig. 3, B and C, show that wild-type $\alpha 4\beta 3\delta$ receptors are sensitive to 30 mM EtOH but insensitive to 1 μM DZ, whereas $\alpha 4\beta 3\delta$H68A receptors are enhanced by both EtOH and 1 μM DZ. Figure 3D depicts summary data showing that the δH68A mutation confers DZ sensitivity to otherwise DZ-insensitive $\alpha 4\beta 3\delta$ wild-type receptors. The δH68A mutation did not lead to significant changes in GABA sensitivity (data not shown) or to differences in Zn^{2+} blockade between recombinant $\alpha 4\beta 3\delta$ and $\alpha 4\beta 3\delta$H68A receptors expressed in oocytes (Table 1).

Diazepam Sensitivity of $\alpha 4\beta 3\delta$H68A-Transfected cells Is Correlated with EtOH and β-CCE Enhancement but Shows Only Poor Correlation with 1 μM Zn^{2+} Blockade. We decided to use the "DZ-sensitive" δH68A-mutated subunit as a tool to determine whether various aspects of pharmacological variability correlated with the presence/absence of δ subunits. If the extent of δ subunit-incorporation into functional surface receptors varies on a cell-to-cell basis, and if this contributes to the pharmacological variability, then in individual $\alpha 4\beta 3\delta$H68A-transfected cells, the extent of enhancement of 300 nM GABA currents by 1 μM DZ should covary with the effects of pharmacological agents selective for δ subunit-containing receptors.

Whole-cell recordings were made from human and rat $\alpha 4\beta 3\delta$H68A-transfected cells, and currents evoked by 300 nM GABA alone were compared with 300 nM GABA responses in the presence of modulators: 30 mM EtOH, 1 μM DZ, 1 μM β-CCE, or 1 μM Zn^{2+}. Figure 4, A to D, shows recordings from individual cells with large (Fig. 4A), inter-

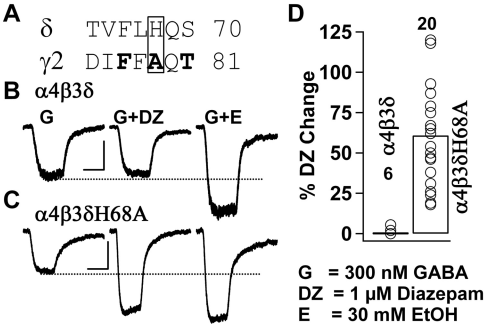

Fig. 3. The δH68A mutation makes $\alpha 4\beta 3\delta$ receptors sensitive to allosteric diazepam enhancement. A shows a protein sequence alignment of a region recognized as important for the binding of ligands (e.g., acetylcholine, nicotine, BZ site ligands) in the extracellular loop of "cysteine-loop" ligand-gated receptors. Three amino acid residues in the γ2 subunit (Phe77, Ala79, and Thr81, bold) contribute to the BZ binding site at the $\alpha+/\gamma2-$ subunit interface. B and C show in representative recordings that 30 mM EtOH-sensitive $\alpha 4\beta 3\delta$ $GABA_AR$s are not enhanced by 1 μM DZ (B), whereas with the δH68A mutation, $\alpha 4\beta 3\delta$H68A receptors are enhanced similarly by both 1 μM DZ and 30 mM EtOH. Summary data are shown in Fig. 3D: mean change in $\alpha 4\beta 3\delta$-transfected cells with 1 μM DZ is −2 ± 9%, $n = 6$ (Fig. 3D), whereas both rat and human $\alpha 4\beta 3\delta$H68A are enhanced similarly by 1 μM DZ [rat, 64 ± 23% ($n = 13$); human, 61 ± 33% ($n = 7$)]; therefore, data were pooled in Fig. 3D. Vertical scale is 100 pA for B and 50 pA for C; all horizontal scales are 5 s.

TABLE 1

The δH68A mutation does not change the Zn^{2+} sensitivity of $\alpha 4\beta 3\delta$ receptors expressed in oocytes

Zn^{2+} sensitivity of agonist (e.g., 300 nM GABA) evoked currents was evaluated at 0.1, 1, and 10 μM Zn^{2+} and data are percent current block with standard deviations. Statistical analysis, with P values calculated using the Student's t test, shows that there is no significant difference in Zn^{2+} block at all three Zn^{2+} concentrations tested.

	[Zn^{2+}]		
	0.1 μM	1 μM	10 μM
$\alpha 4\beta 3\delta$ ($n = 6$) % block	0.8 ± 1.4	26.3 ± 2.1	88.8 ± 11.8
$\alpha 4\beta 3\delta$H68A ($n = 7$) % block	1.4 ± 1.9	27.1 ± 6.7	81.4 ± 10.7
P value, δ versus δH68A	0.54	0.95	0.26

mediate (Fig. 4, B and C), and not detectable (Fig. 4D) 300 nM GABA current modulation by EtOH, DZ, or β-CCE. Figure 4E shows summary data in a scatter plot format. Note that four $\alpha4\beta3\delta$H68A-transfected cells showed little EtOH/DZ/β-CCE enhancement but significant block by 1 μM Zn^{2+} (Fig. 4D). Data points obtained from individual HEK cell experiments shown in Fig. 4, A to D, are indicated with filled symbols (marked with a–d in Fig. 4, E–H).

Figure 4, F to H, shows correlation plots with 300 nM GABA current enhancement (in percent) by 1 μM diazepam plotted against enhancement by 30 mM EtOH (F), 1 μM β-CCE (G), and percentage blockade of currents by 1 μM Zn^{2+} (H). Correlation coefficients (r^2) obtained from linear regression indicate that large fractions of the variability in EtOH (63%) and β-CCE (58%) responsiveness can be explained by the level of δ subunit incorporation. In contrast, Zn^{2+} inhibition is relatively poorly correlated (37%). This can be appreciated by examining cells in which δ incorporation appears incomplete, as indicated by submaximal enhancement by DZ, EtOH, or β-CCE (e.g., the cells labeled b and c in Fig. 4), yet 1 μM Zn^{2+} inhibition in these cases is minimal.

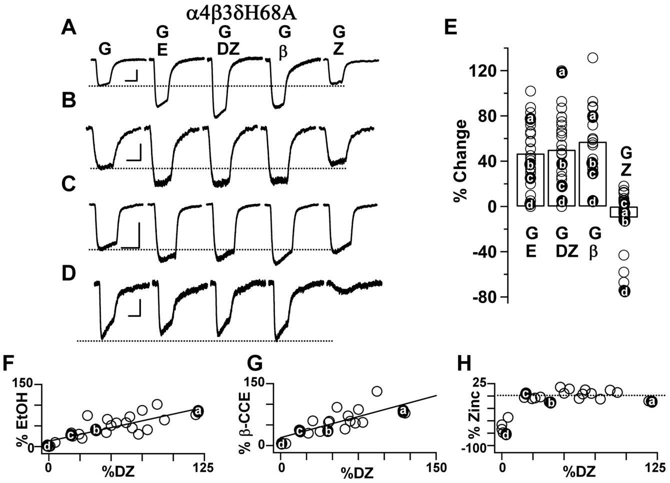

Fig. 4. Correlation analysis suggests that δ subunit incorporation is necessary for EtOH and β-CCE sensitivity. A to D show original recordings from human and rat $\alpha4\beta3\delta$H68A-transfected cells to demonstrate the variability of 300 nM GABA current (G, dotted line) modulation by 30 mM ethanol (G+E), 1 μM DZ (G+DZ), 1 μM β-CCE (G+β), and 1 μM Zn^{2+} (G+Z). A shows an example of near-maximal enhancement by 30 mM EtOH, 1 μM DZ, and 1 μM β-CCE, whereas Fig. 4D shows a $\alpha4\beta3\delta$H68A-transfected cell with no detectable enhancement by EtOH, DZ, and β-CCE, but considerable block by 1 μM Zn^{2+}, indicating lack of δ subunit expression. Recordings shown in Fig. 4, B and C, show GABA responses from individual $\alpha4\beta3\delta$H69A-transfected cells with intermediate responses to EtOH, DZ, and β-CCE but no detectable current suppression by 1 μM Zn^{2+}. E shows summary data for the enhancement of GABA responses of $\alpha4\beta3\delta$H68A-transfected HEK cells with data from the original recordings shown in A to D indicated by filled circles labeled with a, b, c, and d. Data from human and rat $\alpha4\beta3\delta$H68-transfected cells were pooled to plot the summary data shown in E to H. Mean values and S.E.M. for E are 46 ± 5% for 30 mM EtOH, 50 ± 7% for 1 μM DZ, 57 ± 7% for 1 μM β-CCE, and −10 ± 7% for 1 μM Zn^{2+}. F to H show correlation plots to compare the enhancement of 300 nM GABA currents by 1 μM DZ with enhancement by 30 mM EtOH (F), 1 μM β-CCE (G), and block by 1 μM Zn^{2+} (H) in individual recordings. The correlation coefficients (r^2) for enhancement are as follows: 30 mM ETOH versus 1 μM DZ, $r^2 = 0.63$; 1 μM β-CCE versus 1 μM DZ, $r^2 = 0.58$; and 1 μM Zn^{2+} versus 1 μM DZ, $r^2 = 0.32$. Vertical scale for A and C is 400 pA and for B and C is 100 pA; horizontal scales are 5 s.

Discussion

In this study we expressed human and rat $\alpha4\beta3\delta$ receptors in a human cell line (HEK 293T) that, despite its human embryonic kidney origin, shares many molecular markers with immature neurons (Shaw et al., 2002). We show that 300 nM GABA responses in $\alpha4\beta3\delta$-transfected cells can be enhanced by 30 mM EtOH in an alcohol antagonist/Ro15-4513-reversible manner. The alcohol concentration of 30 mM is reached in humans after high levels of alcohol consumption, and it is close to the peak blood alcohol concentration reached after 2 g/kg EtOH applied intraperitoneally in rodents, a dose at which important aspects of intoxication are reversed by the BZ alcohol antagonist Ro15-4513 in rats (Suzdak et al., 1986).

Given the high evolutionary protein sequence conservation of GABA$_A$R subunit proteins among mammals (percentage identity between rat and human proteins are 90.1, 94.8, and 97.1% for $\alpha4$, δ, and $\beta3$ subunits, respectively), the similarity of human and rat $\alpha4\beta3\delta$ receptors in pharmacological and biophysical properties is not surprising.

Although we show here that 30 mM EtOH enhancement shows considerable variability, the maximum enhancement (60–70% increase of GABA-evoked currents) of human and rat $\alpha4\beta3\delta$ GABA$_A$Rs in HEK cells by 30 mM EtOH is similar to what we have previously reported with rat $\alpha4/6\beta3\delta$ receptors expressed in oocytes (Wallner et al., 2003, 2006b; Hanchar et al., 2006). This is consistent with the notion that the unique alcohol/Ro15-4513/β-CCE pharmacology of $\alpha4\beta3\delta$ GABA$_A$Rs is, like BZ sensitivity of classic γ subunit-containing receptors, an intrinsic property of $\alpha\beta3\delta$ GABA$_A$R subtypes. This is not to say that the pharmacological properties of these receptors could not be further modulated. For example, it is possible that δ subunit incorporation is necessary, but not sufficient, for the formation of highly alcohol sensitive receptors and that further modifications of receptors triggered, for example, by phosphorylation (Choi et al., 2008), could provide an explanation for some of the variability in the data reported here. In this context, we would like to note that, like our native receptor alcohol study (Hanchar et al., 2005), our pipette solution in this study included 2 mM ATP and 0.2 mM GTP.

It is believed that most, but probably not all, native neuronal GABA$_A$ receptors have either γ, δ, or ε subunits incorporated into the receptor pentamer (Bencsits et al., 1999; Mortensen and Smart, 2006; Meera et al., 2009). It has been known since shortly after the first GABA$_A$R cDNAs were cloned that GABA$_A$Rs composed of only α and β subunits readily form functional receptors in recombinant systems. In addition, γ subunit incorporation into functional GABA$_A$R subtypes is often incomplete in recombinant expression systems, resulting in mixtures of true $\alpha\beta\gamma$ receptors and receptors formed by α and β subunits without γ subunits (Boileau et al., 2002; Baburin et al., 2008). Reconstitution of δ and ε subunit-containing GABA$_A$Rs might be even more problematic given that different groups have published contradictory results concerning alcohol or anesthetic sensitivity conferred by δ and ε subunits, respectively (Davies et al., 1997; Thompson et al., 2002; Wallner et al., 2003, 2006; Borghese et al., 2006; Yamashita et al., 2006).

Here we tested the hypothesis that difficulties in δ subunit incorporation into functional receptors, resulting in "contamination" by functional binary $\alpha\beta$ receptors, might explain divergent results. We introduced a mutation into the δ subunit that conferred DZ sensitivity to these otherwise DZ-insensitive $\alpha4\beta3\delta$ $GABA_A$Rs and thereby functionally tagged δ subunit-containing receptors. Together with $\gamma2$ subunits, $\alpha4$ and $\alpha6$ subunits render $GABA_A$Rs insensitive to benzodiazepines (Benson et al., 1998), and so the enhancement by 1 μM DZ in $\alpha4\beta3\delta$H68A $GABA_A$Rs described here was somewhat unexpected. It suggests that the arginine residue at position 100 in the $\alpha4$ subunit (a histidine in $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$) that prevents diazepam sensitivity of $\alpha4/6\beta x\gamma2$ subunits does not prevent high-affinity diazepam binding when present in context with the δH68A subunit.

We show that the amount of alcohol enhancement in $\alpha4\beta3\delta$H68A receptors is correlated with DZ and β-CCE sensitivity in individual recordings. This implies that δ subunit incorporation is incomplete and is a limiting factor for endowing recombinant $GABA_A$Rs with low concentration alcohol sensitivity. In other words, our results are consistent with the idea that variability in the amount of EtOH enhancement arises from mixtures of EtOH-insensitive $\alpha4\beta3$ and EtOH-sensitive $\alpha4\beta3\delta$ receptors, even when δ subunit cDNA/cRNA is transfected/injected in excess. It is noteworthy that our results and conclusions with the δH68A mutation are similar to those of previous work with "synaptic" γ subunit-containing $GABA_A$Rs showing that the considerable variability in BZ and GABA responses is due mainly to the contamination of γ subunit-containing receptors by BZ-insensitive and highly GABA-sensitive "binary" $\alpha\beta$ receptors, even under conditions in which nucleic acids coding for γ subunits are coinjected or cotransfected in excess in recombinant systems (Boileau et al., 2002; Baburin et al., 2008). The possibility that an excess of δ subunit expression results in unnatural subunit assembly seems unlikely because the low concentration alcohol sensitivity matches that of native δ subunit-containing $GABA_A$Rs (Wei et al., 2004; Hanchar et al., 2005; Fleming et al., 2007; Glykys et al., 2007; Liang et al., 2007; Jia et al., 2008).

Although we show that δ subunit incorporation, EtOH enhancement, and β-CCE enhancement tightly covary, we found that insensitivity to 1 μM Zn^{2+}, believed to accompany δ (as well as γ and ε) subunit incorporation into functional $GABA_A$Rs, showed only poor correlation with diazepam enhancement in $\alpha4\beta3\delta$H68A receptors. These data suggest that even a small amount of δ subunit expression, as judged by minimal enhancement by 30 mM EtOH and 1 μM β-CCE (and 1 μM DZ in $\alpha4\beta3\delta$H68A receptors), leads to an essentially complete loss of 1 μM Zn^{2+} inhibition and that loss of 1 μM Zn^{2+} sensitivity might not be a good indicator for homogeneous populations of alcohol-sensitive $\alpha4\beta3\delta$ receptors.

There are a number of possible explanations for the tight covariance of diazepam modulation and allosteric modulator actions but weak covariance between diazepam and Zn^{2+} sensitivity. One possibility is that Zn^{2+} inhibition may depend on an interchannel or "clustering" mechanism. In this scenario, Zn^{2+} inhibition could be disrupted by a small fraction of δ subunit-containing receptors in a channel cluster. Another possibility is that free δ subunits in the membrane somehow interact with functional $\alpha\beta$ receptors to render them insensitive to Zn^{2+} blockade. A third possibility is that δ incorporation might be sufficient for conferring Zn^{2+} resistance but might not by itself be sufficient for enhancement by EtOH, β-CCE, and diazepam. For example, there could be posttranslational modifications on $\alpha4\beta3\delta$ receptors that confer sensitivity to modulators such as alcohol and diazepam. Finally, although it is considered likely that receptors are formed in a 2α, 2β, and 1δ stoichiometry, with a δ subunit replacing the γ subunit in a functional pentamer (Olsen and Sieghart, 2008), there is evidence that other subunit arrangements might be possible (Baur et al., 2009). Alternative subunit stoichiometries involving multiple δ subunits receptors could explain the discrepant Zn^{2+} block and allosteric modulator enhancement if a single δ subunit abolished zinc block, but multiple δ subunits are required for alcohol sensitivity.

Incorporation of γ subunits is associated with low GABA sensitivity (Baburin et al., 2008) and incorporation of δ subunits is associated with high GABA sensitivity, leading to the notion that δ subunit incorporation into functional receptors increases the GABA sensitivity of $\alpha4\beta3$ receptors (Brown et al., 2002; Wallner et al., 2003). Our data suggest that incorporation of $\gamma2$ subunits is responsible for most of this difference, because $\alpha4\beta3\delta$ and $\alpha4\beta3$ receptors are similar in terms of their GABA sensitivity (Fig. 1).

The variable δ subunit incorporation that we describe here has relevance to the controversy surrounding EtOH actions on recombinant $GABA_A$Rs. One premise of the study challenging the EtOH sensitivity of δ subunit-containing $GABA_A$Rs (Borghese et al., 2006) is that a homogeneous pool of δ subunit-containing receptors was being studied. Considering that $\alpha4\beta3$ and $\alpha4\beta3\delta$ receptors have similar GABA sensitivity (see Fig. 1), and that Zn^{2+} inhibition does not correlate tightly with δ subunit incorporation or EtOH modulation, this premise is in question. It is also worth noting that $\alpha4\beta3$ and $\alpha4\beta3\delta$ receptors cannot be easily distinguished on the basis of responsiveness to GABA-active anesthetics etomidate, propofol, and the neurosteroid tetrahydrodeoxycorticosterone (Meera et al., 2009). Further studies may make use of the functional tagging strategy described here to determine the relative fractions of $\alpha\beta\delta$ and $\alpha\beta$ receptors in mixed populations.

Future work may also identify proteins and mechanisms that in native neurons ensure the assembly of mature homogenous γ, δ, and ε subunit-containing receptor populations. Finding conditions and accessory proteins that help in the formation of homogeneous receptor populations in recombinant systems that resemble native receptor subtypes in their pharmacological properties may lead to dramatically reduced variability in pharmacological and biophysical properties. Reliable expression of defined $GABA_A$ receptor subtypes is essential for understanding the contribution that distinct $GABA_A$R subtypes make to neuronal signaling, to studying the actions of pharmacological modulators, and for revealing detailed molecular mechanisms.

In summary, we confirm here that human $\alpha4\beta3\delta$ $GABA_A$Rs expressed in a human cell line are sensitive to alcohol and to Ro15-4513. A functional tagging strategy shows that despite a 5-fold excess of δ subunits in transfection, sensitivity to the allosteric modulators varies, which is consistent with the notion that δ subunit incorporation varies in $\alpha4\beta3\delta$ subunit-transfected cells. In addition, the essentially identical results seen with rat and human receptor subunits support the notion that these receptors could make important contributions to behavioral actions of alcohol in humans. However, the alcohol pharmacology of δ subunit knockout mice remains somewhat ambiguous. Compared with controls, δ-deficient mice show reduced alcohol consumption, attenuated withdrawal from long-term ethanol exposure, and reduced seizure-protective alcohol effects. However, they show unaltered anxiolytic and hypothermic ethanol responses and develop both long- and short-term alcohol tolerance (Mihalek et al., 2001). Furthermore, to our knowledge, there are no reports showing that Ro15-4513 is an alcohol antagonist in humans; therefore, it will be important in the future to determine whether these receptors mediate important aspects of alcohol actions in humans.

Acknowledgments

We thank Dr. Jake Hanchar (Carnegie Mellon University, Pittsburgh, PA) for sharing unpublished data on the δH68A mutation.

References

Baburin I, Khom S, Timin E, Hohaus A, Sieghart W, and Hering S (2008) Estimating the efficiency of benzodiazepines on GABA$_A$ receptors comprising $\gamma1$ or $\gamma2$ subunits. *Br J Pharmacol* **155:**424–433.

Baur R, Kaur KH, and Sigel E (2009) Structure of $\alpha6\beta3\delta$ GABA(A) receptors and their lack of ethanol sensitivity. *J Neurochem* **111:**1172–1181.

Bencsits E, Ebert V, Tretter V, and Sieghart W (1999) A significant part of native aminobutyric acid$_A$ receptors containing $\alpha4$ subunits do not contain γ or δ subunits. *J Biol Chem* **274:**19613–19616.

Benson JA, Löw K, Keist R, Mohler H, and Rudolph U (1998) Pharmacology of recombinant aminobutyric acid$_A$ receptors rendered diazepam-insensitive by point-mutated α-subunits. *FEBS Lett* **431:**400–404.

Boileau AJ, Baur R, Sharkey LM, Sigel E, and Czajkowski C (2002) The relative amount of cRNA coding for $\gamma2$ subunits affects stimulation by benzodiazepines in GABA$_A$ receptors expressed in *Xenopus* oocytes. *Neuropharmacology* **43:**695–700.

Borghese CM, Stórustovu S, Ebert B, Herd MB, Belelli D, Lambert JJ, Marshall G, Wafford KA, and Harris RA (2006) The delta subunit of aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. *J Pharmacol Exp Ther* **316:**1360–1368.

Brown N, Kerby J, Bonnert TP, Whiting PJ, and Wafford KA (2002) Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ GABA$_A$ receptors. *Br J Pharmacol* **136:**965–974.

Choi DS, Wei W, Deitchman JK, Kharazia VN, Lesscher HM, McMahon T, Wang D, Qi ZH, Sieghart W, Zhang C, et al. (2008) Protein kinase Cdelta regulates ethanol intoxication and enhancement of GABA-stimulated tonic current. *J Neurosci* **28:**11890–11899.

Davies PA, Hanna MC, Hales TG, and Kirkness EF (1997) Insensitivity to anaesthetic agents conferred by a class of GABA$_A$ receptor subunit. *Nature* **385:**820–823.

Farrant M and Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA$_A$ receptors. *Nat Rev Neurosci* **6:**215–229.

Fleming RL, Wilson WA, and Swartzwelder HS (2007) Magnitude and ethanol sensitivity of tonic GABA$_A$ receptor-mediated inhibition in dentate gyrus changes from adolescence to adulthood. *J Neurophysiol* **97:**3806–3811.

Glykys J, Peng Z, Chandra D, Homanics GE, Houser CR, and Mody I (2007) A new naturally occurring GABA$_A$ receptor subunit partnership with high sensitivity to ethanol. *Nat Neurosci* **10:**40–48.

Hanchar HJ, Chutsrinopkun P, Meera P, Supavilai P, Sieghart W, Wallner M, and Olsen RW (2006) Ethanol potently and competitively inhibits binding of the alcohol antagonist Ro15-4513 to $\alpha4/6\beta3\delta$ GABA$_A$ receptors. *Proc Natl Acad Sci USA* **103:**8546–8550.

Hanchar HJ, Dodson PD, Olsen RW, Otis TS, and Wallner M (2005) Alcohol-induced motor impairment caused by increased extrasynaptic GABA$_A$ receptor activity. *Nat Neurosci* **8:**339–345.

Hanchar HJ, Wallner M, and Olsen RW (2004) Alcohol effects on γ-aminobutyric acid type A receptors: are extrasynaptic receptors the answer? *Life Sci* **76:**1–8.

Jia F, Chandra D, Homanics GE, and Harrison NL (2008) Ethanol modulates synaptic and extrasynaptic GABA$_A$ receptors in the thalamus. *J Pharmacol Exp Ther* **326:**475–482.

Khiabani HZ, Opdal MS, and Mørland J (2008) Blood alcohol concentrations in apprehended drivers of cars and boats suspected to be impaired by the police. *Traffic Inj Prev* **9:**31–36.

Kucken AM, Teissére JA, Seffinga-Clark J, Wagner DA, and Czajkowski C (2003) Structural requirements for imidazobenzodiazepine binding to GABA$_A$ receptors. *Mol Pharmacol* **63:**289–296.

Liang J, Suryanarayanan A, Abriam A, Snyder B, Olsen RW, and Spigelman I (2007) Mechanisms of reversible GABA$_A$ receptor plasticity after ethanol intoxication. *J Neurosci* **27:**12367–12377.

Liljequist S and Engel J (1982) Effects of GABAergic agonists and antagonists on various ethanol-induced behavioral changes. *Psychopharmacology* (*Berl*) **78:**71–75.

Lovinger DM and Homanics GE (2007) Tonic for what ails us? High-affinity GABA$_A$ receptors and alcohol. *Alcohol* **41:**139–143.

Meera P, Olsen RW, Otis TS, and Wallner M (2009) Etomidate, propofol and the neurosteroid THDOC increase the GABA efficacy of recombinant $\alpha4\beta3\delta$ and $\alpha4\beta3$ GABA$_A$ receptors expressed in HEK cells. *Neuropharmacology* **56:**155–160.

Meera P, Wallner M, Song M, and Toro L (1997) Large conductance voltage- and calcium-dependent K$^+$ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0–S6), an extracellular N terminus, and an intracellular (S9–S10) C terminus. *Proc Natl Acad Sci USA* **94:**14066–14071.

Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL, and Homanics GE (2001) GABA$_A$-receptor δ subunit knockout mice have multiple defects in behavioral responses to ethanol. *Alcohol Clin Exp Res* **25:**1708–1718.

Mortensen M and Smart TG (2006) Extrasynaptic $\alpha\beta$ subunit GABA$_A$ receptors on rat hippocampal pyramidal neurons. *J Physiol* **577:**841–856.

Olsen RW and Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of γ-aminobutyric acid$_A$ receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev* **60:**243–260.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, and Seeburg PH (1989) Importance of a novel GABA$_A$ receptor subunit for benzodiazepine pharmacology. *Nature* **338:**582–585.

Santhakumar V, Wallner M, and Otis TS (2007) Ethanol acts directly on extrasynaptic subtypes of GABA$_A$ receptors to increase tonic inhibition. *Alcohol* **41:**211–221.

Shaw G, Morse S, Ararat M, and Graham FL (2002) Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *Faseb J* **16:**869–871.

Smart TG, Moss SJ, Xie X, and Huganir RL (1991) GABA$_A$ receptors are differentially sensitive to zinc: dependence on subunit composition. *Br J Pharmacol* **103:**1837–1839.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, and Smith SS (2002) Hormonally regulated $\alpha_4\beta_2\delta$ GABA$_A$ receptors are a target for alcohol. *Nat Neurosci* **5:**721–722.

Suzdak PD, Glowa JR, Crawley JN, Schwartz RD, Skolnick P, and Paul SM (1986) A selective imidazobenzodiazepine antagonist of ethanol in the rat. *Science* **234:**1243–1247.

Thompson SA, Bonnert TP, Cagetti E, Whiting PJ, and Wafford KA (2002) Overexpression of the GABA$_A$ receptor ε subunit results in insensitivity to anaesthetics. *Neuropharmacology* **43:**662–668.

Uusi-Oukari M and Korpi ER (1989) Cerebellar GABA$_A$ receptor binding and function in vitro in two rat lines developed for high and low alcohol sensitivity. *Neurochem Res* **14:**733–739.

Wallner M, Hanchar HJ, and Olsen RW (2003) Ethanol enhances $\alpha4\beta3\delta$ and $\alpha6\beta3\delta$ aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci USA* **100:**15218–15223.

Wallner M, Hanchar HJ, and Olsen RW (2006a) Low dose acute alcohol effects on GABA$_A$ receptor subtypes. *Pharmacol Ther* **112:**513–528.

Wallner M, Hanchar HJ, and Olsen RW (2006b) Low dose alcohol actions on $\alpha4\beta3\delta$ GABA$_A$ receptors are reversed by the behavioral alcohol antagonist Ro15-4513. *Proc Natl Acad Sci USA* **103:**8540–8545.

Wallner M and Olsen RW (2008) Physiology and pharmacology of alcohol: the imidazobenzodiazepine alcohol antagonist site on subtypes of GABA$_A$ receptors as an opportunity for drug development? *Br J Pharmacol* **154:**288–298.

Wei W, Faria LC, and Mody I (2004) Low ethanol concentrations selectively augment the tonic inhibition mediated by δ subunit-containing GABA$_A$ receptors in hippocampal neurons. *J Neurosci* **24:**8379–8382.

Yamashita M, Marszalec W, Yeh JZ, and Narahashi T (2006) Effects of ethanol on tonic GABA currents in cerebellar granule cells and mammalian cells recombinantly expressing GABA$_A$ receptors. *J Pharmacol Exp Ther* **319:**431–438.

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