Alcohol- and Alcohol Antagonist-Sensitive Human GABA<sub>A</sub> Receptors: Tracking δ Subunit Incorporation into Functional Receptors

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**ABSTRACT**

GABA<sub>A</sub> receptors (GABA<sub>A</sub>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<sub>A</sub>Rs is uniquely sensitive to ethanol and enhanced at concentrations relevant for human alcohol consumption. Ethanol enhancement of recombinant α4β3δ receptors is blocked by the behavioral alcohol antagonist 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-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 α4β3δ 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 δ-H68A substitution confers diazepam sensitivity to otherwise diazepam-insensitive α4β3δ receptors. The extent of enhancement of α4β3δ-H68A receptors by 1 μM diazepam, 30 mM EtOH, and 1 μM β-carboline-3-carboxy ethyl ester (but not 1 μM Zn<sup>2+</sup> block) is correlated in individual recordings, suggesting that δ subunit incorporation into recombinant GABA<sub>A</sub>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<sub>A</sub>Rs.

**Introduction**

Classic synaptic GABAergic inhibition is characterized by the pulsatile release of the neurotransmitter GABA onto a molecularly distinct subset of GABA<sub>A</sub>Rs that contain γ2 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<sub>A</sub>R subtypes that exhibit high affinity for GABA and slow desensitization, with much of this tonic inhibition mediated by δ subunit-containing GABA<sub>A</sub>Rs (Farrant and Nusser, 2005).

Although GABA<sub>A</sub>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<sub>A</sub>Rs have been elusive (Wallner et al., 2006a). A possible solution for this conundrum was provided by findings that extrasynaptic δ subunit-containing GABA<sub>A</sub>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 α6 gene (α6R100Q), initially identified in rats with increased alcohol-induced motor impairment (also known as alcohol nontolerant rats) (Usui-Oukari and Korpi, 1989), further increases the EtOH sensitivity of α6β3δ receptors in vivo and in vitro (Hanchar et al., 2005). In addition, ethanol enhancement of δ subunit-containing re-
ceptors is generally believe 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_A Rs with distinct pharmacological properties (Davies et al., 1997; Wallner et al., 2003). GABA_A Rs constituted 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_A Rs, and benzodiazepine sensitivity is conferred by γ2 subunit coexpression (Pritchett et al., 1989). It has been shown that the formation of “binary” αβ receptors leads to pharmacologically heterogeneous receptor populations when “synaptic,” γ subunit-containing GABA_A Rs are expressed in recombinant systems (Boileau et al., 2002; Baburin et al., 2008). To mitigate such problems with heterogeneous populations of αβ and αβγ GABA_A Rs 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 γ2 subunit, transfection of HEK 239 T cells with human and rat α4, β3, and the δ subunit results in the formation of heterogeneous, pharmacologically distinct populations of functional α4β3 and α4β3δ receptors. We show here that δ subunit coexpression led to functional rat and human GABA_A Rs 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 α4β3δH68A 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 α4β3δ 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_A Rs.

Materials and Methods

Diazepam and β-CCE were gifts from Hoffman-La Roche (Nutley, NJ) and Ferrostan (Soeborg, Denmark), respectively. Most other standard chemicals, including EtOH, were obtained from Sigma (St. Louis, MO). Human α4, β3, and ε 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_A R 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 α4 and β3 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 α4, 4 μg of β3, and 20 μg of δ or δH68A (δ cDNA omitted for αβ 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^+, 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 on-set 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]_H^{1/H}))), 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 $n_{50}$ is the Hill coefficient. $I$ is the peak current evoked by a given concentration of GABA. $I_{\text{max}}$ 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 $\delta$ subunit-containing GABA$_A$Rs are EtOH- and Ro15-4513-sensitive (Lovingier and Homianics, 2007), we decided to test human and rat $\alpha_4$, $\beta_3$, and $\delta$ 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 $\alpha_4$, $\beta_3$, and $\delta$ 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 $\alpha_4\beta_3$ and $\alpha_4\beta_3\delta$ Receptors in HEK Cells.** Figure 1 compares GABA dose-response curves of HEK cells transfected with human $\alpha_4$ and $\beta_3$ subunit alone with cells cotransfected with $\alpha_4$ and $\beta_3$ subunits and a 5-fold excess of $\delta$ subunit cDNA. GABA responses were evoked by perfusion of GABA from a threshold concentration of 100 nM to a saturating concentration of 30 $\mu$M. In both $\alpha_4\beta_3$- and $\alpha_4\beta_3\delta$-transfected cells, currents are substantially activated by a GABA concentration of 300 nM (300 nM GABA is $\sim EC_{150}$ for $\alpha_4\beta_3$-transfected cells and $\sim EC_{50}$ for $\alpha_4\beta_3\delta$-transfected cells). Responses from human and rat $\alpha_4\beta_3\delta$-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 $\delta$ subunit coexpression leads to a small yet statistically significant ($p = 0.008$) increase in GABA sensitivity of $\alpha_4\beta_3\delta$ receptors (EC$_{50} = 0.53 \pm 0.04$ $\mu$M, $n = 7$) compared with binary $\alpha_4\beta_3$ GABA$_A$Rs (EC$_{50} = 1.3 \pm 0.1$ $\mu$M, $n = 8$).

**Recombinant $\alpha_4\beta_3\delta$ 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 $\mu$M, 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 $\mu$M).

GABA currents in human and rat $\alpha_4\beta_3$- and $\alpha_4\beta_3\delta$-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-

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**Fig. 2.** Alcohol and alcohol antagonist-related pharmacology of human and rat $\alpha_4\beta_3\delta$ GABA$_A$Rs. A to C show current traces recorded from three individual HEK cells transfected with human $\alpha_4\beta_3$ (A), rat $\alpha_4\beta_3\delta$ (B), and human $\alpha_4\beta_3$ (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 + 1 $\mu$M flumazenil (G+E+R), 4) 30 mM EtOH + 300 nM Ro15-4513 + 1 $\mu$M flumazenil + 1 $\mu$M Zn$^{2+}$ (G+E+R+F), 5) 1 $\mu$M $\beta$-CCE (G+β), and 6) 1 $\mu$M 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 $\alpha_4\beta_3\delta$-transfected cells that showed no enhancement by 30 mM EtOH and/or 1 $\mu$M $\beta$-CCE were, when tested, inhibited by 1 $\mu$M Zn$^{2+}$, indicative of $\alpha_4\beta_3\delta$ expression (E) and lack of $\delta$ 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 $\alpha_4\beta_3\delta$ (n = 8) and 43 ± 23% for human $\alpha_4\beta_3\delta$ (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 $\alpha_4\beta_3\delta$-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 $\alpha_4\beta_3\delta$-transfected cells, the modulation was as follows: 30 mM EtOH, 1 ± 3%; (n = 5); 1 $\mu$M $\beta$-CCE, 0.1 ± 5% (n = 8); 1 $\mu$M Zn$^{2+}$, −70 ± 6% (n = 8). Vertical scale is 100 pA for A and B and 200 pA for C; horizontal time scale is 5 s for all panels.
The δ Subunit Is Necessary for High Alcohol Sensitivity

dilication of 30 mM EtOH together with 300 nM Ro15-4513 (G+EtOH). In four recordings, we also tested for Ro15-4513 alcohol antagonism reversal by 1 μM flumazenil (G+EtOH+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 Zn²⁺ (G+Z⁺).

Mean enhancement of 300 nM GABA responses by 30 mM ethanol showed no statistically significant differences in human and rat α4β3δ-transected 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 α4β3δ-transected cells showed considerable variability, as illustrated in the scatter plot, in which each point represents the percentage change in an individual α4β3δ-transected 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 α4β3δ-transected cells were enhanced by 1 μM β-CCE (p < 0.01, G versus G+B, n = 7) but not blocked by 1 μM Zn²⁺ (p = 0.4, G versus G+Z, n = 6). In contrast, recordings from cells transfected with α4 and β3 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 Zn²⁺ (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 α4β3δ and α4β3δ receptors. On the other hand, the lack of inhibition by 1 μM Zn²⁺ implies that there was very little “contamination” by αβ 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 RRs. 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 α+γ/γ- subunit interfaces (Kucken et al., 2003). Our attention was drawn to a histidine residue in the δ subunit (H68) 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 α4 and β3 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 α4β3δ receptors are sensitive to 30 mM EtOH but insensitive to 1 μM DZ, whereas α4β3δH68A 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 α4β3δ wild-type receptors. The δH68A mutation did not lead to significant changes in GABA sensitivity (data not shown) or to differences in Zn²⁺ blockade between recombinant α4β3δ and α4β3δH68A receptors expressed in oocytes (Table 1).

Diazepam Sensitivity of α4β3δH68A-Transfected Cells Is Correlated with EtOH and β-CCE Enhancement but Shows Only Poor Correlation with 1 μM Zn²⁺ 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 α4β3δH68A-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 α4β3δH68A-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 Zn²⁺. Figure 4, A to D, shows recordings from individual cells with large (Fig. 4A), inter-
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 α4β3δH68A-transfected cells showed little EtOH/DZ/β-CCE enhancement but significant block by 1 μM Zn2+ (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 Zn2+ (H). Correlation coefficients (r2) 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, Zn2+ 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 Zn2+ inhibition in these cases is minimal.

Discussion

In this study we expressed human and rat α4β3δ 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 α4β3δ-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 intraintraperitoneally 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 GABAR subunit proteins among mammals (percentage identity between rat and human proteins are 90.1, 94.8, and 97.1% for α4, δ, and β3 subunits, respectively), the similarity of human and rat α4β3δ 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 α4β3δ GABARs in HEK cells by 30 mM EtOH is similar to what we have previously reported with rat α4β6δ3 receptors expressed in oocytes (Wallner et al., 2003, 2006b; Hancock et al., 2006). This is consistent with the notion that the unique alcohol/Ro15-4513/β-CCE pharmacology of α4β3δ GABARs is, like BZ sensitivity of classic γ subunit-containing receptors, an intrinsic property of α4β3δ GABAR 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 (Hancock 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 GABAR receptors have either γ, δ, or ε subunits incorporated into the receptor pentamer (Benes et al., 1999; Mortensen and Smart, 2006; Meera et al., 2009). It has been known since shortly after the first GABAR cDNAs were cloned that GABARs composed of only α and β subunits readily form functional receptors in recombinant systems. In addition, γ subunit incorporation into functional GABAR subtypes is often incomplete in recombinant expression systems, resulting in mixtures of pure αβγ 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 GABARs 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; Yamasita et al., 2006).

Here we tested the hypothesis that difficulties in δ subunit incorporation into functional receptors, resulting in "contam-
inatation” by functional binary αβ receptors, might explain divergent results. We introduced a mutation into the δ subunit that conferred DZ sensitivity to these otherwise DZ-insensitive α4β3δ GABA_ARs and thereby functionally tagged δ subunit-containing receptors. Together with γ2 subunits, α4 and α6 subunits render GABA_ARs insensitive to benzodiazepines (Benson et al., 1998), and so the enhancement by 1 μM DZ in α4β3δH68A GABA_ARs described here was somewhat unexpected. It suggests that the arginine residue at position 100 in the α4 subunit (a histidine in α1, α2, α3 and α5) that prevents diazepam sensitivity of α4/6βxy2 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 α4β3δH68A 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 endorsing recombinant GABA_ARs 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 α4β3 and EtOH-sensitive α4β3δ 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_ARs 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” αβ receptors, even under conditions in which nucleic acids coding for γ subunits are cojected 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_ARs (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_ARs, showed only poor correlation with diazepam enhancement in α4β3δH68A 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 α4β3δH68A 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 α4β3δ 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 αβ 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 α4β3δ receptors that confer sensitivity to modulators such as alcohol and diazepam.

Finally, although it is considered likely that receptors are formed in a 2α, 2γ, and 1 ε 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 α4β3 receptors (Brown et al., 2002; Wallner et al., 2003). Our data suggest that incorporation of γ2 subunits is responsible for most of this difference, because α4β3δ and α4β3 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_ARs. One premise of the study challenging the EtOH sensitivity of δ subunit-containing GABA_ARs (Borghese et al., 2006) is that a homogeneous pool of δ subunit-containing receptors was being studied. Considering that α4β3 and α4β3δ 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 α4β3 and α4β3δ receptors cannot be easily distinguished on the basis of responsiveness to GABA-active anesthetics etomidate, propofol, and the neurosteroid tetrahydroxydortocosterone (Meera et al., 2009). Further studies may make use of the functional tagging strategy described here to determine the relative fractions of αβδ and αβ 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_AR receptor subtypes is essential for understanding the contribution that distinct GABA_AR 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 α4β3δ GABA_ARs 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 α4β3δ 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.

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