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Alcohol- and alcohol antagonist-sensitive human $GABA_A$ receptors: tracking δ subunit incorporation into functional receptors

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The δ subunit is necessary for high alcohol sensitivity

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List of non-standard abbreviations: BAC, blood alcohol concentration; β-CCE, β-carboline-3-carboxy ethyl ester; BZ, benzodiazepine; DZ, diazepam; EtOH, ethanol; $GABA_AR$, $GABA_A$ receptors; HEK cells, human embryonic kidney cells

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Abstract

$GABA_A$ receptors ($GABA_ARs$) have long been a focus as targets for alcohol actions. Recent work suggests that tonic GABAergic inhibition mediated by extrasynaptic δ subunit-containing $GABA_ARs$ 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 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 use 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 α4β3δ receptors. The extent of enhancement of α4β3δH68A receptors by 1 μM diazepam, 30 mM EtOH and 1 μM β-CCE (but not 1 μM Zn^{2+} block) is correlated in individual recordings, suggesting that δ subunit incorporation into recombinant $GABA_ARs$ 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 necessary for high ethanol sensitivity, one of the features of native δ subunit-containing $GABA_ARs$.

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Introduction

Classical synaptic GABAergic inhibition is characterized by the pulsatile release of the neurotransmitter GABA onto a molecularly distinct subset of $GABA_A$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 $GABA_A$R subtypes which 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 classical 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 (Hanchar et al., 2004; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). 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 (a.k.a. alcohol non-tolerant (ANT) rats) (Uusi-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 receptors is competitively antagonized by the rat behavioral alcohol antagonist Ro15-4513 (Hanchar et al., 2006; Wallner et al., 2006b). These results provided evidence that EtOH/Ro15-4513-sensitive $GABA_A$R 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). Importantly, the extrasynaptic $GABA_A$R hypothesis has been extensively corroborated by

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recordings from δ subunit-containing native neurons (Fleming et al., 2007; Glykys et al., 2007; Hanchar et al., 2005; Jia et al., 2008; Liang et al., 2007; Santhakumar et al., 2007; Wei et al., 2004). Yet despite the abundant evidence that native extrasynaptic $GABA_A$Rs are ethanol sensitive, the hypothesis has been challenged by groups unable to replicate the high ethanol sensitivity of δ subunit-containing $GABA_A$Rs reconstituted using recombinant receptor cDNAs in Xenopus oocytes (Baur et al., 2009; Borghese et al., 2006) or in mammalian cells (Yamashita et al., 2006).

Functional $GABA_A$Rs are pentamers, with prototypical synaptic $GABA_A$Rs in vertebrate brains generally thought 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 distinctive pharmacological properties (Davies et al., 1997; Wallner et al., 2003). $GABA_A$Rs 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 $GABA_A$Rs, and benzodiazepine sensitivity is conferred by γ2 subunit co-expression (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 (Baburin et al., 2008; Boileau et al., 2002). 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 test 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

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the formation of heterogeneous, pharmacologically distinct populations of functional α4β3 and α4β3δ receptors. We show here that δ subunit co-expression leads to functional rat and human $GABA_A$Rs which are enhanced by 30 mM EtOH, and that this EtOH enhancement is blocked by the behavioral BZ alcohol antagonist Ro15-4513. However, we find substantial variability in the amount of EtOH enhancement among individual recordings, with 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 exploit a "functional tag", a mutation in the δ subunit (δH68A) that confers diazepam sensitivity to α4β3δH68A receptors with no changes in EtOH or GABA sensitivity.

Using the δH68A mutation we find 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 co-vary in individual recordings. This is consistent with our hypothesis that incomplete δ subunit incorporation causes variability in EtOH responses in recombinant systems. Interestingly, we find that the extent of inhibition by 1 μM Zn^{2+} is 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, while 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 Hoffmann La-Roche and Ferrosan. Most other standard chemicals, including EtOH, were obtained from Sigma. Human α4, β3, and δ cDNAs

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were either cloned by RT-PCR using human total brain mRNA (Invitrogen) as previously described (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 (CMV) promoter as well as a T7 RNA polymerase promoter. Oocyte expression methods and the rat clones used are as previously described (Wallner et al., 2003). HEK-293 T cells (ATCC) were transfected using a dextran-transfection method as described (Meera et al., 1997). Co-transfections 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 GFP epifluorescence. Total amounts of plasmid DNA were 4 μg α4, 4 μg β3, 20 μg δ or δH68A (δ cDNA omitted for αβ receptors) together with 0.4 μg of eGFP-plasmid DNA for each 10 cm diameter plate. Whole cell electrophysiological recordings were performed between 70 and 150 hours post transfection. Recordings were made from individual cells plated on poly-lysine coated cover slips at room temperature. Voltage was clamped using an Axopatch 200B amplifier (Molecular Devices, USA) at a holding potential of –60 mV. The external solution was (in mM): 142 NaCl, 1 $CaCl_2$, 6 $MgCl_2$, 8 KCl, 10 glucose, and 10 HEPES, pH 7.4 (327–330 mOsm). The pipette internal solution (in mM): 140 CsCl, 4 NaCl, 0.5 $CaCl_2$, 10 HEPES, 5 EGTA, 2 Mg^{2+} ATP, and 0.2 GTP. Drug solutions were applied using a multi-barrel pipette driven by a stepper motor (SF77B, Warner Instruments) 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, USA). The normalized concentration-response data were least-square fitted (with the "Solver" function in Microsoft EXCEL) using the Hill equation: $I/I_{max}= (1/(1 + (EC_{50}/[A])^n)$ where EC_{50} represents

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the concentration of the agonist ([*A*]) inducing 50% of the maximal current evoked by a saturating concentration of the agonist and *n* 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, USA).

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 co-expressed in a mammalian cell line (HEK 293T) for EtOH sensitivity and unique alcohol-related pharmacology (Hanchar et al., 2005; Suzdak et al., 1986; Wallner et al., 2003; Wallner et al., 2006b). Human α4, β3, and δ cDNAs were sub-cloned into the same vectors that we previously used to express rat subunits in HEK 293T cells (Hanchar et al., 2006) and 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 co-transfected with α4 and β3 subunits and a five-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 GABA. 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 and ~EC_{30} for α4β3δ transfected cells). Responses from human and rat α4β3δ-transfected cells did not appear to be different in their GABA sensitivity (p

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= 0.98; see Fig. 1C) or in their sensitivity to other modulators (discussed below). On this basis and considering the fact that rat and human sequences show 90-97% 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 co-expression leads to a small, yet statistically significant ($p = 0.008$), increase in GABA sensitivity of α4β3δ receptors (EC_{50} = 0.53 ± 0.04 μM, n = 7) when compared to binary α4β3 $GABA_A$Rs (EC_{50} = 1.3 ± 0.1 μM, n = 8).

Recombinant α4β3δ $GABA_A$Rs are enhanced by 30 mM EtOH, an effect blocked by 300 nM of the behavioral alcohol antagonist Ro15-4513

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 (BAC) reported for authority-apprehended intoxicated suspects (Khiabani et al., 2008) and less than twice the legal driving limit for adult drivers in the US (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 application of 30 mM EtOH together with 300 nM of the alcohol antagonist Ro15-4513 (G+E+R). In four recordings, we also tested for Ro15-1513's alcohol antagonism reversal by 1 μM flumazenil (G+E+R+F); data points from individual experiments where Ro15-4513's alcohol antagonism was tested for reversal by 1 μM flumazenil are connected by lines in figure 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 did not show statistically significant differences in human and rat α4β3δ-transfected cells ($p = 0.95$; human = 33 ± 9 %, n = 6, rat = 34 ± 5 %, n = 8, mean ± S.D.), and therefore data were pooled in summary figure 2D. Data obtained from individual α4β3δ-transfected cells showed considerable

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variability as illustrated in the scatter plot format in which each point represents the percent change in an individual α4β3δ-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 α4β3δ 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 α4 and β3 subunits alone, exhibited GABA responses that were neither enhanced by 30 mM EtOH (p = 0.45 versus G+E, n = 5) nor by 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.2A and Fig. 2B), an observation consistent with the notion that individual HEK cells have variable fractions of α4β3 and α4β3δ receptors. On the other hand, the lack of inhibition by 1 μM Zn^{2+} 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 $GABA_A$Rs

In order to find differences that might be responsible for lack of effects of classical BZs (like 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- subunit

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interfaces (Kucken et al., 2003). Our attention was drawn to a histidine residue in the δ subunit (δH68), which 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, co-transfected 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 figures 3B and 3C show that wild type α4β3δ receptors are sensitive to 30 mM EtOH but insensitive to 1 μM DZ, while 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 (not shown), nor did this mutation lead to differences in Zn^{2+} blockade between recombinant α4β3δ and α4β3δH68A receptors expressed in oocytes (see table 1).

Diazepam sensitivity of α4β3δH68A-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 α4β3δH68A transfected cells the extent of enhancement of 300 nM GABA currents by 1 μM DZ should co-vary with the effects of pharmacological agents selective for δ subunit-containing receptors.

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Whole cell recordings were made from human and rat α4β3δH68A-transfected cells and currents evoked by 300 nM GABA alone were compared to 300 nM GABA responses in the presence of modulators: 30 mM EtOH, 1 μM DZ, 1 μM β-CCE, or 1 μM Zn^{2+}. Figures 4A-D show recordings from individual cells with large (Fig. 4A), intermediate (Fig. 4B and C), and no detectable 300 nM GABA current modulation by EtOH, DZ, or β-CCE (Fig. 4D). 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 Zn^{2+} (Fig. 4D). Data points obtained from individual HEK cell experiments shown in Figures 4A-D are indicated with filled symbols (marked with a to d in Figs. 4E-H).

Figure 4F-H show correlation plots with 300 nM GABA current enhancement (in %) by 1 μM diazepam plotted against enhancement by 30 mM EtOH (F), 1 μM β-CCE (G), and percent 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 sub-maximal enhancement by DZ, EtOH or β-CCE (e.g., the cell labeled b and c in Fig. 4) yet 1 μM Zn^{2+} 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

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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 $GABA_AR$ subunit proteins among mammals (percent identity between rat and human proteins are 90.1 %, 94.8%, 97.1% for α4, δ, and β3 subunits, respectively), the similarities of human and rat α4β3δ receptors in pharmacological and biophysical properties is not surprising.

While 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 $GABA_ARs$ in HEK cells by 30 mM EtOH is similar to what we have previously reported with rat α4/6β3δ receptors expressed in oocytes (Hanchar et al., 2006; Wallner et al., 2003; Wallner et al., 2006b). This is consistent with the notion that the unique alcohol/Ro15-4513/β-CCE pharmacology of α4β3δ $GABA_ARs$ is, like BZ sensitivity of classical γ subunit-containing receptors, an intrinsic property of αβ3δ $GABA_AR$ 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 by, for example 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 similar to 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 thought that most, but likely not all, native neuronal $GABA_A$ receptors have either γ, δ or ε subunits incorporated into the receptor pentamer (Bencsits et al., 1999; Meera et al., 2009; Mortensen and Smart, 2006). It has been known since shortly after the first $GABA_AR$ cDNAs were cloned that $GABA_ARs$ composed of only α and β subunits readily form functional

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receptors in recombinant systems. Also, γ subunit incorporation into functional $GABA_AR$ subtypes is often incomplete in recombinant expression systems resulting in mixtures of true αβγ receptors and receptors formed by α and β subunits without γ subunits (Baburin et al., 2008; Boileau et al., 2002). Reconstitution of δ and ε subunit-containing $GABA_ARs$ might be even more problematic as different labs have published contradictory results concerning alcohol or anesthetic sensitivity conferred by δ and ε subunits respectively (Borghese et al., 2006; Davies et al., 1997; Thompson et al., 2002; Wallner et al., 2003; Wallner et al., 2006b; Yamashita et al., 2006).

Here we tested the hypothesis that difficulties in δ subunit incorporation into functional receptors resulting in "contamination" by functional binary αβ receptors might explain divergent results. We introduced a mutation into the δ subunit that confers DZ sensitivity to these otherwise DZ-insensitive α4β3δ $GABA_ARs$ and thereby functionally tags 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 is somewhat unexpected. It suggests that the arginine residue at position 100 in the α4 subunit (is a histidine in α1, α2, α3 and α5) that prevents diazepam sensitivity of α4/6βxγ2 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 endowing 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.

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Notably, our results and conclusions with the δH68A mutation are similar to previous work with "synaptic" γ subunit-containing $GABA_A$Rs showing that the considerable variability in BZ and GABA responses is mainly due to the contamination of γ subunit-containing receptors by BZ-insensitive and highly GABA-sensitive "binary" αβ receptors, even under conditions when nucleic acids coding for γ subunits are co-injected or co-transfected in excess in recombinant systems (Baburin et al., 2008; Boileau et al., 2002). The possibility that an excess of δ subunits expression results in unnatural subunit assembly seems unlikely as the low concentration alcohol sensitivity matches that of native δ subunit-containing $GABA_A$Rs (Fleming et al., 2007; Glykys et al., 2007; Hanchar et al., 2005; Jia et al., 2008; Liang et al., 2007; Wei et al., 2004).

While we show that δ subunit incorporation, EtOH enhancement, and β-CCE enhancement tightly co-vary, we found that insensitivity to 1 μM Zn^{2+}, thought to accompany δ (as well as γ and ε) subunit incorporation into functional $GABA_A$Rs, shows 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, 1 μM β-CCE (and 1 μM DZ in α4β3δH68A receptors), leads to an essentially complete loss of 1 μM Zn^{2+} inhibition and suggest 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 possibilities that could explain the tight covariance of diazepam modulation and allosteric modulator actions but a weak covariance between diazepam and Zn^{2+} sensitivity. One possibility is that Zn^{2+} inhibition may depend on an inter-channel 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+} block,

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but may not by itself be sufficient for enhancement by EtOH, β-CCE, and diazepam. For example, there could be posttranslational modifications, only possible on α4β3δ receptors that confer sensitivity to modulators like alcohol and diazepam. Finally, while 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 abolishes 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 with high GABA sensitivity, leading to the notion that δ subunit incorporation into functional receptors increases 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 as 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 $GABA_A$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 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 discriminated on the basis of responsiveness to GABA-active anesthetics etomidate, propofol, and the neurosteroid THDOC (Meera et al., 2009). Further studies may make use of the functional

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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 will hopefully 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 $GABA_AR$ subtypes make to neuronal signaling, to study 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 knock-out mice remains somewhat ambiguous. Compared to controls, δ-deficient mice show reduced alcohol consumption, attenuated withdrawal from chronic ethanol exposure, and reduced seizure-protective alcohol effects. However, they show unaltered anxiolytic and hypothermic ethanol responses and develop both chronic and acute alcohol tolerance (Mihalek et al., 2001). Furthermore, to our knowledge there are no reports showing that Ro15-4513 is an alcohol antagonist in humans and therefore it will be important in the future to determine if these receptors mediate important aspects of alcohol actions in humans.

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Figure legends

Fig. 1. Co-expression of the δ subunit increases GABA sensitivity. Figures 1A and 1B 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 transfected cells are 0.54 ± 0.05 μM (n = 3) and for human α4β3 are 1.3 ± 0.1 μM (n = 8). Because rat α4β3δ receptors (EC_{50} = 0.53 ± 0.02 μM (n = 4)), are similar in their GABA sensitivity (C) to human clones (p = 0.98) 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 co-expression leads to a small, yet significant (**p < 0.01), increase in GABA sensitivity (C). Horizontal time scale for both A and B is 5 seconds and the vertical scale in A is 200 pA and 50 pA in B.

Fig. 2. Alcohol and alcohol antagonist related pharmacology of human and rat α4β3δ $GABA_A$Rs. Figures 2A 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 Figs. 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). Figure 2D and 2E 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

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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 for expression of α4β3 expression (see Fig. 2 E) and lack of δ subunit expression, and 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 (in percent ± S.D.) with rat and human clones was for rat α4β3δ (n = 8) 42 ± 21 % and human α4β3δ (n = 6) 43 ± 23 % (p = 0.98) and 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 percent ± S.D. are for 30 mM EtOH: 43 ± 21 % (n = 14); for 30 mM EtOH + 300 nM Ro15-4513, 2 ± 6 (n = 10). For α4β3 transfected cells the modulation was: with 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 for 2A and 2B is 100 pA and 200 pA for 2C, horizontal time scale is 5 seconds for all panels.

Fig. 3. The δH68A mutation makes α4β3δ receptors sensitive to allosteric diazepam enhancement. Figure 3A 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 (F77, A79 and T81, bold) contribute to the BZ binding site at the α+/γ2- subunit interface. Fig. 3B and C show in representative recordings that 30 mM EtOH-sensitive α4β3δ $GABA_A$Rs are not enhanced by 1 μM DZ (B), whereas with the δH68A mutation, α4β3δH68A receptors are enhanced similarly by both 1 μM DZ and 30 mM EtOH. Summary data are shown in Fig. 3D: mean change in α4β3δ transfected cells with 1 μM DZ is -2% ± 9%, n = 6 (Fig. 3D), whereas both rat and human α4β3δH68A are enhanced similarly by 1 μM DZ (rat

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64 ± 23% (n = 13), human 61 ± 33% (n = 7)) and therefore data were pooled in Fig. 3D. Vertical scale is 100 pA for 3B and 50 pA for 3C, whereas all horizontal scales are 5 seconds.

Fig. 4. Correlation analysis suggests that δ subunit incorporation is necessary for EtOH and β-CCE sensitivity. Figures 4A-D show original recordings from human and rat α4β3δH68A 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). Figure 4A shows an example of near maximum enhancement by 30 mM EtOH, 1 μM DZ and 1 μM β-CCE, whereas Fig. 4D shows a α4β3δH68A 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 Figs. 4B and 4C show GABA responses from individual α4β3δH69A transfected cells with intermediate responses to EtOH, DZ, β-CCE, but no detectable current suppression by 1 μM Zn^{2+}. Figure 4E shows summary data for the enhancement of GABA responses of α4β3δH68A transfected HEK cells with data from the original recordings shown in figure 4A-D indicated by filled circles labeled with a, b, c and d. Data from human and rat α4β3δH68 transfected cells were pooled to plot the summary data shown in Figs. 4E-H. Mean values and standard error of mean (SEM) for Fig. 4E are, 46 ± 5 % for 30 mM EtOH, 50 ± 7 % for 1 μM DZ, 57 ± 7 % for 1 μM β-CCE, -10 ± 7 % for 1 μM Zn^{2+}. Figures 4 F-H show correlation plots to compare the enhancement of 300 nM GABA currents by 1 μM DZ with enhancement by 30 mM EtOH (4F), 1 μM β-CCE (4G) and block by 1 μM Zn^{2+} (4H) in individual recordings. The correlation coefficients (r^2) for enhancement by 30 mM ETOH versus 1 μM DZ is r^2 = 0.63, for 1 μM β-CCE versus 1 μM DZ r^2 is 0.58, and for 1 μM Zn^{2+} versus 1 μM DZ r^2 is 0.32. Vertical scale for 2A and 2C is 400 pA and 100 pA for 2B and 2C, horizontal scales are 5 seconds.

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Table legend

Table 1: The δH68A mutation does not change the Zn^{2+} sensitivity of α4β3δ 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.

TABLE1

[Zn^{2+}]	0.1 μM	1 μM	10 μM
α4β3δ (n = 6) % block	0.8 ± 1.4	26.3 ± 2.1	88.8 ± 11.8
α4β3δH68A (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

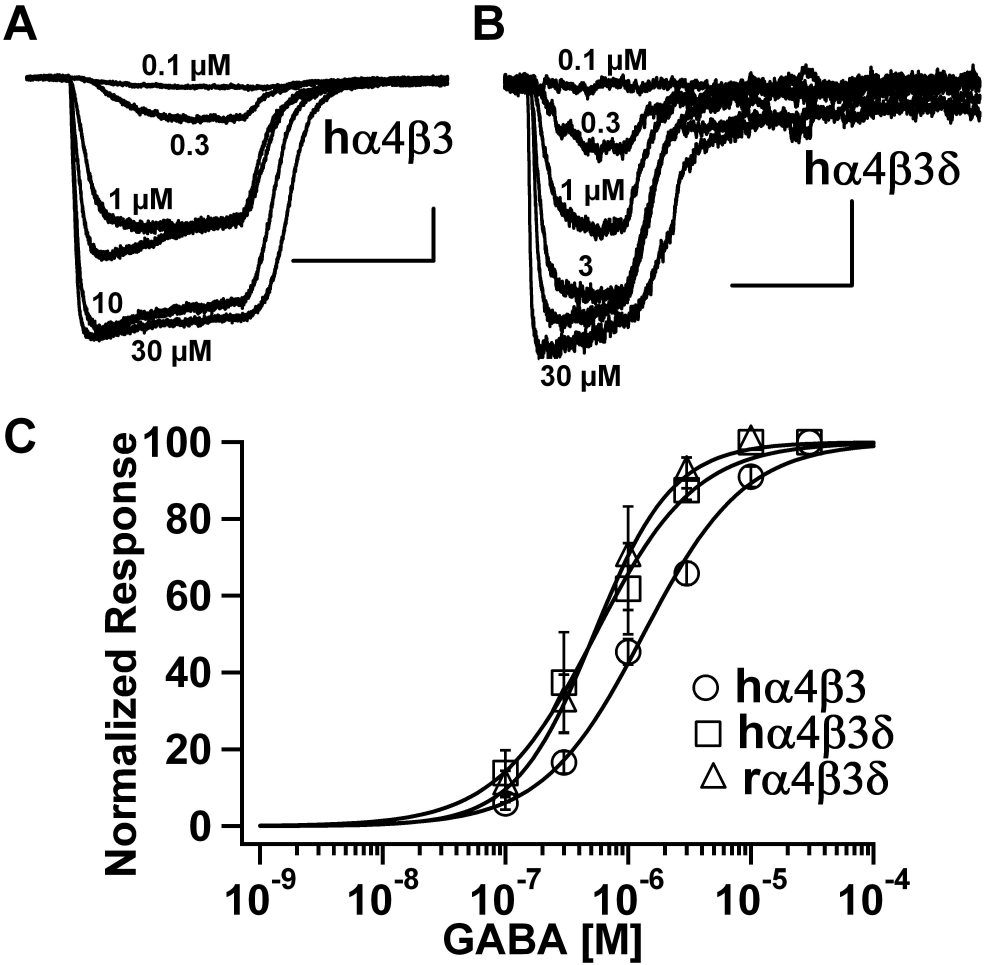
A
0.1 μM
0.3
1 μM
10
30 μM
hα4β3
B
0.1 μM
0.3
1 μM
3
30 μM
hα4β3δ
C
Normalized Response
100
80
60
40
20
0
10-9
10-8
10-7
10-6
10-5
10-4
GABA [M]
hα4β3
hα4β3δ
rα4β3δ

Figure 1

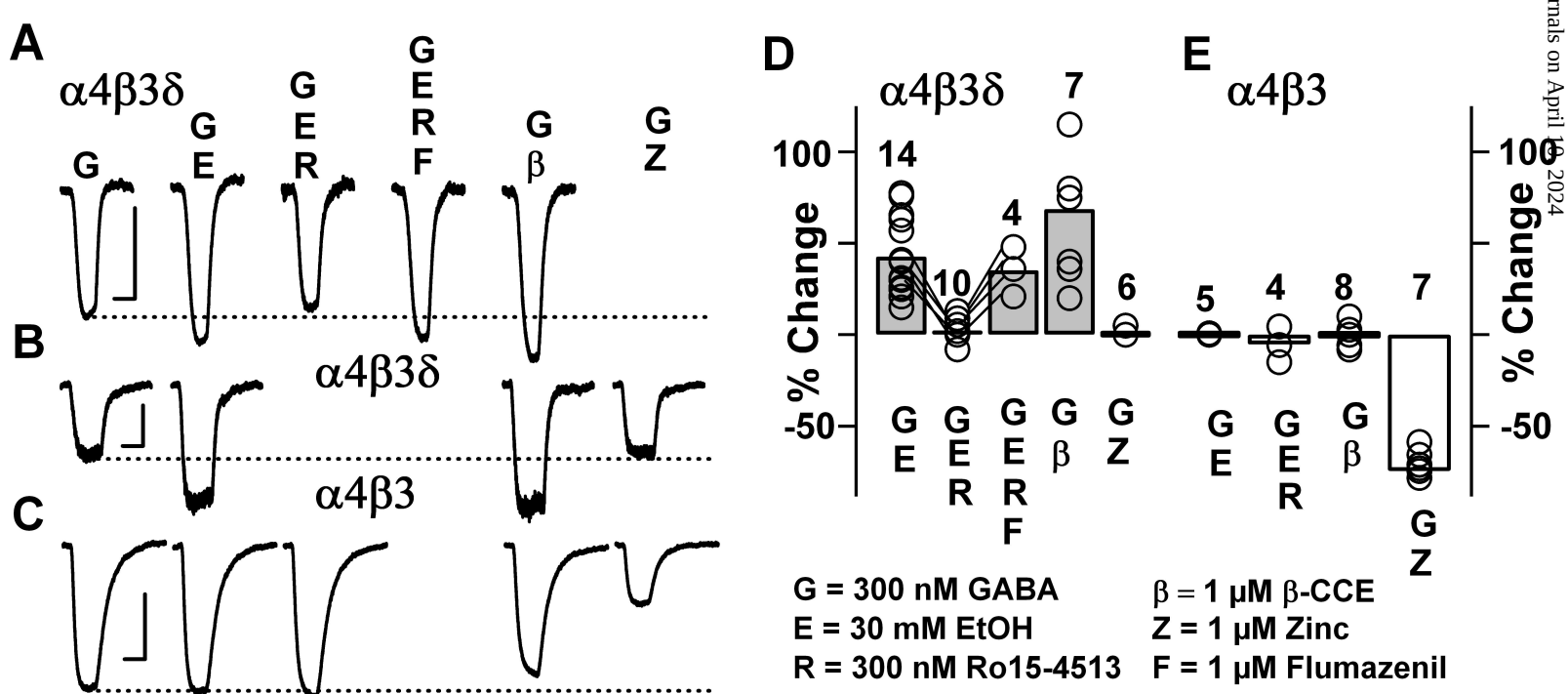

Figure 2

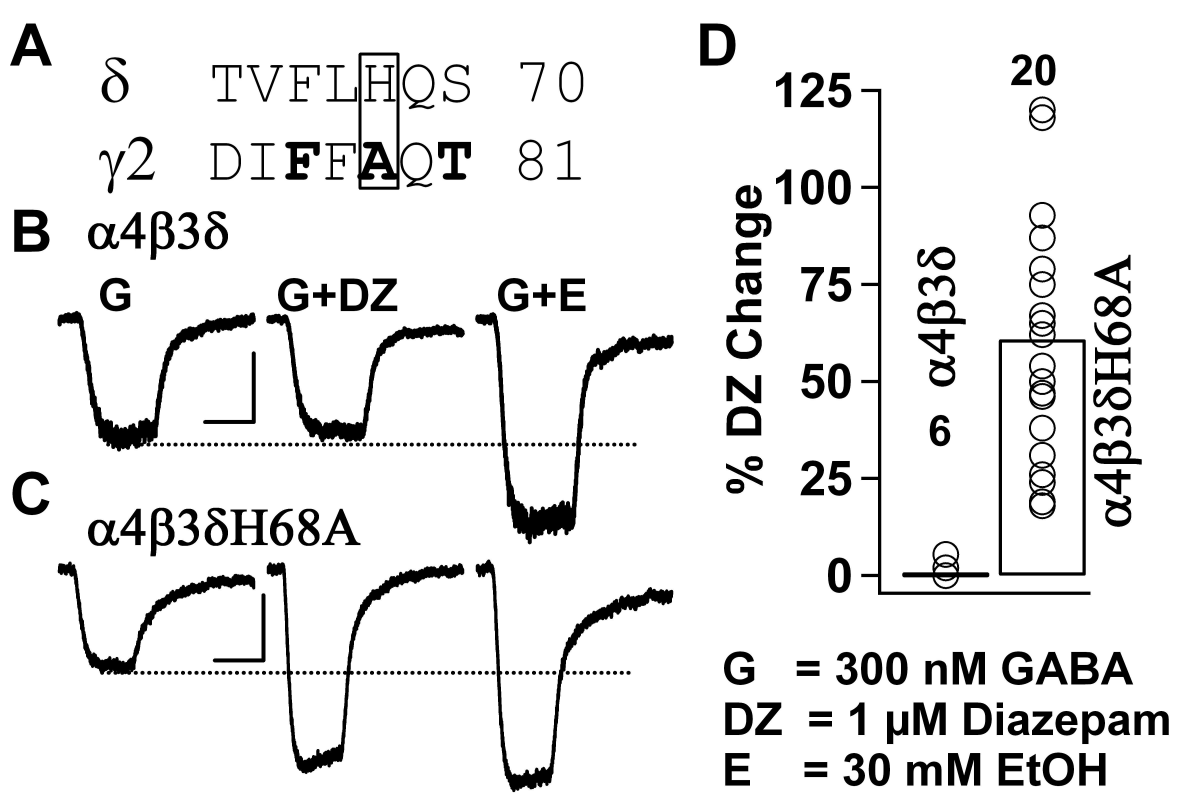
A
δ TVFLHQS 70
γ2 DIFFAQT 81
B α4β3δ
G
G+DZ
G+E
C
α4β3δH68A
D
% DZ Change
125
100
75
50
25
0
6
20
α4β3δ
α4β3δH68A
G = 300 nM GABA
DZ = 1 μM Diazepam
E = 30 mM EtOH

Figure 3

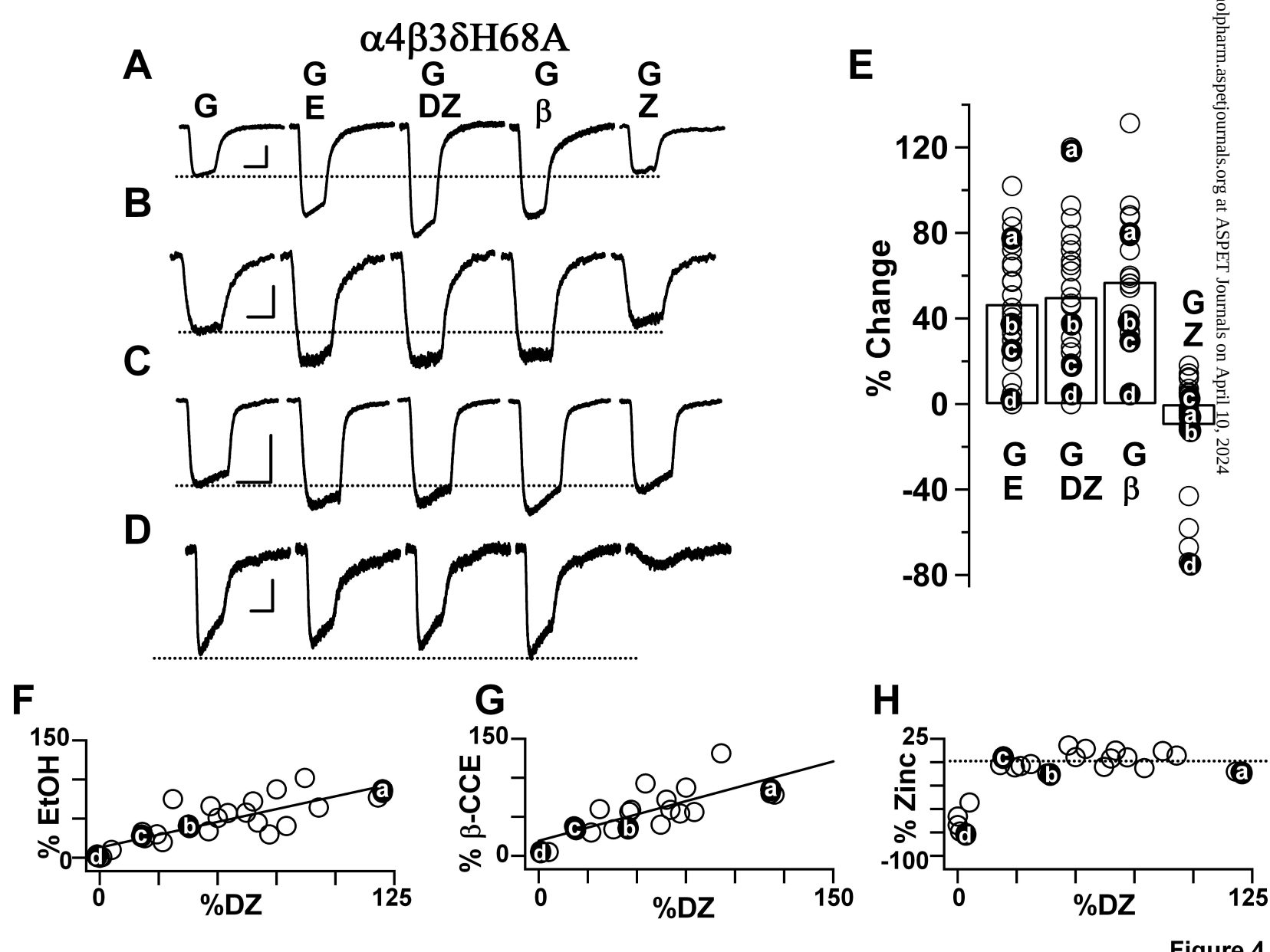

Figure 4